Structural and Thermodynamic Parameters Associated with the Interaction of Cobalt (III) Complexes and Duplex DNA

Jaime M. Ferreira
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Structural and Thermodynamic Parameters

Associated with the Interaction of Cobalt (III) Complexes and Duplex DNA

Jaime M. Ferreira

This dissertation submitted to the Department of Chemistry and Biochemistry of Seton Hall University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

May, 2006

Mentor: Dr. Richard D. Sheardy
Certification

We certify that we have read this thesis and that in our opinion it is adequate in scientific scope and quality as a dissertation for the degree of Doctor of Philosophy.

APPROVED

Richards D. Sheardy, Ph.D.

George Turner, Ph. D.

Alexander Fadeev, Ph. D.

Nicholas Snow, Ph. D.
Chair, Department of Chemistry and Biochemistry
Dedication
To my parents, Lillian and Frans Ferreira.

"Be not afraid. Do not be afraid of man’s weakness or his grandeur. Man does not cease to be great, not even in his weakness."

Pope John Paul II

"Deus Quere, O Homen Sonha, A Obra Nasce."

Fernando Pessoa

“Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light, not our darkness that most frightens us."

Nelson Mandela

"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning."

Albert Einstein
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I would like to thank past and present members of the Seton Hall University Biochemistry Research Group. Dr. Tony Paiva provided guidance in properly operating a wide range of research instrumentation including the CD spectropolarimeter, UV/VIS, and DSC. He provided valuable input and guidance on a variety of topics including DNA thermodynamics, and the synthesis of DNA oligomers.

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I would like to thank my professors, Dr. Richard Sheardy, Dr. George Turner, Dr. Mark Chiu, Dr. Nicholas Snow, Dr. Alex Fadlev, and Dr. Rorer Murphy for taking time to share their knowledge and wisdom pertaining to scientific inquiries. My mentor, Dr. Richard Sheardy offered continuous guidance, encouragement, and provided an advanced research laboratory to facilitate intellectual discovery.

Finally, I would like to extend warm thanks to my family, Lillian, Frank, and Adamo for their unconditional love and support. To Christine who has provided me with constant support, advice, and understanding throughout this endeavor, I am truly thankful for all who have contributed to the completion of this dissertation.
1. AA = atomic absorbance spectrophotometry, graphite furnace analysis
2. A.A; C.C; G.G; T.T; N.N = Nucleotide; Nucleotide on opposite strands, not necessarily base-paired. The common practice is to denote base-paired nucleotides on opposite strands with a center dot as in "A·T". However, the nucleotides referred to with this abbreviation are not necessarily base-paired in a Watson-Crick sense, so a unique abbreviation was adopted to avoid confusion with the abbreviation commonly used in the DNA field.
3. ACN = acetonitrile
4. $A_{260} = $ Absorbance, 260 nm; This abbreviation refers to the assay (as in "...the absorbance was measured with the $A_{260}$ assay.") and not the actual absorbance at 260 nm ($A_{260}$).
5. AEC = Averaged Extinction Coefficient
6. ALFS = Absorbance Units Full Scale
7. AXHPLC = Anion Exchange High Performance Liquid Chromatography
8. bp = basepair; This abbreviation is commonly used with a numeral designating a specific length of duplex DNA such as “10-bp” meaning a 10-basepair segment.
9. BSA = bovine serum albumin
10. Carbonato = pentaaamminecarbonato-o-balt (III) nitrate, $[\text{Co(NH}_3)_5\text{CO}_3\text{NO}_3]$
11. CD = Circular Dichroism – instrument used to detect structural changes in chiral molecules
12. Cisplatin = cis-diaminedichloroplatinum (II), cis-Pt(NH$_3$)$_2$Cl$_2$
13. Cohex – hexaamminecobalt(III) chloride, \([\text{Co(NH}_3_\text{)}_6\text{Cl}_3]\)
14. Coper – pentaammineaquocobalt (III), \([\text{Co(NH}_3_\text{)}_5\text{OH}_2\text{]}\text{ClO}_4\)
15. Cotar – short for an oligomer sequence to determine a cobalt (III) target site.
16. CPG = Controlled Pore Glass
17. DIBT = Differential Ion Binding Term
18. DMT = Dimethoxytrityl
19. DNA = Deoxyribonucleic acid
20. DSC = Differential Scanning Calorimetry
21. \(\Delta G_m\) – the free energy of duplex formation via a van’t Hoff Analysis
22. \(\Delta H_m\) – the enthalpy of duplex formation via a van’t Hoff Analysis
23. HPLC = High Performance Liquid Chromatography
24. i.d. = inner diameter
25. ITC = Isothermal Titration Calorimetry
26. \(K_b\) = binding constant
27. kD = kilo Dalton
28. MWCO = Molecular Weight Cut-Off – the MW required to prevent DNA from escaping dialysis chamber.
29. \(n\) = number of bound cobalt per DNA molecule
30. \(\Delta n\) – the differential ion binding term, representing the number of sodium ions released per duplex during a duplex to single strand transition.
31. o.d. = outer diameter
32. OD – optical density unit that represents an amount of DNA in 1 ml, at wavelength 260 nm read in a 1 cm path length.
33. Oligo – a synthetic DNA of known length in base pairs
34. PIPES = Piperazine – N,N’-bis[2-ethane-sulfonic acid]
35. psi = pounds per square inch
36. RP-HPLC = Reversed Phase High Performance Liquid Chromatography
37. ΔSθm – the entropy of duplex formation
38. Δθm – the fraction of single strands DNA still in duplex state at a particular thermal denaturation temperature.
39. TAE = Tris Acetate EDTA (solution)
40. TBE = Tris Borate EDTA (solution)
41. TCA = Trichloroacetic Acid
42. TEAA – triethylammoniumacetate
43. TGA = Thermal Gravimetric Analysis
44. TEMED – N,N,N’,N’- tetramethylethylenediamine
45. THF – tetrahydrofuran
46. Tm – the duplex to single strand transition temperature for DNA also called the melting temperature.
47. UV/Vis – Ultraviolet Visible Spectrophotometer
48. μW = microwatt
49. Z8A – an alternating pyrimidine-purine eight base pair oligomer of cytosine and guanosine, [(dCdG)₈]₂
50. Z8M – an alternating pyrimidine-purine eight base pair oligomer of 5’ methyl cytosine and guanosine, [(5’me-dCdG)₈]₂

vii
## PHYSICAL CONSTANTS

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## ENERGY CONVERSION FACTORS

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# Table of Contents

1. Abstract .............................................................................................................. 1
2. Introduction ....................................................................................................... 4
   2.1 DNA Structure and Biological Function ...................................................... 4
      2.1.1 Nucleotide Structure and Function .................................................... 4
      2.1.2 Nucleic Acids .................................................................................. 5
   2.2 Secondary and Tertiary Structure of Nucleic Acids .................................... 9
      2.2.1 Hydrogen Bonds and Stacking Forces that Stabilize the Double Helix ... 11
   2.3 Alternative Nucleic Acid Structures ......................................................... 13
      2.3.1 Z-Forms DNA ................................................................................ 14
      Sugar Pucker ......................................................................................... 19
   2.4 Proposed Function of altered DNA Structures ......................................... 20
      2.4.1 Overview ....................................................................................... 20
      2.4.2 Quadruplex .................................................................................... 21
      2.4.3 Left-handed Z-DNA ....................................................................... 22
   2.5 Biophysical Studies of Nucleic Acid Conformational Changes ............... 24
      2.5.1 Overview ....................................................................................... 24
      2.5.2 Pohl and Jovin - Van't Hoff Analysis ................................. 27
      2.5.3 Klump - Raman Spectroscopy ......................................................... 24
      2.5.4 Chaires and Sturtevant - Differential Scanning Calorimetry ... 26
      2.5.5 Felgner - Nuclear Magnetic Resonance (NMR) .......................... 28
   2.6 Small metal complexes that induce structural changes in nucleic acid ....... 29
      2.6.1 Overview ....................................................................................... 29
      2.6.2 cis-Dichloro Diammine Platinum (II) ............................................ 30
      2.6.3 Cobalt (III) Molecules .................................................................. 32
   2.7 Metal complexes involved in Biological activity ....................................... 33
      2.7.1 Overview ....................................................................................... 33
      2.7.2 Transition Metal’s Involved in Biological Activity ......................... 34
   2.8 Project Aims, Purpose, and Rationale ....................................................... 35
      2.8.1 Overview and Purpose .................................................................... 35
      2.8.2 The Cobalt (III) Complexes Investigated ..................................... 36
      2.8.3 The Relevance of the Sequences Studied ....................................... 36
      2.8.4 Characterization of the Structural Properties .............................. 37
      2.8.5 Characterization of the Thermodynamic Properties .................... 39
      2.8.6 Reactive Properties of Cobalt (III) Complex ................................ 41
   3. Materials and Methods ................................................................................. 43
      3.1 Definition of Equipment and Principles of Operation ......................... 43
         3.1.1 High Performance Liquid Chromatography (HPLC) System ........ 43
         3.1.2 Circular Dichroism Spectropolarimeter (CD) ......................... 43
         3.1.3 Differential Scanning Calorimetry (DSC) ............................ 44
         3.1.4 Isothermal Titration Calorimetry (ITC) ................................ 44
         3.1.5 UV/VIS Spectrophotometer .................................................... 47
         3.1.6 DNA Synthesizer ....................................................................... 47
         3.1.7 Gel Electrophoresis Equipment ............................................. 49

ix
3.1.8 Poly Pak II Cartridge Column (PPII) ............................................ 50
3.1.9 Digital Photography Equipment .................................................. 52
3.1.10 Dialysis Equipment ............................................................... 52
3.1.11 Nuclear Magnetic Resonance .................................................... 53
3.1.12 Infrared Spectrophotometer ...................................................... 53
3.2 Definition of Common Solutions and Reagents .................................. 54
3.2.1 SHU Denaturing Gel Sample Buffer ........................................... 54
3.2.2 SHU Native Gel Sample Buffer .................................................. 54
3.2.3 Tris-Borate-EDTA (TBE) ........................................................... 54
3.2.4 Standard Phosphate Solution (SPS) ............................................. 54
3.2.5 10X TPS (Stock solution used for sample preparation) ................. 54
3.2.6 5M Sodium Chloride ............................................................... 55
3.2.7 Acrylamide Stock Solution ......................................................... 55
3.2.8 2.0 M Triethylamine Acetate ................................................... 55
3.2.9 0.1 M Triethylamine Acetate .................................................... 55
3.2.10 1% Trifluoroacetic Acid .......................................................... 55
3.2.11 0.1 M TEA/A ......................................................................... 55
3.2.12 2.0 M TEA/A .................................................................... 56
3.2.13 0.1% STAINS .................................................................. 56
3.2.14 5 mM Trizma-HCl ................................................................. 56
3.2.15 Gel Staining Solution ............................................................... 56
3.3 Overview of Oligomer Synthesis ..................................................... 56
3.3.1 Introduction to DNA Synthesis .................................................... 56
3.3.2 ABI 380B DNA Synthesizer ....................................................... 57
3.3.3 Overview of the Phosphoramidite Synthesis Procedure ............... 62
3.3.4 Monitoring the Synthesis and Determining Crude Oligomer Yield .... 72
3.4 Oligomer Purification Methods ....................................................... 75
3.4.1 Purification Overview ................................................................. 75
3.4.2 PolyPak II Purification ............................................................... 76
3.4.3 Preparative Reverse-Phase HPLC .............................................. 78
3.4.4 Dialysis .............................................................................. 84
3.4.5 Lyophilization ................................................................... 86
3.5 Oligomer Characterization ............................................................... 86
3.5.1 Analytical RP-HPLC ................................................................. 86
3.5.2 Analytical Gel Electrophoresis: Urea-PAGE Electrophoresis .......... 88
3.5.3 DNA Concentration Determination – The A260 Assay ................. 93
3.6 Cobalt (III) Complex Synthesis ...................................................... 97
3.6.1 Overview of Synthetic Approach .............................................. 97
3.6.2 Procedure for the Synthesis of Simple Cobalt (III) Complexes ...... 98
3.6.2.2 cis-Cobalt (III) Tetramine Diaquio (cis-[Co(NH3)4(H2O)2]3+) ....... 100
3.7 Cobalt (III) Complex Characterization ......................................... 102
3.7.1 Complex Characterization by UV/VIS Spectroscopy ...................... 102
3.7.2 Characterization by Infrared Spectroscopy .................................. 104
3.8 DNA Structural Characterization .................................................. 107
3.8.1 Circular Dichroism Spectra ..................................................... 107
3.8.2 Circular Dichroism Titrations .................................................... 110

x
3.8.3 UV-Thermal Melts as a Function of [Salt] (Cobalt(III) Complex) .... 112
3.8.4 Singular Value Decomposition ........................................... 114
3.9 Thermodynamic Characterization ........................................... 116
3.9.1 UV-Thermal Melts (Optical Melts) ..................................... 116
3.9.3 Differential Scanning Calorimetry (DSC) ............................. 120
3.10 Algorithmic Determination of Free Energy from CD Data ............. 126
3.10.2 Overview of ITC .......................................................... 126
3.10.3 Analysis of Experiments ................................................. 127
3.11 Isothermal Titrations Calorimetry ......................................... 128
3.11.2 Overview of ITC .......................................................... 128
3.11.3 The ITC experiment ..................................................... 129
3.11.3.2 Free Energy Changes .............................................. 135
4 Results ................................................................................. 146
4.1 Structural Studies ............................................................. 146
4.1.1 Cobalt(III) –DNA Complexes ........................................... 146
4.1.2 UV/VIS Spectroscopy ....................................................... 146
4.1.3 Circular Dichroism ......................................................... 146
4.1.4 Effects of [Na+] by CD Spectroscopy ................................... 147
4.1.5 Effects of Cobalt(III) Complexes by CD Spectroscopy ... 152
4.1.6 Single Value Decomposition ............................................. 155
4.1.7 CD Studies of Co(III) –DNA Complexes ............................. 161
4.1.8 Temperature Dependence CD Spectroscopy ......................... 167
4.1.9 Differential Ion Binding Term ........................................... 176
4.1.10 Native Gel Electrophoresis .............................................. 178
4.2 Thermodynamic ............................................................... 180
4.2.2 Thermodynamic Parameters (DSC) .................................... 198
4.2.3 Thermodynamic Parameters (ITC) ..................................... 217
4.3 The B-to-Z Transition ......................................................... 213
4.3.3 Differential Scanning Calorimetry ..................................... 219
4.3.4 Isothermal Titration Calorimetry ...................................... 220
4.3.5 Heat Capacity ............................................................... 225
4.3.6 Enthalpic Thermodynamic Cycle ...................................... 231
4.3.7 Osmolality Studies (NaCl) ................................................. 234
5 Discussion/Conclusions ......................................................... 239
5.1 Structural Stages ............................................................... 239
5.1.1 Purpose ........................................................................ 239
5.1.2 Differences Between the ZRA and ZRM Oligomer ................. 240
5.1.3 Temperature Dependent CD ........................................... 242
5.1.4 Singular Value Decomposition ........................................ 242
5.2 Thermodynamic ............................................................... 243
5.2.1 Purpose ........................................................................ 243
5.2.2 UV Melts vs Calorimetry ................................................. 243
5.2.3 Isothermal Titration Calorimetry ...................................... 247
5.2.4 The B-to-Z Transition .................................................... 248
5.2.5 Enthalpic, Entropic and Free Energy Correlations ................. 252
5.2.6 Heat Capacity ............................................................... 254
xi
List of Tables

Table 1: A Table of pHa values for Mononucleotides...........................................9
Table 2: Structural Features of Ideal A-, B-, and Z-DNA.............................................19
Table 3: Thermodynamic results for the B-to-Z transition.........................................25
Table 4: Previously published results for the enthalpy of the B-to-Z transition.............27
Table 5: Flow rate specifications for the ABI model 380B DNA synthesizer.................57
Table 6: Reagent shelf-life for the Expedite 8909 and ABI 380B...............................60
Table 7: Typical synthesis yield for ABI 380B and Expedite 8909..............................74
Table 8: Trityl-ON DNA, Prep-RP-HPLC...............................................................79
Table 9: Trityl-ON DNA – high CG rich and methylated sequences............................80
Table 10: Trityl-Off DNA – RP-HPLC Protocol..........................................................82
Table 11: A guide to the optimal acrylamide concentration for PAGE...........................89
Table 12: UV/Vis values for cobalt (III) complexes.....................................................101
Table 13: Table of Major Infrared Peaks for Cobalt (III) Complexes...........................103
Table 14: Results of SVD of the CD spectra for the B-to-Z transition.........................160
Table 15: Differential Ion Bonding Values for NaCl and (Co(NH3)6)3+..........................177
Table 16: Thermodynamic Properties for the Z8M Oligomer.......................................181
Table 17: Thermodynamic Parameters for the ZBA Oligomer....................................182
Table 18: DSC Values Obtained for the ZBA - Cobalt (III) Molecules.........................201
Table 19: DSC Values Obtained for the Z8M - Cobalt (III) Molecules..........................202
Table 20: ITC Values for various Cobalt (II) complexes (ZIA Z8M).........................212
Table 21: The Fitting Parameters for the B-to-Z transition..........................................219
Table 22: Free Energy Change for the B-to-Z transition (NaCl)..................................219
Table 23: Free Energy Changes of the B-to-Z transition ([Co(NH3)6]3+..........................220
Table 24: The DSC thermodynamic properties for ZBA and Z8M..................................220
Table 25: The ΔH, ΔG, TASS values obtained by ITC at 25°C for NaCl and 
(\text{Co(NH}_{3})_{6}\text{Cl}_{3})^{3+}...........................................................................225
Table 26: The Enthalpy of the B-to-Z transition determined by ITC............................226
Table 27: The ΔH, ΔS, ΔG, and n values at 25°, 35°, 45°, and 55 °C obtained by 
ITC for the ZBA and Z8M Oligomer in the presence [Co(NH3)6]3+.........................227
Table 28: The ΔH, ΔS, ΔG, and n values at 25°, 35°, 45°, and 55 °C obtained by 
ITC for the ZBA and Z8M Oligomer (NaCl).......................................................228
Table 29: The ΔAH at 25°, 35°, 45°, and 55 °C obtained by ITC for the ZBA and 
Z8M Oligomer (NaCl).........................................................................................230
Table 30: The ΔAH at 25°, 35°, 45°, and 55 °C obtained by ITC for the ZBA and 
Z8M Oligomer ([Co(NH3)6]3+)............................................................................231
Table 31: Heat Capacity Values for ZBA and Z8M (NaCl and [Co(NH3)6]3+)..............232
Table 32: Calorimetric Enthalpy Values Obtained for the B-to-Z transition at 
Various Osmolyte Concentrations (NaCl)...........................................................238
Table 33: Values For the Number of Water Molecules Released..................................240
Table 34: Comparison between van’t Hoff and calorimetric enthalpies (Z8M)............245
Table 35: Comparison between van’t Hoff and calorimetric enthalpies (Z8).............247
Table 36: The Enthalpy Contributes from an osmolyte..............................................258
Figure 44: The Normalized Spectra Response for NaCl (ZBM)........................................ 152
Figure 45: The CD spectra of the ZBM oligonucleotides at various [Co(NH₃)₆]³+ .......... 154
Figure 46: The NSR as a function of [Co(NH₃)₆]³⁺ for the ZBM oligomer ............... 155
Figure 47: Matrix A of the measured CD spectra for the NaCl titrations of ZBM ........ 157
Figure 48: Matrix S of the measured CD spectra for the NaCl titrations of ZBM .......... 158
Figure 49: Matrix V of the measured CD spectra for the NaCl titrations of ZBM ...... 159
Figure 50: Significant Bnus Spectra.......................................................... 159
Figure 51: Residual Plots ........................................................................... 160
Figure 52: CD Spectra of ZBA and ZBM in the presence of [Co(NH₃)₆(H₂O)]³⁺ ...... 162
Figure 53: CD Spectra of ZBA and ZBM in the presence of [Co(NH₃)₆Cl]²⁻ ........ 164
Figure 54: CD spectra of cis-[Co(NH₃)₆(H₂O)]³⁺ for ZBA and ZBM Oligomer....... 165
Figure 55: CD spectra of Cobalt (III) Complexes and NaCl.............................. 167
Figure 56: Temperature Dependent CD Spectrum of [Co(NH₃)₆(H₂O)]³⁺ ............ 169
Figure 57: Temperature Dependent CD Spectrum of [Co(NH₃)₆(H₂O)]³⁺ ............ 170
Figure 38: Temperature Dependent CD Spectrum of cis-[Co(NH₃)₆(H₂O)]³⁺ .... 171
Figure 59: Temperature Dependent CD Spectrum of cis-[Co(NH₃)₆(H₂O)]³⁺ .... 172
Figure 60: Temperature Dependent CD Spectrum of [Co(NH₃)₆Cl]²⁻ (ZBA) ...... 174
Figure 61: Temperature Dependent CD Spectrum of [Co(NH₃)₆Cl]²⁻ (ZBM) ...... 175
Figure 62: Differential Ion Binding Plots for NaCl and [Co(NH₃)₆Cl]²⁻ .......... 177
Figure 63: Native Gel Electrophoresis of DNA—Cobalt (III) Complexes ............ 179
Figure 64: Absorbance vs Temperature for the ZBA and ZBM in SPB Conditions .... 183
Figure 65: Plot of Tm versus pH for the ZBA and ZBM oligomer in SPB Solution .... 184
Figure 66: Absorbance vs Temperature for the ZBA in 2 M and 4 M NaCl Conditions 185
Figure 67: Absorbance vs Temperature for the ZBM in 2 M and 4 M NaCl .......... 186
Figure 68: Plot of Tm versus pH for the ZBA and ZBM oligomer in NaCl ............. 187
Figure 69: Absorbance vs Temperature for the ZBA in 200 µM [Co(NH₃)₆Cl]²⁻ .... 189
Figure 70: Absorbance vs Temperature for the ZBM in [Co(NH₃)₆Cl]²⁻ .......... 189
Figure 71: Plot of Tm versus pH for the ZBA/ZBM oligomers in [Co(NH₃)₆Cl]²⁻ .... 190
Figure 72: Absorbance vs Temperature for the ZBM in [Co(NH₃)₆(H₂O)]³⁺ .... 196
Figure 73: Plot of Tm versus pH for the ZBA/2M oligomers in [Co(NH₃)₆(H₂O)]³⁺ ... 193
Figure 74: Absorbance vs Temperature for the ZBM in 200 µM [Co(NH₃)₆(H₂O)]³⁺ .... 194
Figure 75: Plot of Tm versus pH for the ZBA/ZBM oligomer in [Co(NH₃)₆(H₂O)]³⁺ ... 195
Figure 76: Absorbance vs Temperature for the ZBM Oligomer in [Co(NH₃)₆Cl]²⁻ .... 196
Figure 77: Plot of Tm versus pH for the ZBA/2M oligomer [Co(NH₃)₆Cl(2Cl)⁻] .... 197
Figure 78: DSC of the ZBA oligomers in 200 and 400 µM [Co(NH₃)₆Cl]²⁻ .......... 203
Figure 79: DSC of the ZBA oligomers in 200 and 400 µM [Co(NH₃)₆(H₂O)]³⁺ .... 204
Figure 80: DSC of the ZBA oligomers in SPB ................................................. 205
Figure 81: DSC of the ZBM oligomers in SPB ................................................. 206
Figure 82: DSC of the ZBA oligomers in 200 µM [Co(NH₃)₆]³⁺ ......................... 207
Figure 83: DSC of the ZBM oligomers in 4 M NaCl ........................................ 208
Figure 84: DSC of the ZBM oligomers in 200 and 400 µM [Co(NH₃)₆Cl]²⁻ .... 209
Figure 85: DSC of the ZBM oligomers in 200 and 400 µM [Co(NH₃)₆(H₂O)]³⁺ .... 210
Figure 86: Representative ITC Binding Isotherms for the cobalt (III) complexes .... 212
Figure 87: Schematic Representation of the Proposed Thermodynamic Cycle .... 215
Figure 88: ITC Titrations of NaCl into ZBA and ZBM Oligomer ....................... 221
Figure 89: ITC Titrations of Cobalt (III) hexammine into ZBA and ZBM Oligomer ... 222
List of Equations

Equation 1: \[ \Delta H_{\text{TH}} = A R T_m^2 \left( C_{\text{trypsin}} / \Delta h_{\text{cal}} \right) \]
Equation 2: \[ 1 / T_m = \left( (n-1) R / \Delta H \right) \Delta C_i + \left[ \Delta S (n-1) R n_{2+} + \Delta n \right] / \Delta H \]
Equation 3: \[ \text{Overall Yield} = \text{Peak Height}_{\text{sim}} \times \Delta h_{\text{cal}}^{*} (100\%) \]
Equation 4: \[ \text{Step Yield} = \left( \text{Peak Height}_{\text{sim}} / \text{Peak Height}_{\text{in}} \right) \times (100\%) \]
Equation 5: \[ \text{Step Yield} = \left( \text{Overall Yield} \right)^{1/n-1} \]
Equation 6: \[ A_{280} \text{ oligomer} = 27 \mu g \text{ DNA} \]
Equation 7: \[ \left( A_{280}\text{ (dilution)} \right) / 0.027 = \mu g \text{ DNA/ml} \]
Equation 8: \[ \% \text{ yield} = [(\text{DNA})_{\text{post diazo}} / (\text{DNA})_{\text{pre diazo}}] \times (100\%) \]
Equation 9: \[ R_t = qE / f \]
Equation 10: \[ \lambda = \omega / \nu \]
Equation 11: \[ E = h \nu \]
Equation 12: \[ \log (I/l_{0}) = -a \theta \text{ or } I/l_{0} = e^{-a \theta} \]
Equation 13: \[ a \theta = \varepsilon c b \]
Equation 14: \[ \log (l/l_{0}) = c \theta b \]
Equation 15: \[ A = c \theta b \]
Equation 16: \[ \varepsilon = (15.200 N_{A} + (7050 N_{C}) + (12010 N_{G}) + (8400 N_{T})) \]
[pmol/\mu l] = A_{280} \times 100 \left( (1.5 N_{A}) + (0.71 N_{C}) + (1.2 N_{G}) + (0.84 N_{T}) \right) \]
Equation 17: \[ \Delta A = (A_{280} \text{ (dilution factor)} / \varepsilon \]
Equation 18: \[ C = (\text{pmol/\mu l} / A_{280}) \times \Delta A \]
Equation 19: \[ \Delta A = (e_{t} - e_{a}) C I \]
Equation 20: \[ \Delta C = (e_{t} - e_{a}) \]
Equation 21: \[ \% \text{ transition} = \delta \theta_{290} \text{ (salt)} / \delta \theta_{290} \text{ (total)} \times 100 \]
Equation 22: \[ \delta \theta_{290} \text{ (Salt)} = \delta \theta_{290} \text{ (low salt)} - \delta \theta_{290} \text{ (salt)} \]
Equation 23: \[ \delta \theta_{290} \text{ (total)} = \delta \theta_{290} \text{ (low salt)} - \delta \theta_{290} \text{ (high salt)} \]
Equation 24: \[ dT_m / \log [M] = \left( 2.303 R T_m^2 / \Delta H_m \right) \Delta n \]
Equation 25: \[ T_m = \left( 2.303 R T_m^2 / \Delta H_m \right) \Delta n \]
Equation 26: \[ C_{\text{X}(t)} = \sum_{i=1}^n x_{i(t)} \]
Equation 27: \[ d[t] / dT = \Delta H / R T^2 \]
Equation 28: \[ K = a / \left( [C/n]^{n-1} (1 - a)^n \right) \]
Equation 29: \[ K = a / \left[ n(C/n)^{n-1} (1 - a)^n \right] \]
Equation 30: \[ H_{\text{TH}} = (2 + 2n) R T_m^2 (2a/5T) \]
Equation 31: \[ \ln K = -\left( \Delta H / RT \right) + \left( \Delta S / R \right) \]
Equation 32: \[ \Delta C_p = 0 \]
Equation 33: \[ H = E + PV \]
Equation 34: \[ \Delta H = \Delta E + \Delta (PV) \]
Equation 35: \[ dH = dE + dP V + V dP \]
Equation 36: \[ dE = dQ + dW \]
Equation 37: \[ dH = dQ + dW + V dP + V dP \]
Equation 38: \[ dP = 0 \]
Equation 39: \[ dW = -P dV \]
\[
\text{Equation 40:} \quad dH_0 = dQ_B - PdV + PdV + VdP \\
\text{Equation 41:} \quad C_p = dQ/dT \\
\text{Equation 42:} \quad dH = C_p dT \\
\text{Equation 43:} \quad \Delta H = \int C_p dT \\
\text{Equation 44:} \quad (dQ/dT)(1/M) = C_p^n \\
\text{Equation 45:} \quad W = \int P \, dV \\
\text{Equation 46:} \quad K_1 = [I] / [B] = [a / a^2] \quad K_2 = [BZ] / [I] = [a / a^2]^{2} \\
\text{Equation 47:} \quad C_l = [B] + [I] + [BZ] \\
\text{Equation 48:} \quad [B] = C_l / (1 + K_1 + K_2) \\
\text{Equation 49:} \quad \theta_{\text{app}} = \{ \theta_0 (B) + \theta_0 (I) + \theta_{\text{int}} (BZ) \} / C_l \\
\text{Equation 50:} \quad \text{NSR} = (\theta_{\text{app}} - \theta_{\text{ref}}) / (\theta_0 - \theta_{\text{as}}) \\
\text{Equation 51:} \quad \Delta G = -RT \ln K \\
\text{Equation 52:} \quad \Delta Q_{\text{int}} = \Delta Q_{\text{a}} + \Delta Q_{\text{b}} \\
\text{Equation 53:} \quad \Delta Q_{\text{app}} = \theta_{\text{a}} - \theta_{\text{b}} \\
\text{Equation 54:} \quad \theta_{\text{app}} = \Delta Q_{\text{b}} / \Delta Q_{\text{int}} + \Delta Q_{\text{as}} \\
\text{Equation 55:} \quad \Delta Q_0 = \Delta Q_{\text{app}} - \Delta Q_{\text{a}} - \Delta Q_{\text{b}} = \text{n[M]} \text{tot} \text{V}_{\text{tot}} \Delta H_{\text{app}} \times R \\
\text{Equation 56:} \quad Y^2 \times Y_i \times \{ 1 + (j/n \text{k}_A \text{[M]tot}) \times ((\text{l}_A \text{[M]tot}) / \text{n[M]} \text{tot}) \} + \text{n[L]tot} \text{[M]tot} = 0 \\
\text{Equation 57:} \quad Q = \frac{\sum_{i=1}^{n} [M] L_i^j}{[M]} - 1 + \frac{\sum_{i=1}^{n} [M] L_i^j}{[M]} = 1 + \frac{\sum_{j=1}^{n} B_j [L]^{j}}{} \\
\text{Equation 58:} \quad \beta_j = \prod_{j=1}^{n} K_j \\
\text{Equation 59:} \quad \beta_j = \frac{n!}{j(n-j)!} \prod_{j=1}^{n} K_j \\
\text{Equation 60:} \quad Q = 1 + K_A [I]^j \\
\text{Equation 61:} \quad Q = (1 + K_A [I]^j)^{ii}}
Equation 62:  \[ Q = \prod_{i=1}^{n} (1 + K_{A, i} [L])^{\gamma_i} \]

Equation 63:  \[ \gamma_i = \frac{\sum_{j} \beta_j [L]^j}{\frac{\partial \ln Q}{\partial \ln [L]}} \]

Equation 64:  \[ \Delta Q_i = M_{T} \times V_{cel} \frac{\sum_{j} \Delta H_{ref,j} \beta_j [L]^j}{Q} \]

Equation 65:  % transition = \( \delta \theta \) (NaCl) / \( \delta \theta \) (total)x100

Equation 66:  \( \delta \theta \) (NaCl) = \( \theta \) (115 mM SPB) - \( \theta \) ([NaCl])

Equation 67:  \( \delta \theta \) (total) = \( \theta \) (115 mM SPB) - \( \theta \) (2.5 M NaCl)

Equation 68:  \[ \Lambda = USV^T \]

Equation 69:  \[ C = \sum_{i=1}^{n} X_i X_{-1} \]

Equation 70:  \[ \frac{dT_{\theta}}{\partial \theta_{\theta \theta}} M[M] = \{2.303RT_n^2/\Delta H_n \} \Delta n \]

Equation 71:  \[ K_1 = [I] / [B] = [(a/a_i)][n_i] \quad K_2 = [Z][I] = [(a/a_2)][n_2] \]

Equation 72:  \[ C_1 = [B] + [I] + [Z] \]

Equation 73:  \[ [B] = C_1 / (1 + K_1 + K_3) \]

Equation 74:  \[ \delta = \{ \delta_{B}(B) + \delta_{Z}(Z) \} / C_1 \]

Equation 75:  \[ \Delta H_{ref} = \Delta H_{ref} + \Delta H_{ref} \]

Equation 76:  \[ \Delta H_{ref} = \Delta H_{ref} + \Delta H_{ref} \]

Equation 77:  \[ C = \left( \frac{\partial Q}{\partial T} \right)_{const} = T \left( \frac{dS}{dT} \right)_{const} \]

Equation 78:  \[ \Delta C_p = \frac{d(\Delta H)}{dT} = T \frac{d(\Delta S)}{dT} \]

xix
Equation 79: \[ \frac{dK_{cat}}{dM_a} = \frac{RT}{55.6 (\Delta N_{sec})} \]
1 ABSTRACT

Structural and Thermodynamic Parameters Associated with the Interaction of Cobalt (III) Complexes and Duplex DNA

DNA molecules can attain various conformations depending on base sequence and the physiological conditions under which they are prepared. The conformational variability of DNA affects biological functions such as mutagenesis, control of transcription, and recognition of regulatory proteins. The right-handed (B-form) conformation first seen by X-ray crystallography in 1953 by James Watson and Francis Crick, reveals a double stranded helix, forming a twisted upright ladder. Two other conformations that been characterized through X-ray crystallography are the A and Z-forms (left-handed).

The Z-form is a left-handed DNA double helix with Watson-Crick base pairing. This structure was first detected by Alex Rich for the duplex strand of d(CpGpCpGpCpG). The biological significance of Z-DNA is unclear. Indeed, several cellular proteins that bind specifically to Z-DNA have been isolated from the nuclei of Drosophila fruit flies suggesting a specific role for the Z DNA conformation. Speculation of the biological roles for the Z DNA conformation have concentrated on gene expression and genetic recombination, but not much advancement has been achieved. Previous studies have shown that small metal complexes such as [Ru(NH$_3$)$_6$(OH)$_2$]$^{2+}$, cis-[Ru(NH$_3$)$_5$Cl]$^{2+}$, cis-Pt(NH$_3$)$_2$Cl$_2$, and [Co(NH$_3$)$_6$]$^{3+}$ induce conformational changes in DNA molecules. Along with ruthenium, platinum, and other metal complexes, cobalt (III) molecules can also interact with DNA oligomers and polymers in a variety of
fashions depending on the sequence of the DNA as well as the nature of the ligands present on the cobalt core. The replacement of one or two of the ammine ligands on [Co(NH₃)₆]³⁺ with more labile ligands such as a chloro and/or an aquo can lead to irreversible binding of the cobalt center to the DNA, presumably at N7 of the guanine bases. We are reporting studies on the effects of NaCl, [Co(NH₃)₆]Cl⁺, [Co(NH₃)₅Cl]⁺, cis-[Co(NH₃)₅Cl]²⁺, [Co(NH₃)₅(OH₂)]²⁺, and cis-[Co(NH₃)₅(OH₂)₂]⁴⁺ on the thermodynamic properties associated with the duplex to single strand transition as well as any conformational transitions for two model oligonucleotides, d(C-G)₄ (Z8A) and d(5-meC-G)₄ (Z8M).

It is well known, that [Co(NH₃)₆]³⁺ can induce the B to Z transition for poly 4(G-C) and poly 4(G-5-meC). In the presence of 200 μM [Co(NH₃)₆]³⁺ methylated G-C rich sequences (e.g., 5(meC-G)₄) assume a double-stranded, left-handed Z conformation at 25°C. We report the thermodynamic properties related to this transition. Thermodynamic properties of the B-to-Z transition have been studied through indirect methods. Although these methods have provided scientist with important information pertaining to B-Z transitions; none of these methods are able to measure the thermodynamic parameters directly. The only method currently available to study the differences in energy directly associated with the conformational change of the B to Z transition is by calorimetry. Calorimetry allows the thermodynamics of a system to be measured directly without analyzing the data with algorithms or proposing models to describe the cooperative thermodynamic effects (i.e., van't Hoff Analysis or model independent methods). We propose a method of calculating the thermodynamic properties of the B-to-
Z transition and other transitions, using a model independent method, via calorimetry.

Each DNA oligomer was incubated with the cobalt (III) complexes at various concentrations and the stabilities of the modified DNA duplexes determined by optical melting studies and Differential Scanning Calorimetry (DSC). The values of $T_m$, $\Delta H_m$, $\Delta H_{gb}$, and $\Delta n$ (differential ion binding term) obtained allow us to compare DNA oligomer stability associated with various cobalt (III) complexes due to different ligand substituents. Any structural changes associated with the binding of the Cobalt (III) complexes were determined by Circular Dichroism (CD) at each concentration. Isotothermal Titration Calorimetry (ITC) was used to measure the enthalpy associated with the initial mode of binding and used for determining thermodynamic properties related to conformational changes in DNA. ITC was also used to monitor water activity related to the transitions. Our work leads to insight into the thermodynamic properties associated with the binding of simple cobalt (III) complexes with oligonucleotides, and into DNA conformational transitions.
2 INTRODUCTION

2.1 DNA Structure and Biological Function

2.1.1 Nucleotide Structure and Function

The biosynthesis of nucleotides is a vital process for the synthesis of both RNA and DNA. Without RNA synthesis, protein synthesis is halted, and unless cells can synthesize DNA, they cannot divide. Nucleotides are also necessary for constant repair of DNA, a process necessary for cell survival. Inhibitors of nucleotide biosynthesis are very toxic to cells. The toxicity has been used to advantage in the treatment of certain diseases resulting from infections by viruses, bacteria, or protozoans. Nucleotides play important roles in all major aspects of metabolism. ATP, an adenine nucleotide, is the major substance used by all organisms for the transfer of chemical energy from energy-yielding reactions to energy-requiring reactions such as biosynthesis. Other nucleotides are activated intermediates in the synthesis of carbohydrates, lipids, proteins and nucleic acids. Adenine nucleotides are components of many major coenzymes, such as NAD⁺, NADP⁺, FAD, and CoA. The critical role played by nucleotides is regulation of metabolism in both prokaryotic and eukaryotic organisms and in signal transduction.

Pathways for the metabolic degradation of nucleotides are also very important to organisms. Generic defects causing blocks in these pathways have serious consequences for the health of humans and other animals. The physiological importance of nucleotides is reflected in the careful regulation of their intracellular levels as well as intra and extracellular levels of nucleosides and nucleobases. This regulation is determined by the concentration and location of the enzymes of nucleotide metabolism and by levels of
substrates, products, and effectors.\textsuperscript{22}

2.1.2 Nucleic Acids

There are two types of nucleic acid, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Each is a polymeric chain in which the monomer units are connected by covalent bonds. Each monomer or nucleotide is composed of three parts: (1) a heterocyclic nitrogenous base, (2) a pentose, and (3) a phosphoryl group. The bases are planar and aromatic moieties.

Figure 1: Typical Nucleotide

The nucleotide base deoxycytosine-5'-phosphate (dCMP) contains a heterocyclic nitrogenous base (red), a pentose (blue), and a phosphoryl group (green). All three components make up a nucleotide base used for all DNA and RNA molecules.

Nucleotide bases belong to two classes: pyrimidines and purines. In pyrimidine nucleotides, N-1 of the pyrimidine base is attached to the pentose C-1'. The common pyrimidine bases in nucleotides are uracil, cytosine, and thymine. Purine bases have a six member pyrimidine ring fused to a five membered imidazole ring, the fused system containing four nitrogen atoms. In purine nucleotides, the glycosidic linkage is between N-9 of the purine and C-1' of the pentose. The common purine bases in nucleotides are
Figure 2: Five Nucleotide Base's
Thymine, Cytosine, Guanine, and Adenine are used for DNA base pairing, while Uracil, Cytosine, Guanine and Adenine are used for RNA base pairing.

The pentose component of naturally occurring nucleotides is ribose or 2-deoxyribose (i.e. ribose with a hydrogen instead of a C-2' - OH). In nucleotides the purine or pyrimidine is attached to C-1' of the pentose in the β-configuration. This means that the base is cis relative to C-5' and trans relative to the C-3' - OH. The major function of deoxynucleotides (those that have 2-deoxyribose as the pentose) is to serve as building blocks for DNA.

The phosphoryl group of nucleotides is most commonly substituted on the C-5' - OH of the pentose. In nucleic acids each nucleotide unit has one phosphoryl group esterified to the C-5' - OH and another at the C-3' - OH, and these phosphoryl groups link the
nucleotide units. Figure 3 shows an example of the structural differences between RNA and DNA.

**Figure 3: Structural Differences between RNA and DNA.**
The structural differences between DNA and RNA lie in the sugar region of the molecule. For DNA, the C-2' of the sugar contains a hydrogen, but for RNA, the C2' of the sugar contains a hydroxyl group. This slight variation in the sugar is responsible for the structural differences between DNA and RNA.

All of the commonly occurring bases in nucleotides are capable of existing in two tautomeric forms, which differ by the placement of a proton and some electrons. An example is guanosine which can undergo a change from keto form to an enol form as shown in Figure 4. The keto form is so strongly favored that it is difficult to detect even trace amounts of the enol form at equilibrium. Similarly, the keto form of thymine and uridine is strongly preferred. Adenosine and cytidine can isomerize to imino forms, but the amino forms are strongly preferred. These unusual tautomers are present in small amounts, and when present in DNA, they contribute to the mutations in the genetic material.
Figure 4: Tautomerism in DNA bases
An example of a conformational change in guanine as the molecule changes from a keto form to an enol form. A similar conformational change can occur for adenine and cytosine, when these molecules can isomerize to the imino form.

Nucleotides are quite strong acids; the primary ionization of the phosphate occurs with a pKa of approximately 1.0. Both secondary ionization of the phosphate and protonation or deprotonation of some of the groups on the bases within the nucleotides can be observed at pH values quite close to neutrality as shown in Table 1. At neutrality there is no charge on any of the bases. Three of the bases, A, C, and G, undergo protonation as the pH is lowered. In guanosine a proton adds to N-7 rather than the amino group, again indicating the unusually low basicity of the amino groups on the nucleotides compared with primary aliphatic amines. On the basic side of neutrality, GMP loses a proton from the imino nitrogen at position N-3 and N-1.
Table 1: A Table of pKₐ values for Nucleotides.
The table indicates the pKₐ values for the 1°, 2° Phosphate, and the base. It also indicates the location the proton is involved in protonation and deprotonation.

<table>
<thead>
<tr>
<th></th>
<th>Primary</th>
<th>Secondary</th>
<th>Base</th>
<th>Loss of Proton</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphate (pKₐ,1)</td>
<td>Phosphate (pKₐ,2)</td>
<td>(pKₐ)</td>
<td></td>
</tr>
<tr>
<td>5’ AMP</td>
<td>0.9</td>
<td>6.1</td>
<td>3.4</td>
<td>N-1</td>
</tr>
<tr>
<td>5’ UMP</td>
<td>1.0</td>
<td>6.4</td>
<td>9.4</td>
<td>N-3</td>
</tr>
<tr>
<td>5’ CMP</td>
<td>0.8</td>
<td>6.3</td>
<td>4.5</td>
<td>N-3</td>
</tr>
<tr>
<td>5’ GMP</td>
<td>0.7</td>
<td>6.1</td>
<td>2.4, 9.4</td>
<td>N-7, N-1</td>
</tr>
</tbody>
</table>

Figure 5: Protonation and deprotonation of nucleotides
The figure 5 illustrates the location at which the protonation would occur for guanosine and adenosine. For guanine, the N-7 of the base is the location responsible for the binding of many molecules due to the lower pKₐ value.

2.2 Secondary and Tertiary Structure of Nucleic Acids
In 1953, Watson and Crick first proposed a correct secondary structure for DNA using X-
ray diffraction data of DNA fibers along with years of chemical data from various research groups. The DNA diffraction patterns were obtained by Rosalind Franklin while working at Cambridge University in England. Watson and Crick recognized that the DNA fiber diffraction exhibited a cross pattern typical of helical secondary structures. The structure consisted of two polymer chains coiled around a single axis. Initially, the intermolecular forces holding together the chains were not clearly understood. The body of chemical information that proved vital to understanding DNA structure came from Erwin Chargaff's analysis of the nucleotide composition of duplex DNA.

Figure 6: Watson and Crick Base-pairing

As shown below, A-T base pairing generates two hydrogen bonds, while G-C base pairing gives three hydrogen bonds. Watson and Crick proposed the idea of a double stranded helix and later through X-ray crystallography was able to determine distances of H-bonding with in the base pair.

Chargaff found that in each of the duplex DNAs analyzed, equality between the Adenine (A) and Thymine (T) and Guanine (G) and Cytosine (C) monomers was present. From this data and Pauling's (H-bonding), Watson and Crick were able to develop a model that proves that two stranded helix could be stabilized by hydrogen bonding between
bases on opposite strands if the bases were aligned in a particular position as shown in Figure 6.

Each hydrogen bonded base pair lies in a plane perpendicular to the helical axis. The DNA base pairs stack on top of one another due to a favorable attractive force between the aromatic ring systems. The base pairing and stacking forces result in a favorable free energy and stability of the DNA double-helical structure at physiological temperature.

2.2.1 Hydrogen Bonds and Stacking Forces that Stabilize the Double Helix

Noncovalent interactions account for the stability of the double-helix structure and the dynamics of the biological macromolecule. The negatively charged phosphoyl groups are all located on the outer surface, where they have a minimum effect on one another. The repulsive electrostatic interactions generated by these charged groups are often partly neutralized by interaction with cations such as Mg$^{2+}$, basic polyamines, and the positively charged side chains of chromosomal proteins. The surface of the helix B form DNA contains two grooves of unequal width known as the minor and major grooves. The surface of the helix is composed of the base pairs held together by the specific hydrogen bonds and also by favorable stacking interactions between the plans of adjacent base pairs.

Hydrogen bonding was originally believed to be the major force involved in the association of two DNA strands to form a double helix. Theoretical calculations of the energy of a hydrogen bond between macromolecules like DNA in aqueous solution
indicate that it is weaker than the energy of a hydrogen bond between two water molecules (5 kcal/mol). Therefore, it is not only the hydrogen bonding that is accounting for the stability of the helix, but it is the additive contributions of hydrogen bonding and other interactions that stabilize DNA helices.

Base stacking is additive, diffusion controlled, and stabilized by weak interactions. A study on association of bases and nucleosides in aqueous solution using osmometric techniques led early to the conclusion that vertical base stacking occurs and goes beyond the dimeric state. Stacking interactions involve dipole-dipole interactions. π-electron systems, dipole-induced dipole movement and London dispersion forces (van der Waals forces) along with hydrophobic forces in aqueous solutions. Base stacking is stabilized by entropic contributions due to the individual solute molecules aggregating, and reducing the number of solute-solvent contacts. This hydrophobic interaction contributes to the stabilization of other nucleic acid secondary structures such as Z-DNA and A-DNA. Hydrophobic interactions alone cannot explain some effects related to specific bases, such as the observation that purines stack better than pyrimidines and methylation enhances stacking. These individual properties are related to the electronic systems of the bases and are mainly due to London dispersion forces and to interactions between dipoles. As London stated, the principal contribution to the van der Waals attractive forces between atoms in close proximity resides in electrokinetic interactions between the systems. Dipoles created in one group of atoms polarize the electronic system of neighboring atoms or molecules, inducing parallel dipoles which attract each other, and which are independent of temperature. The bases possess permanent dipole moments: the
two electronic effects, London dispersion and permanent dipole, combine and lead to appreciable effects which are more pronounced in purine than in pyrimidine bases. All of these interactions contribute to the stability of nucleic acid formation and contribute to the stability of their secondary structures.\(^3\)

2.3 Alternative Nucleic Acid Structures

At the time Watson and Crick proposed their model, two quite different x-ray diffraction patterns had already been obtained for DNA, indicating that the molecule can exist in more than one form. We now know that the same base pairing arrangement found in B-DNA is found in all naturally occurring double-helix structures including A-DNA and Z-DNA.

Figure 7: Sugar Pucker

There are four sugar pucker conformations for the sugars in nucleotides. This conformational change in the sugar pucker is responsible for many of the various structures DNA can assume.
The flexibility in the furanose ring of the sugar and the degree of freedom generated by several rotatable single bonds per residue—six in the sugar phosphate backbone and one in the C-1' N-glycosidic linkage—lead to considerable variations in the conformations adopted by the double helix structure. Four puckered conformations for the sugar, with small differences in stability are shown in Figure 7.

In A-form DNA the furanose rings are in the C-2' endo conformation, but when hydration occurs the double helix pushes even closer together and the furanose rings change their pucker to the C-3' endo conformation. Therefore B-form DNA is prepared under conditions of high humidity while A-form DNA is prepared under low humidity. This is why the proper solvent is essential for the formation of a double helix. An aqueous solution with a millimolar concentration of a monovalent or divalent cation as found in in vivo provides an optimum environment for B-DNA formation. Extensive solvent hydrogen bonding and cation condensation allows DNA to form very ordered secondary structures in solution.26

2.3.1 Z-Form DNA

The most striking conformational variant observed for a DNA double helix with Watson-Crick base pairing is referred to as the Z-form. This structure was first detected by Alex Rich and his co-workers in 1979 for the deoxyoligonucleotide d(CpGpCpGpCpGp), which crystallizes into an antiparallel double helix with a left-handed rather than a right-handed helix. Years before Rich observed the Z-form crystallographically, Pohl and Jovin (1972), suggested a left-handed helix or Z-DNA on the basis of ultraviolet and
Circular Dichroism (CD) changes at high salt buffers (2.5 M NaCl, 1.8 M NaClO₄, 0.7 M MgCl₂).²³ Pohl and Jovin reported an intramolecular isomerization of double-stranded oligomer (dCdG) and poly (dCdG). The UV absorption spectrum showed a red shift in the λmax and a change in the A250/A280 ratio from 8.5 in low salt to 3.2 in high salt. It was observed that the low salt CD/ORD spectrum was nearly inverted in high salt and these spectra were designated as the R and L forms. Each electrolyte produced the same transition only at different concentrations. Overlays of CD/ORD spectra taken while titrationing in salt showed a transition from R → L with the presence of isotropic points indicating an all or non-transition between two species. Plots of specific optical rotation vs percent NaCl at 280 and 310 nm give a sigmoidal plot indicating a cooperative transition. The changes in the high salt UV and CD/ORD spectra were completely reversible upon dialysis or dilution to low salt concentration. It was suggested that the spectral changes observed were due to helical changes in the DNA molecule.

The Z form is a considerably slimmer helix than the B form and contains 12 base pairs per turn rather than ten. In the Z form, the planes of the base pairs are rotated approximately 180° with respect to the helix axis from their orientation in the B form. The flipping of the base pairs involves different conformational changes in the G and C residues the base is rotated 180° about the glycosidic bond, a change resulting in a transition from the anti conformation found in B-DNA to the syn conformation.
Figure 8: Syn- versus Anti- Conformation
Rotation around the glycosidic bond between the sugar and nucleotide can arrange the molecule in a syn- or an anti- conformation.

The anti conformation of the nucleotide found in B DNA has less steric crowding than the syn conformation but it is far easier for a purine nucleotide to adopt the syn conformation than the pyrimidine nucleotide. In Z DNA, cytidine remains in the anti conformation, and the flipping of the base in going from the B to Z conformation involves rotation of the entire base while maintaining the anti conformation. This effect causes the sugar phosphate backbone to follow a zig-zag shape. The Z-form of DNA requires that the sequence of purine and pyrimidine bases be strictly alternating. Other
Arrangements of T and G and A and C have been found to adopt the Z conformation, but A and T DNA sequence do not. Therefore, the repeating unit in Z-DNA is a CpG step. The base stacking in the CpG steps differs from the stacking in the GpC steps. CpG are not stacked directly on top of each other like the GpC steps. In the GpC steps the guanine are stacked over the cytosine of the same chain below them while the cytosines are stacked over the guanines of the same chain below them.

Figure 9: Differences in Stacking between B- and Z-DNA
This illustration is a view looking down the helix of B- and Z-DNA. The angle of the CpG basepair under left-handed conditions is much less than the CpG basepair in B-DNA. On the contrary, the angle of the GpC, for the Z-DNA is greater than the angle seen in B-DNA for the same basepair.

Z-DNA has a single deep groove, which extends down to the helical axis. The deep
groove in Z-DNA is formed from the B-DNA minor groove upon conversion from B → Z DNA while the B-DNA major groove converts into the convex outer surface of Z-DNA. While the base pairs in B DNA are located at the bottom of the major groove, the base pairs in Z-DNA make up the convex surface of the helix. The imidazole rings of the guanine expose the N7 and the C8 while in the Z conformation but are shielded in the B conformation. A number of structural parameters change as B DNA converts to Z DNA and A DNA. These differences in structural parameters are summarized in the table below.

Figure 10: Structure of A-, B-, and Z-DNA
The structure of A-, B-, and Z-DNA are shown here for an eight base pair oligonucleotide sequence. The view is a side view, outlining the phosphate backbone (pink).

Z-DNA
B-DNA
A-DNA

A factor that effects the ability of DNA to adopt the Z conformation is the methylation of the 5 position of cytosine residues. Behe and Felsenfeld studied the effects on the B → Z transition upon methylating the cytosine. The transition midpoint of the B-to-Z transition
for poly(dG-m3' dC) occurs at 0.7 M NaCl or at 0.6 mM MgCl2. The transition occurred at a much lower ionic strength than Pohl and Jovin reported for poly(dGdC). More recently, Cherrak et al. (2003) discovered methylating the 8 position of the guanine (m3'G) promotes left-handed helix under physiological salt conditions.

Table 2: Structural Features of Ideal A-, B-, and Z-DNA.

<table>
<thead>
<tr>
<th>Helix Sense</th>
<th>B DNA</th>
<th>Z DNA</th>
<th>A DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residues per turn</td>
<td>10</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Rise per residue</td>
<td>3.4 Å</td>
<td>3.7 Å</td>
<td>2.55 Å</td>
</tr>
<tr>
<td>Helix pitch</td>
<td>34 Å</td>
<td>45 Å</td>
<td>28 Å</td>
</tr>
<tr>
<td>Base-pair tilt</td>
<td>6°</td>
<td>7°</td>
<td>29°</td>
</tr>
<tr>
<td>Rotation per residue</td>
<td>36°</td>
<td>-60°</td>
<td>33°</td>
</tr>
</tbody>
</table>

Glycosidic Conformation

<table>
<thead>
<tr>
<th>Sugar Pucker</th>
<th>Deoxycytidine</th>
<th>Deoxyguanosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti</td>
<td>Anti</td>
<td>Anti</td>
</tr>
<tr>
<td>Syn</td>
<td>Anti</td>
<td>Anti</td>
</tr>
</tbody>
</table>

Using NMR and circular Dichroism, the DNA sequence, d(Cm3'GCGGCGCGCG), will adopt a left-handed helix at 0.1 M NaCl, and under physiological pH. Another Z-DNA
inducer is ethanol. The unmethylated polymer poly(dGdC) requires 60% ethanol in order to observe a B-to-Z transition, while in the methylated polymer poly(dG-m^3dC) a transition midpoint was reached at 20% concentration of ethanol. Using the trivalent cobalt hexamine, only 20 mM and 5 mM [Co(NH_3)_6]^{3+} concentrations were needed to reach the midpoint for the methylated and unmethylated polymers. Many other multivalent cations effect the formation of Z-DNA, such as calcium, nickel, and aluminum. Other potential stabilizing factors for Z-DNA are naturally occurring polyamines, spermidine and spermine. Behe and Felsenfeld (1981) studied the effects of polymer DNA with spermine and found that millimolar concentrations of spermidine and spermine cause Z-DNA to be stabilized.

To summarize the three major stabilizing factors of Z-DNA to be; (1) the relatively high levels of unconstrained negative supercoiling, (2) the presence of Z-DNA forming DNA sequences (methylations of the 5 position of cytosine), and (3) the presence of Z-DNA stabilizing multivalent cations (i.e. NaCl, Cobalt (III) Hexammine, spermidine).

2.4 Proposed Function of Altered DNA Structures

2.4.1 Overview

Biological information is encoded by DNA in two different ways; (1) through nucleotides: it specifies the composition of proteins, (2) through its shape: DNA can provide information that is used directly or indirectly by a variety of macromolecules to regulate the assembly of cellular machineries. DNA is capable of assuming many shapes, such as quadruplexes, left-handed Z-DNA, and hairpin-type structures. All of these
conformational changes are depended on sequence and environmental effects. The biological functions of these altered DNA structures are currently not fully understood but much advancement has been done relating the function to the structure.

2.4.2 Quadruplex

G-quadruplex is a unique structure, formed by Hoogsteen-style base pairing between four guanines and involving chelation of a metal cation. G-quadruplexes can be formed by intramolecular folding of guanine-rich sequences or by intermolecular association of two or four sequences.

Figure 11: G-Quartet structure

G-quadruplex structures are involved in Hoogsteen base pairing, and are formed by intramolecular folding, either with two or four sequences.

![G-Quartet structure](image)

The G-quadruplexes are formed in the telomeres that constitute the extremities of the chromosomes and are important to the integrity of the genome. Telomeric DNA consists of tens of thousands of repeats of a short sequence in which one strand shows a preference for guanines. This G-rich strand has a single strand overhang, which can form
G-quadruplexes. Normal somatic cells progressively lose telomeric repeats during successive cycles of cell division. Human telomerase has been proposed as a novel and potential highly selective target for antitumor drug design because of its specific expression in tumor cells.

The human sequence TTAGGG forms G-quadruplex structure very readily in humans and its structure is a potent telomerase inhibitors that can cause mutations in the genetic sequence leading to cancer and other genetic disorders (i.e. Cystic fibrosis). The geometrical features of G-quadruplex DNA allow specific recognition by small ligands that can intercalate or stack on the terminal G-tetrad, or even bind in the grooves.

Possible biological roles for G-quartets have only been given serious consideration with the more recent discoveries that quadruplexes composed of G quartets are formed by DNA oligomers with repeat sequences from chromosomal telomeres and other biologically relevant sources. G-quadruplex formation and function in vivo is still lacking, but the discovery that proteins associated with chromosomes telomeres can act as molecular chaperones for the formation of G quadruplexes is highly suggestive of the presence of G quadruplexes in vivo.

2.4.3 Left-handed Z-DNA

Z-DNA is very difficult to directly demonstrate but a number of early observations clearly suggest its existence. Z-DNA is highly polyclonal and immunogenic and antibodies can be made to recognize left-handed conformation. The first proof that Z-
DNA existed \textit{in vivo} were from sera taken from patients with autoimmune diseases, such as lupus erythematosis, showing these patients produced antibodies that were specific to Z-DNA.\textsuperscript{47}

Recent studies have shown that gene mutations are involved in the pathology of neurological disorders. CCG repeats cause genetic instability and are localized at the 5' end of the non-coding regions of the FMRI gene in fragile X syndrome.\textsuperscript{48} Studies have shown that aluminum (Al) levels were elevated in serum samples of fragile X syndrome and its interaction with the (CCG)$_n$ repeat causes the B-DNA to convert to a Z-DNA conformation at low concentration (10$^{-3}$ M) of aluminum.\textsuperscript{49}

Another study using Alzheimer patients, showed evidence for altered DNA conformation in the hippocampus Alzheimer’s disease affecting the brain.\textsuperscript{50} The study showed that severely affected AD DNA showed a typical left-handed Z-DNA conformation while the young, healthy, and aged brain have a typical right-handed B-DNA conformation. Elevated aluminum levels in the patients, causing typical right-handed B-DNA to change to a left-handed Z-DNA conformation, cause both disorders. Aluminum plays the role in switching off the gene by inducing the helical transition from B to Z-DNA. Ultimately it’s the DNA topology in the repeats that play the important role in the expression of the gene responsible for the disorder. With the advancement of technology, scientists are now pursuing the idea that Z-DNA may play a more important role in other disorders.\textsuperscript{51} Understanding the structural transition of different nucleic acid conformations will give scientists the ability to better establish the link between gene and disease.
2.5 Biophysical Studies of Nucleic Acid Conformational Changes

2.5.1 Overview

The thermodynamic properties associated with the conformational change from a right-handed B-DNA to a left-handed Z-DNA has been studied using different types of Z induced molecules (i.e. Mg\textsuperscript{2+}, Na\textsuperscript{+}, Ni\textsuperscript{2+}, spermidine).\textsuperscript{52,53} Each study results in a different value obtained for the B-to-Z transition. Of all the studies done, all but one have been done using a model dependent method (i.e. van’t Hoff Analysis) to determine the enthalpy of the B-to-Z transition, ΔH\textsubscript{B-Z}.

2.5.2 Pohl and Jovin - Van’t Hoff Analysis\textsuperscript{14}

Pohl and Jovin (1972) reported that varying the temperature for poly (dG-dC) in 2.5 M NaCl between 20° and 50° C did not change the ORD spectrum. The enthalpies of the B-to-Z transition were calculated by van’t Hoff Analysis. The enthalpy, ΔH\textsubscript{RL} = RT\textsubscript{d}(ΔlnK\textsubscript{AT}/ΔT) values calculated were zero (±1 kcal/mol). Therefore, the B-to-Z transition Pohl and Jovin studied is entropically driven. More recent studies, however, have shown that the transition is more enthalpically driven than previously assumed.\textsuperscript{54,55}

2.5.3 Klump - Raman Spectroscopy\textsuperscript{48}

Klump (1993) studied the energetics of Z-DNA formation in poly d(AT), poly d(GC), and poly d(AC) poly d(GT) in the presence of Na\textsuperscript{+}, Mg\textsuperscript{2+} or Ni\textsuperscript{2+}. With the use of Raman spectroscopy, UV spectroscopy, and adiabatic differential scanning calorimetry (DSC), Klump investigated the role of temperature in driving the B-to-Z and the Z-DNA to coil

24
transition. The results show a hypochromic shift at around 260 and 280 nm for the
differential UV spectra, and thus a conformational change to the left-handed
conformation was observed as temperature is increased in the presence of 100 mM Ni²⁺
and 5 M NaCl for the poly d(AT). The left-handed conformation was confirmed using
Raman spectroscopy by the drastic decrease of the band at 680 cm⁻¹ and the appearance
of a new band at 828 cm⁻¹ due to the purine bases in the syn conformation upon inversion
of the helix. The true transition enthalpy ΔH for the B-to-Z transition for the poly d(AT)
was determined to be 4 ± 0.4 kcal/mbp by DSC. The transition entropy ΔS at 55° C Tₘ
were calculated from the ratio of ΔH/Tₘ to be 12.2 cal/mbp and the ΔG value was
calculated to be 0.366 kcal/mbp. The ΔH determined by calorimetric methods is shown in
table 3 for poly d(AT), poly d(GC), poly d(AC) and poly d(GT).

Previously, Klump (1986) and Loffler (1985) studied another set of oligomers, poly (dG-
dC) and poly (m'dG-dC), and found no detectable difference between the enthalpies of
transition for the B and Z forms, and obtained a value of 11.6 kcal/mol at 120.8° C for the
left handed helix to coil transition. This discrepancy between their past results and
current work could be related to more advanced calorimetric methods, and different
buffer conditions.
Table 3: Thermodynamic results for the B-to-Z transition.

Klump, et al. used Raman Spectroscopy, UV/VIS spectroscopy and Adiabatic Differential Scanning Calorimetry to calculate thermodynamic values for the B-to-Z conformational change.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Transition</th>
<th>Na⁺ (M)</th>
<th>Ni²⁺ (M)</th>
<th>Tm (°C)</th>
<th>ΔH (kcal/mol)</th>
<th>ΔS (cal/mol K)</th>
<th>ΔG° (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly d(AT)</td>
<td>B-helix/coil</td>
<td>0.1</td>
<td>-</td>
<td>54</td>
<td>7.46</td>
<td>22.8</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>Z-helix/coil</td>
<td>5.0</td>
<td>0.15</td>
<td>71</td>
<td>10.50</td>
<td>30.5</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>helix/helix</td>
<td>5.0</td>
<td>0.15</td>
<td>42</td>
<td>4.0</td>
<td>12.7</td>
<td>0.22</td>
</tr>
<tr>
<td>poly d(GC)</td>
<td>B-helix/coil</td>
<td>0.01</td>
<td>-</td>
<td>97</td>
<td>9.50</td>
<td>25.8</td>
<td>1.85</td>
</tr>
<tr>
<td></td>
<td>Z-helix/coil</td>
<td>1.5</td>
<td>0.04</td>
<td>110</td>
<td>11.04</td>
<td>28.7</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>helix/helix</td>
<td>1.5</td>
<td>0.04</td>
<td>85</td>
<td>2.0</td>
<td>5.6</td>
<td>0.34</td>
</tr>
<tr>
<td>poly d(AC)</td>
<td>B-helix/coil</td>
<td>0.1</td>
<td>-</td>
<td>83</td>
<td>8.56</td>
<td>24.0</td>
<td>1.39</td>
</tr>
<tr>
<td>poly d(GT)</td>
<td>Z-helix/coil</td>
<td>7.0</td>
<td>0.04</td>
<td>99</td>
<td>11.3</td>
<td>30.4</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>helix/helix</td>
<td>2.0</td>
<td>0.04</td>
<td>73</td>
<td>3.1</td>
<td>9.0</td>
<td>0.43</td>
</tr>
</tbody>
</table>

2.5.4 Chairies and Sturtevant – Differential Scanning Calorimetry

Chairies and Sturtevant (1986) determined the thermodynamics of the B-to-Z transition in poly (m⁶dG-dC) using DSC, temperature dependent absorbance, and Circular Dichroism (CD) spectroscopy. Chairies found three distinct peaks in the thermograms of the poly (m⁶dG-dC) in a sodium phosphate buffer containing 50 mM NaCl and 1.0 mM MgCl₂. The first peak centered at 38.2° C, from the reversible B-to-Z transition, the second is the transition centered at 53.6° C from the Z-form to an unknown structure, and the third peak is observed at 120.9° C and is due to the left-handed helix to coil transition. The
enthalpic contributions of the B-to-Z transition were calculated by planimeter integration of the DSC curve, and the van’t Hoff enthalpy, $\Delta H_{\text{vH}}$ in cal/mol, was calculated using the expression:

\[
\Delta H_{\text{vH}} = -A R T_m^2 \left( C_{\text{vH,app}} / \Delta h_{\text{cal}} \right),
\]

where the value of $A$ appropriate for a simple two state process is 4.00, $R$ is the gas constant, and $T_m = T_a + 273.15$ is the absolute temperature at which the apparent excess specific heat has its maximal value $C_{\text{vH,app}}$. The results reported are a direct estimation, by DSC, of the enthalpy of the B-to-Z transition in poly (m$^3$dG-dC). In BP buffer and at a heating rate of 0.25 K/min, the transition is characterized by a calorimetric enthalpy, $\Delta H_{\text{cal}} = 0.61 \pm 0.07$ kcal/mol bp and a van’t Hoff enthalpy, $\Delta H_{\text{vH}} = 68 \pm 7$ kcal/mol. Their results indicate that the B-to-Z transition is enthalpically driven, in contrary to previous studies that the process is entropically driven. Previous estimates for the enthalpy of the B-to-Z transition have been based on van’t Hoff analysis of temperature dependent optical or NMR data assuming a two state model. The thermodynamic parameters for temperature-dependent B-to-Z transitions in DNA polymers and oligomers obtained from van’t Hoff analysis of spectroscopic and DSC data are presented in Table 4.
Table 4: Previously published results which compare various techniques used to obtain the energetics related to the B-to-Z, conformational change.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Tm (°C)</th>
<th>ΔH_{VH} (kcal/mol)</th>
<th>ΔH_{mol} (kcal/mol bp)</th>
<th>NaCl m/M</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly d(G-C)</td>
<td>20-50</td>
<td>9</td>
<td>-</td>
<td>2250</td>
<td>Pohl and Jovin 1972; Pohl 1982; Marky et al. 1982</td>
</tr>
<tr>
<td>Poly d(G-C)</td>
<td>140-200</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>Hill &amp; Maestre 1984</td>
</tr>
<tr>
<td>Poly d(m^2G-C)</td>
<td>32.7</td>
<td>68</td>
<td>0.61</td>
<td>50</td>
<td>Chaires; Sturtevant 1986</td>
</tr>
<tr>
<td>Poly(dG-m^2dC)</td>
<td>40</td>
<td>-</td>
<td>1.0</td>
<td>720</td>
<td>Behe et al. 1985</td>
</tr>
<tr>
<td>Poly d(A-m^2C) d(G-T)</td>
<td>57</td>
<td>220</td>
<td>1.2</td>
<td>5390</td>
<td>Jovin et al. 1983; L.M. McIntosh and T.M. Jovin</td>
</tr>
<tr>
<td>Poly d(A-br^2C) d(G-T)</td>
<td>40</td>
<td>195</td>
<td>0.9</td>
<td>4430</td>
<td>Jovin et al. 1983; L.M. McIntosh and T.M. Jovin</td>
</tr>
<tr>
<td>d(CGm^2CGCG)</td>
<td>40</td>
<td>-</td>
<td>-1.25</td>
<td>1800</td>
<td>Cavailles et al. 1984</td>
</tr>
<tr>
<td>d(m^2CGCGm^2CG)</td>
<td>29</td>
<td>-</td>
<td>-1.33</td>
<td>2000</td>
<td>Tran-Dinh et al. 1984</td>
</tr>
<tr>
<td>d(m^2CGm^2CGm^2CG)</td>
<td>61</td>
<td>-</td>
<td>-1.17</td>
<td>100</td>
<td>Feigou et al. 1984</td>
</tr>
</tbody>
</table>

2.5.5 Feigou – Nuclear Magnetic Resonance (NMR)

Feigou et al. used NMR spectroscopy in order to calculate the thermodynamic parameters associated with the B-to-Z transition in the presence of methanol at various temperatures (20°, 30° and 40° C). They used the oligomer, d(m^2C-G)_n, a short molecule to ensure that the conformation observed would be all Z or none. There results conclude that the B-to-Z transition is temperature dependent in that the percentage of Z-DNA decreased with increasing temperature. They constructed Arrenius plots of ln P_T vs 1/T, where ln
$P_\theta/P_2$ is the fraction of B vs Z, was calculated to be 6-8 kcal/mol depending on the temperature the transition occurs at. An entropy change of 21-27 cal/mol K was observed at each of the temperatures. The results conclude that the percentage of Z-DNA increases in a cooperative manner as a function of methanol concentration and decreases with increasing temperature.

2.5.6 Irikura – Molecular Modeling and NMR

Irikura calculated the entropy of the B-to-Z transition by looking at vibrational analysis. The vibrational entropy associated with the B-to-Z transition was found to be 22 cal/mol K for the d(C-G) oligomer, which is the same results obtained by Feigon and others who performed NMR spectroscopy to determine entropy of the transition.

2.6 Small metal complexes that induce structural changes in nucleic acid

2.6.1 Overview

A number of small metal complexes have been shown to interact and cause structural changes in nucleic acids. As previously described, cobalt (III) hexammine is a small metal complex consisting of six ammine substituents. The interaction associated with $[\text{Co(NH}_3)_3]^{3+}$ causes right-handed B-DNA to invert to a left-handed Z-Form causing different structural characteristics possibly interfering with biological activity. This interaction is the basis of future work being done to look at other small metal complexes that potentially bind to DNA, interfering with transcription or the functionality of certain DNA sequences.
The development of new molecules that bind selectively to nucleic acids expands the range of potential pharmaceutical agents whose mode of bioactivity is through interaction with DNA or RNA. Transition metal complexes such as [Co(NH₃)₆]²⁺ are perfect candidates for therapeutical agents because of their ability to alter their structure and their high reactivity. These agents provide the ability to selectively bind to significant portions of the nucleic acid surface due to the large size of these metals. Another factor that can potentially increase the ability of these metal complexes to bind to DNA is the positive charge associated with transition metals. The charged species of the metals provide an excellent companion for the negatively charge phosphate backbone of the DNA. A number of these small metal complexes have been shown to interact with the major groove N7 of the guanine, and other locations throughout a DNA strand. Whether they are binding electrostatically or covalently with the DNA, these complex-nucleic acids molecules have shown to be very promising in the field of medicine and in the interest of scientists.

2.6.2 cis-Dichloro Diammine Platinum (II)

A transition metal that has gained notoriety for its ability to treat cancer patients is cis-platin. Its effectiveness is directly connected to the covalent modification of DNA to the complex. The binding associated with cisplatin causes a local intrastrand crosslinking at GpG or GpA sites. When this intrastrand crosslinking occurs, the duplex is bent and alters the DNA’s reactivity with high mobility group (HMG) proteins, while still allowing the DNA to be repaired. When small molecules such as cisplatin interact with DNA, various modes of interaction must be considered. Another factor that must be
considered is the sequence specificity and the conformational alterations.

There are several modes of covalent Pt attaching to the DNA bases. The first interaction is known as monofunctional.\textsuperscript{65} This occurs with the release of one of the chloro groups and covalently binding to the N7 of the guanine of either strand as depicted in Figure A. The second mode of binding is known as interstrand crosslinking and occurs when both chloro groups dissociate forming two bonds between two bases of two separate strands in the same helix. The third mode of binding is known as intrastrand, and this occurs when the two chloro groups are released and two bonds from two adjacent bases on the same strand are formed.

The conformational changes due to DNA-metal interactions, determined by gel electrophoresis, enzymatic digestion, RP-HPLC, and NMR led to discovery of the guanine intrastrand adducts [Pt(NH\textsubscript{3})\textsubscript{2}(d(GpG))], [Pt(NH\textsubscript{3})\textsubscript{2}(d(ApG))], [Pt(NH\textsubscript{3})\textsubscript{2}(d(GMP))\textsubscript{2}] and the monofunctional adduct [Pt(NH\textsubscript{3})\textsubscript{2}(d(GMP))].\textsuperscript{66} The results suggest that cisplatin preferentially binds to guanine rich sequences. The findings also suggest that intrastrand crossing favors a d(GG)\textsubscript{2} sequence while intrastrand crosslinking favors d(GC)\textsubscript{2} sequences.\textsuperscript{67}

Thermal denaturation is a common method used to determine mode of binding in nucleic acids. DNA melting or thermal denaturation, measures the absorbance at a particular wavelength as the temperature is increased, revealing changes in the duplex to single strand transition temperature (T\textsubscript{m}). Therefore, a decrease in the T\textsubscript{m} value when a metal
complex is introduced is believed to be the result of intrastrand complexation of the metal, while an increase in the Tm value is believed to be the result of interstrand crosslinking.

2.6.3 Cobalt (III) Molecules

Pentaammineaquocobalt (III) [Co(NH3)5OH2]3+ is a transition metal, low spin d6 complex, studied by our lab. Shearay and Hicks reported that pentammineaquocobalt (III) interacts with DNA irreversibly through an intrastrand crosslinking. [Co(NH3)5OH2]3+ lowers the d(GG) duplex stability upon binding suggesting that the binding is between the complex and DNA bases and is covalent. The enzyme digestion studies show that, due to the localized binding caused by [Co(NH3)5OH2]3+, the cutting efficiency is decreased for BamH1 at the 5'-GGATCC-3' restriction site. This suggests that [Co(NH3)5OH2]3+ coordinates to the -GG- sites of guanine rich sequences. The complex binds to guanine bases by a combination of coordination covalent and hydrogen bonding to the GN7 and O6 of the guanines with the loss of the aquo group. Atomic Absorption results show a delayed binding of the complex to d(GC) sites that may involve a metal complex dimerization before interacting with this DNA sequence. When the metal complex dimerized, the reactivity of Hind III is unhindered for the 5'-AAGCTT-3' restriction site due to intrastrand crosslinking. The data acquired for [Co(NH3)5OH2]3+ reacting with guanine rich sequences play an important role in understanding the thermodynamic relationship between cobalt-DNA adducts. Cobalt (III) complexes possessing labile groups show surprisingly interesting DNA binding properties, therefore further investigation using other cobalt (III) complexes with a labile
ligand could help understand the nature of the cobalt-DNA adducts.

2.7 Metal complexes involved in Biological activity

2.7.1 Overview

Biochemistry involves elaborate organic molecules, and some simple ones, along with the many organic reactions, but biochemistry is not only organic chemistry. Metallic elements play a very important role in biological activity, without these metals, life would not exist. Obvious elements essential for all forms of life are Na, K, Mg, Ca, C, N, O, P, S, and Cl, while others have trace amounts found in the body (i.e. Fe, Cu, Zn), and some have specific biological role such as Co, Ni, Li, B, F, Si, V, Cr, and I. The role played by some of these metallic elements in biochemistry is for the metal ion to bind to an enzyme to perform the necessary task for that enzyme giving rise to what is known as a metalloenzyme. Other enzymes such as hemoglobin, myoglobin, cytochrome, and other respiratory proteins require metal as a normal part of their structure. These metal elements prove to be essential for daily biological functions in most living things. Cobalt plays an important biochemical role in vitamin B12. This vitamin is a cofactor for a number of enzymes that catalyze the reaction illustrated in Figure 12.

Figure 12: Cobalt's biochemical role in vitamin B12

\[
\begin{align*}
&\text{R} \quad \text{H} \\
&\text{C} \quad \text{C} \\
&\text{H} \quad \text{H} \\
\text{H} \quad \text{H} \\
\end{align*}
\]

The human body contains 2 to 5 mg of vitamin B12 and its derivatives in the liver. The
molecule of vitamin B₁₂ is bonded at the 5' carbon atom of the deoxyribose moiety and is attached to cobalt forming a cyanocobalamin as illustrated in Figure 12. By means of the Co-C hemolytic bond cleavage, a formal Co²⁺ form of the coenzyme and the adenosyl radical is formed. Once this step has occurred, the adenosyl radical accepts a hydrogen atom from the substrate to generate the substrate radical. The enzyme causes this to rearrange to the product radical and this then abstracts a hydrogen atom from RCH₂ to give product and the coenzyme is recovered, ready for another cycle. One important aspect to point out is that Co²⁺ can be substituted by Zn²⁺ in a number of enzymes without change in activity but is not found naturally.

2.7.2 Transition Metal's Involved in Biological Activity

Another important transition metal that has gained popularity for its ability to activate folding in nucleic acids binding proteins ("zinc fingers") is zinc (II) (Zn). The nucleoacapcid (NC) binding protein of human immunodeficiency virus (HIV-1) is a zinc-activated protein (ZAP). The Zn binding and the associated folding is required for activity of the viral gag proteins which control RNA packaging and stabilization in the NC of HIV. The Zn binding sites provide a novel target for HIV therapy, which can be carried out by interfering with metal binding and associated folding. Isothermal Titration Calorimetry (ITC) studies from McLendon and Chang (1999) show a strong binding affinity ( log Keq = 12.0, log Kznc ~ 15.0) for Zn (II) and Co (II) metals to NC's. Since metal binding and folding are formally thermodynamically coupled, understanding of folding to the active state requires thermodynamic characterization of metal binding.

Other transition metals that are interesting complexes to study and have important
biological traits are iron, and copper, two of the most abundant metals in the human body. Their role is to assist in the transport of oxygen from the lungs to the rest of the body, and aid with electron transport.74

2.8 Project Aims, Purpose, and Rationale

2.8.1 Overview and Purpose

The purpose of this study is to investigate various cobalt (III) complexes possessing labile groups as potential drug candidates for nucleic acid research. The project entails investigating the thermodynamic properties associated with the binding of NaCl, \([\text{Co(NH}_3)_6]^{3+}\), \([\text{Co(NH}_3)_2\text{Cl}]^{2+}\), \(\text{cis-}[\text{Co(NH}_3)_2\text{Cl}_2]^{2+}\), \([\text{Co(NH}_3)_3\text{(OH}_2)_2]^{3+}\), and \(\text{cis-}[\text{Co(NH}_3)_3\text{(OH}_2)_2]^{3+}\) with two self-complementary DNA oligomers, \(d\text{(CG)}_4\) (Z8A) and \(d\text{(mCmCG)}_4\) (Z8M). These experiments were designed to give additional insight into the nature of the cobalt-DNA coordination and how its coordination alters the reactive properties of these two oligomers. The cobalt-DNA complexes were reacted and compared by several methods. The thermodynamic properties of the duplex to single strand equilibria were evaluated by UV spectroscopy and differential scanning calorimetry (DSC). Circular dichroism, native-PAGE, UV/VIS spectrosnopy, and \(^{31}\text{P}\) NMR monitored the structural changes due to the binding of these transitions metals with DNA. Isothermal titration calorimetry allowed the initial ion binding energetics to be calculated by a model independent method. Using all of these methods to understand the changes brought about from cobalt reacting with DNA can lead to the discovery of specifically tailored DNA therapeutic agents via other transition metal complexes.
2.8.2 The Cobalt (III) Complexes Investigated

The cobalt (III) complexes studied are [Co(NH₃)₆]³⁺, [Co(NH₃)₅Cl]²⁺, cis-[Co(NH₃)₅Cl₂]⁴⁺, [Co(NH₃)₅(OH₂)]³⁺, and cis-[Co(NH₃)₆(OH₂)]⁴⁺. The cobalt (II) transition metal serves as an excellent model for other transition metals that could be used for therapeutic agents. Each complex has one or more substituents that possess a labile ligand. Our report investigates how the ligand on the cobalt complex may affect the type of binding associated with DNA-cobalt complexes. Each cobalt (III) complexes has a different ligand that will react differently with the DNA duplex depending on the labiality of the ligand, and the sequence of the DNA. A trend pertaining to the labiality of the ligand’s can be generated (diaquo > dichloro > aquo > chloro > ammine). Therefore, depending on how labile the ligand will affect how the cobalt complex reacts with DNA (i.e. intrastrand, interstrand, electrostatic).

2.8.3 The Relevance of the Sequences Studied

The sequences d(CG₄) and d(5-meCG)₄ were studied for this project to compare two similar but very different molecules. For simplification purposes, d(CG₄) will be known as Z8A, and d(5-meCG)₄ will be known as Z8M. Both DNA molecules possess the ability for induced conformational changes by environmental effects. They both have alternating purine-pyrimidine bases, with the same number of hydrogen bonding, similar molecular weights, and virtually identical structures. The differences between these two sequences are that Z8M has a bulky methyl group on each of the cytosines at the 5' position. This large bulky group plays an important role in causing molecules to undergo structural changes with little amounts of an inducer. The inability of Z8A to undergo a structural
change, and the ability of Z8M to undergo a structural change is the main reason for choosing these two sequences. Our lab has been involved in using Z8M in many other experiments, and the information gained from this project could be very beneficial to understanding conformational changes in high GC rich sequence.

2.8.4 Characterization of the Structural Properties

The structure of the DNA oligomers Z8A and Z8M were studied with and without the addition of cobalt (III) complexes. The structural changes induced by the cobalt (III) complexes can be monitored by circular dichroism (CD), native gel electrophoresis (native-PAGE), UV/VIS spectroscopy and nuclear magnetic resonance (NMR). Secondary structures of DNA are defined by the number of base pairs per turn, the rise per base pair, the handedness of the helix and the distance of each base from the helix axis. The ability for DNA to assume different secondary structures is known as polymorphism, and is dependent on sequence and environment effects. Circular dichroism spectroscopy is sensitive to these changes and can therefore be utilized to monitor the changes in DNA secondary structure. Circular dichroism can detect changes to any of the base-base parameters, such as the base-to-base distance, or stacking interactions, brought on by changes in the DNA that correspond to the change in the CD spectrum.

The CD spectra of each DNA-cobalt complex were measured over a range of cobalt concentration, and as a function of temperature. The CD spectra of the DNA at various cobalt concentrations were compared to a reference spectrum to determine if a transition
exists and determine the structure observed. The CD data is a weighted average of the relative structure of the DNA and the cobalt-DNA duplex.

Performing concentration dependence experiments provide the melting temperature of the DNA at various concentrations which allows the structure of the DNA-cobalt complex to be determined. The arrangement of the metal center and the hydrogen bonds of the DNA can take on multiple transitions such as unimolecular, bimolecular, and tetramolecular transitions. A method developed by Marky and Breslauer can be utilized to determine the molecularity of the transition with the following equation, where \( n \) is the molecularity, \( R \) is the universal gas constant, and \( C_i \) is the total oligomer concentration. By plotting \( 1/T_m \) vs \( \ln C_i \), a plot with a slope equal to \( (n-1)R/\Delta H \) and a y-intercept equal to \( \Delta S/\Delta H \) is obtained. A unimolecular transition will give a slope equal to \( 0 \) and all other molecularities will give a slope with some negative value.

Equation 2: \[ 1/T_m = [(n-1)R/\Delta H]\ln C_i + [(\Delta S -(n-1)R)\ln 2 + R\ln(n)]/\Delta H \]

Native Polyacrylamide gel electrophoresis (native-PAGE) was utilized to further clarify the nature of the structures formed when cobalt (III) molecules bind DNA. Native gel electrophoresis separates on the basis of both size and charge, and can give information about the relative size of biomolecules with good resolution. When separated under native conditions, the solution structure of the DNA-Cobalt complex can be compared with the native-PAGE data to determine the structural state of a complex.
2.8.5 Characterization of the Thermodynamic Properties

The thermodynamic properties of a DNA transition can be monitored by UV melting and calorimetry studies. UV optical melting is a technique that monitors optical absorbance as a function of temperature. More ordered DNA, such as duplex, have a lower extinction coefficient than single stranded or denatured DNA. Due to the π-π stacking interactions, the UV extinction coefficient of single stranded DNA is higher than, the more ordered double stranded DNA. This shift in the absorbance is known as a hypochromic effect, and is useful in monitoring the double-stranded to single stranded transition. Melting experiments are used to calculate the van’t Hoff transition enthalpy as detailed by Marky and Breslauer. The nature of the structure-coil transition can be determined by comparing the model-dependent van’t Hoff transition enthalpy with the model-independent calorimetrically derived enthalpy. Therefore, if a transition were a "true two-state transition", the magnitude of the calorimetric enthalpy and van’t Hoff enthalpy values would be comparable to each other (± 5%), otherwise a two-state transition does not occur. Marky and Breslauer's approach states a sequence must undergo a two-state transition in order for the theoretical equations to be applied. In order to determine if the sequence under went a two-state transition, the cobalt-DNA complexes were measured by differential scanning calorimetry. DSC provides a model-free approach to determining thermodynamic information and represents the most accurate approach.

Isothermal titration calorimetry is another model-independent method available to perform experiments that measure the enthalpy of binding or enthalpy of a reaction. The initial ion binding caused by the positively charged cobalt (III) molecules interacts with
the negatively charged phosphate backbone of the nucleic acid. This electrostatic interaction is always the initial form of binding in all cationic molecules with DNA. ITC provides information on how the initial ion binding energy differs between each of the cobalt (II) compounds. By varying the temperature of the ITC instrument, we are able to observe enthalpy as a function of temperature. By plotting the relationship between enthalpy and temperature, entropy can be observed, and the heat capacity can be calculated by determining the slope of the best-fit line. The UV-melt derived van’t Hoff thermodynamic data and the calorimetric data will be used to correlate the stability associated with each cobalt-DNA with either one of the two sequences, Z8A and Z8M. The stability (ΔG) determined would provide valuable information into what parameters are responsible for the cobalt-DNA structural stability, and provide a correlation between the enthalpy, and entropy of the system.

The B-to-Z transition induced by sodium chloride (NaCl) and cobalt (III) hexammine has been observed using model dependent methods, such as van’t Hoff analysis. Another goal of this project is to use calorimetry to determine the thermodynamic parameters related to the B-to-Z transition. This is the first recorded attempt to observe the thermodynamic parameters responsible for the B-to-Z transition in small oligomers using cobalt (II) hexammine and NaCl by a model dependent method such as ITC. This conformational change is due to the dehydration brought about from the perturbation of the water molecules located on the phosphate backbone. This hydration effect is tested using other cobalt (III) molecules to observe any structural changes due to binding effects brought upon by the cobalt (III) molecule.
2.8.6 Reactive Properties of Cobalt (III) Complex

Hicks and Shearoy (1995) used cobalt (III) pentammineaqquo, and observed very interesting reactivity of the cobalt (III) complex and Z8M. The reaction of cobalt (III) pentammineaqquo with Z8M was determined by atomic absorption to be completed within 48 hours under standard conditions (pH 7.0, 50 mM NaCl, extensively dialyzed). The calculated cobalt bound per base pair was determined to be 1:4. The AA results showed the removal of the electrostatically bound complexes from the Z8M DNA oligomer by a decrease in the amount of cobalt bound. An increase in the amount of cobalt bound depicted a tighter bound molecule, not typical of an electrostatic interaction. They determined the sequences with 5'GG- and 5'GC- (5'GG -> 5'GC-) showed an increase in the amount of cobalt retained.

Atomic absorption experiments provide a useful method to determine the reactivity of the cobalt (III) molecules and DNA. The experiments can distinguish between the mode of binding associated with the cobalt molecules and DNA. An electrostatic interaction would have a very low reactivity versus a covalent linkage on the N7 of the guanine molecules. A comparison between a -GC-rich oligomer versus a -G5'meC-rich sequence made by an AA experiment can provide information pertaining to the effect's caused by the methylation of a DNA oligomer, and how this effect could manipulate the mode of binding for cobalt (III) molecules. This information along with the thermodynamic and structural information will provide an insight into the effects of cobalt (III) molecules and short DNA oligomers.
3 MATERIAL AND METHODS

3.1 Definition of Equipment and Principles of Operation

3.1.1 High Performance Liquid Chromatography (HPLC) System

The HPLC system consisted of a Waters Alliance HPLC system with Waters’s proprietary 2690 Separation Module, a Waters Lambda-Max model 481 variable wavelength detector using TurboChrom 4.1 software and a Phenomenex 5 μm C18 (RP-HPLC) (4.6 mm x 150 mm) column (Torrance, CA) utilized as the stationary phase. Data was collected by Turbochrom software detected by a Waters Lambda-Max model 481 variable wavelength set at 280 nm for preparative HPLC or 260 nm for analytical HPLC. The detector was interfaced with a Nelson Analytical series 900 A/D converter and the absorbance versus time data was analyzed by Turbochrom software. The mobile phase was 0.1 M TEAA (pH 7.0) and the organic modifier was 20% HPLC grade acetonitrile. All buffer solutions were filtered through a 0.45 μm filter, and thoroughly degassed prior to use by either vacuum or under helium.

3.1.2 Circular Dichroism Spectropolarimeter (CD)

A 62 ADS AVIV CD spectropolarimeter equipped with a multi-cell holder, a Peltier heating/cooling device with nitrogen purging capabilities was used for all CD measurements. The spectrophotometer was interfaced to a DOS-based computer running AVIV version 3.14 software for data collection, data processing, and command/control options. The temperature of the Peltier heating/cooling device was controlled by an Iso Temp water bath model 9100 (Fisher Scientific). An external fan is necessary to provide
a cooling device for the Peltier heating/cooling system and prevent the instrument from “freezing”. Nitrogen liquid was used to eliminate any moisture on the optics and provide a spectra with little to no background noise. A cold water supply circulates around the Xeon lamp to prevent the lamp from overheating. Prior to the experiments, cuvettes were scanned versus water to ensure no strain in the quartz or film on the surface.

3.1.3 Differential Scanning Calorimetry (DSC)

A Nano-D power compensation differential scanning calorimeter from Calorimetry Science Corporation (Ohio) was used to obtain all heat capacity measurements. The calorimeter contained two cells, a reference and a sample compartment, made up of a platinum alloy material, housed in a Peltier heating/cooling block. The calorimeter is interfaced directly to a Gateway Pentium III Windows-based computer. “DSCRun” version 2.1 software (Calorimetric Sciences Corp) was used for both data collection and DSC control. Data analysis was done by “CpCal” version 2.1 (Calorimetric Science Corp) and Origin version 6.1 software. Each experiment required the instrument to be calibrated depending on the scan rate and temperature range. By balancing the cells and providing a linear residual baseline, the data obtained from the calorimeter will provide more accurate and precise measurements of the heat capacity for the specific temperature range and scan rate of the experiments.

3.1.4 Isothermal Titration Calorimetry (ITC)

Calorimetry measurements were carried out using the Calorimetric Science Corporation
(CSC) Model 4200 Isothermal Titration Calorimeter that is interfaced to a Gateway 2000 Pentium II MMX PC. "CSC Run", "winworks" and Origin Version 4.0 software was used for data acquisition and analysis of all the ITC data. The ITC is calibrated by two methods. These two ligand-binding reactions are used as test reactions in titration microcalorimetry (for the simultaneous determination of enthalpy change and equilibrium constant, $K_c$, the binding reactions between $Ba^{2+}$ and the macrocyclic compound 18-crown-6 (1,4,7,10,13,26-hexaoxaacyclooctadecane) and between 2'-CMP (cytidine 2'-monophosphate) and bovine pancreatic ribonuclease A (RNaseA). Both reactions are conducted in aqueous solution and are 1:1 binding reactions. No test reactions have been proposed for more complex binding models or for reactions in organic solvents. The binding of $Ba^{2+}$ ($BaCl_2$ solution) to 18-crown-6 forms a convenient and reliable test reaction involving inexpensive and stable compounds that are easily available in sufficiently pure form. The values for $\Delta H_m$ and $K_c$ are of a magnitude suitable for calculation of precise results. A small correction should normally be applied for the enthalpy of dilution of the injected barium salt solution. No significant variation in the derived thermodynamic quantities was observed when the concentration $c$ for the crown ether in the vessel was varied from 0.001 to 0.01 mol dm$^{-3}$ or for a variation in $c$ (BaCl$_2$) in the injection syringe from 0.01 to 0.1 mol dm$^{-3}$. Values tentatively recommended for the thermodynamic quantities at 298.15 K are: $\Delta H_m = (-31.42 \pm 0.20)$ kJ mol$^{-1}$, $K_c (\text{mol} \cdot \text{dm}^{-3}) = (5.90 \pm 0.20) \cdot 103$, and $C_p = 126 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ (288 to 310 K).$^{79}$

The CSC Isothermal Titration Calorimeter (ITC) is designed specifically for the study of biopolymer ligand interactions. The ITC can detect heat effects as small as 0.1 yecal
allowing a titration to be done with as little as 1 nmole of biopolymer. Equilibrium constants in the range of $10^2$ to $10^8 \text{ M}^{-1}$ are easily determined for almost any interaction. Experiments can be done with as little as 0.5 μl of a very dilute solution.

Equipped with removable cells, the CSC ITC can be used to study interactions of solutes with immobilized enzymes, tissue samples, or other solid materials in suspension. The ITC can also be used to obtain analytical or kinetic data (for slow reactions) in addition to its principle use in the measurement of thermodynamic parameters ($K_{eq}$, $\Delta H$, $\Delta S$, and $n$) for binding interactions.

Each titration experiment was set up such that 5-10 μl injections of the Z-inducer was added every 600 seconds to ~128 μmol/μl solution of DNA. Temperature dependent experiments allowed us to determine how temperature played a role in the binding of the cobalt complex. At each temperature, the instrument was allowed to equilibrate until the heat exchange between the sample and the reference cell was no more than ± 5 μW. The heat associated with each injection of the cobalt (III) complex is observed as a peak that corresponds to the power required to keep the sample and the reference cells at identical temperatures. The peaks produced over the course of a titration can be converted to heat output per injection by integration and correcting for cell volume and sample concentration. The enthalpies of dilution of the solutions of DNA by standard phosphate buffer/water and the enthalpies of dissolution were determined in separate experiments.
3.1.5 UV/VIS Spectrophotometer

A Cary model 300E Bio or Cary model 100E spectrophotometer from Varian, Inc. was used for all spectroscopy studies. Both the Cary 300E and the Cary 100E were interfaced to a Windows based computer with Cary/Varian WinUVBio version 2.0 software that allowed for data acquisition and data analysis. The software provides the ability for various types of experiments such as, kinetic experiments, thermal or temperature dependent studies, or simple absorbance readings. Both spectrophotometers are equipped with a Peltier thermoelectric heating/cooling block, a multi-cell device and a sample compartment capable of purging. The Peltier heat sink is controlled by an external water bath manufactured by Varian. The spectrophotometers have the capabilities of using either a external probes to measure the temperature inside of a sample cuvette or the ability to use the cell block to measure the temperature of the sample compartment.

3.1.6 DNA Synthesizer

Either an Applied Biosystems Inc. (currently ABI-Perkin Elmer) automated DNA synthesizer model 380B or an Expedite™ model 8909 Nucleic Acid Synthesis System from Millipore was used for all DNA synthesis reactions.

The Expedite™ model 8909 contains a trityl monitor that allows the detection of the dimethoxytrityl (DMT) group as it is removed during the de-blocking step of the synthesis. This instrument has the ability to synthesize using the β-cyanoethyl phosphoramidite DNA and RNA protocols at 0.05, 0.2, 1, and 15 µmole scales. It also enables two syntheses to be performed at the same time using the same or different
sequence. An argon tank was connected to the rear of the instrument and set for 20 psi to ensure the reagents stored on the instrument are kept free from moisture. The instrument can work as a stand-alone or it can be interfaced with a Windows-based computer using the Expedite Workstation software. The software allows the trityl history to be saved, and more advanced control of the system. The Expedite model 8909 requires you to perform a deblocking step after each synthesis. DNA was collected in safety-coated 10 x 130 mm screw-capped culture tubes (Corning Scientific, cat no. 69825-13) and placed in a 55°C water bath for at least 24 hours.

The Applied Biosystems automated DNA synthesizer model 380B also contains a trityl monitor composed of a Pharmacia “Fast Protein Liquid Chromatography (FPLC) Single-Path UV” absorbance detector set at 280 nm at 2.0 absorbance units full scale (AUFS). The output was connected to a chart recorder set for 0.25-0.5 mm/min. A low moisture, high purity argon tank (N2 Welding Supply) set at 70 psi provided the instrument with a blanket of argon to prevent moisture and controls the flow rate of the reagents during the synthesis. The ABI380B has a built in deprotection step that allows the DNA to be cleaved from the CPG-column and collected in safety-coated 10 x 130 mm screw-capped culture tubes (Corning Scientific, cat no. 69825-13) and placed in a 55°C water bath for at least 24 hours.

The oligomers used in these experiments utilized the β-cyanethyl phosphoramidites and the chemistry associated with Glen Research, Inc for both DNA synthesizers. The anhydrous acetonitrile was obtained from Aldrich Chemical Company, Inc at 99.9999%
free of water. The reagent grade ammonium hydroxide was purchased from Fischer Scientific and stored at 5°C. A Schlenk manifold was used to transfer the anhydrous acetonitrile in an argon chamber to glass bottles that fit the ABI 380B instrument, while the acetonitrile used in the Expedite were purchased from Glen Research, Inc “as is” and fit on the instrument. All reagents used for either of the two instruments were stored under desiccation and dated to note the shelf time. To ensure the instruments are working efficiently, a purge was performed on the instrument each day in operation to ensure that both instrument lines were kept free from blockage.

3.1.7 Gel Electrophoresis Equipment

ExCell II electrophoresis unit from Novex, Inc. was used for both the native and denaturing gel set-ups. The Novex Pre-Cast Gels was purchased from Invitrogen in 15% acrylamide Solution for the TBE-Urea Gels, and 20% for the TBE Gels. It is necessary to keep the temperature of the electrophoresis unit between 50–60°C for denaturing conditions. In order to keep the temperature around 55°C, the whole ExCell II electrophoresis unit is placed in a 75°C water bath. When running under native conditions, the ExCell II electrophoresis unit is placed in a freezer at 2°C and allowed to equilibrate prior to running the experiments. The unit was connected to a Bio-Rad S500 Power supply.

For larger numbers of samples, or samples requiring greater resolution, a Hoeffer model SE600 electrophoresis unit equipped with a heating/cooling core was utilized. The electrophoresis tank was connected to a Fisher Scientific Isotemp model 2016D water
bath for heating and cooling operations. A magnetic stirrer was used to ensure even buffer distribution and temperature equilibration. An IsoLab, Inc. model CVR-500 power supply was used to supply electrical power to the unit. The acrylamide, bis-acrylamide and other electrophoresis reagents were purchased from Sigma Chemical Co, or Bio-Rad, Inc.

3.1.3 Poly Pak II Cartridge Column (PPII)

Poly-Pak™ Cartridges from Glen Research were used to purify synthetic oligonucleotides on a small scale using the DMT-on purification strategy. The Poly-Pak II cartridges can purify oligonucleotides on a synthesis scale up to 1 μmole. While using Poly-Pak II, Glen Research states that virtually the entire DMT-on product from a 1-μmole synthesis is absorbed during the loading process, and the yields are up to 150 A260 units of a purified 20 oligomer can be expected. This purification technique allows you to purify the oligomer with the DMT group on or off. Depending on the number of oligomers needed to purify, two methods were used with the Poly-Pak II for the purification process.

The first method incorporated using a 10-nl syringe, held by a ring stand, connected to the female luer of the Poly-Pak II cartridge as illustrated in Figure 13. The reagents are pushed through the cartridge at a rate of 1-2 drops/sec. This method proved to be very tedious and time consuming, but produced high DNA yield, high purity, and only required small amount of reagents. The second method is using a modified Econo-Pump System from Bio-Rad, Inc. enabling a semi-automated approach to oligomer purification.
with Poly-Pak II cartridge columns. The modified pump setup consisted of an eluent switching valve, a buffer reservoir, a peristaltic pump (Bio-Rad model EM-1), an ultraviolet absorbance detector (Bio-Rad model B-1M-1) and a Kipp-Zonen model BD-11 strip chart. The reservoir, switching valve, and pump were connected in series ahead of the Poly-Pak II cartridge. The absorbance detector was set for 1.0 absorbance unit full scale at 254 nm and connected to the strip chart recorder. The internal diameter of the tubing and the rotational speed of the pump rotor affect the flow rate of the pump, which was set to 1.6 ml/min. This method proved to be least time consuming, but required higher volumes of reagents than the manual method.

Figure 13: Schematic of the Econo pump used for purification technique via the Poly Pak II protocol.
3.1.9 Digital Photograph Equipment

Photographs of the gels were obtained by a conventional Sony CyberShot DSC-P100 digital camera (Sony, Inc.) or an Alphalmaager digital camera, (Alpha Innovitech Inc.) model 4912-2810 that was mounted to a Polaroid MP-7 copy stand. The Cybershot had a built-in UV-cut-off filter to allow clear and accurate photos while UV-transilluminator is present. The conventional digital camera stored the images on its hard drive and was put into Adobe Photoshop for editing. The Alphalmaager digital camera was directly interfaced to a Windows-based computer running AlphaEase version 3.3d software for control of the camera, image capture, densitometry, and image manipulation. Included with this camera was a UV-cut-off filter, and a +2 close-up diopter lens enabling macrophotography. The light sources were a UV-transilluminator, a fluorescent transilluminator, or overhead 500 W reflector floods.

3.1.10 Dialysis Equipment

Dialysis tubing from Spectro-Pro Industries with a 1000 molecular weight cutoff (MWCO) pore size, and a 15 mm diameter were used for the dialysis of DNA oligonucleotides. The dialysis tubing was heated to a boil, and Sodium Carbonate was added, along with EDTA for approximately 1 hour. After the solution was cooled, the solution was changed to ddH₂O and the solution was mixed with a magnetic stirrer. This procedure was done several times for the next 2 days to ensure no metals, or other ions are trapped in the tubing pores. Once the dialysis tubing is treated, they can be either stored in 4°C refrigerator for future use or they can be used as is. In addition, unwaxed and unflavored dental floss, magnetic stir plate, and refrigerator were utilized.

52
3.1.11 Nuclear Magnetic Resonance

VARIAN \textsuperscript{\textregistered}INOV\textsuperscript{A} 500 MHz NMR Spectrometer System consists of four major units: the host computer, the NMR system console, the superconducting magnet and the probe. The NMR spectrometer is interfaced with a Senc Microsystems workstation that controls the NMR and runs the Varian software called VNMR. The NMR system console is the main unit of the spectrometer system, and contains all of the electronics required to allow the system to work properly, along with some external accessories such as the channel selector switch, and the magnet console interface. The superconducting magnet holds the probe and provides a stable magnetic field. The probe transmits rf power to the sample and detects minute voltages in return. This is the key to this instrument, and many different types of probes are available to use depending on the nuclei you are studying.

The broadband probe was utilized in order to obtain $^3$P NMR spectra. The NMR has the capabilities to perform all modern one- and multi-dimensional experiments. It has both direct and indirect detect-pulse field gradients — with a broadband probe capable of observing resonance frequencies between $^{31}$P and $^{15}$N. The probe has the ability to vary temperature between $-150^\circ$ to $200^\circ$C.

3.1.12 Infrared Spectrophotometer

Infrared spectroscopy experiments were analyzed by a Nicolet 8700 FT-IR spectrometer with the Vector-Piezo\textsuperscript{TM} interferometer along with a step-scan spectroscopy. The scans observed spectral changes in the spectral range from 25,000 to 20 cm$^{-1}$. IR spectra's were used predominately to monitor and characterize cobalt (II) molecules. Each scan was taken at room temperature, using a concentration of 0.1 - 1.0 nM of the cobalt (III).
complex. Though these compounds have been previously characterized, it is important to the study, that each of the compounds are fully characterized to ensure the reliability of the synthesis.

3.2 Definition of Common Solutions and Reagents

3.2.1 SHU Denaturing Gel Sample Buffer (for TBE-based PAGE) - 1X Tris-Borate-EDTA (TBE defined below) solution pH 8.0, 90% v/v formamide, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol.

3.2.2 SHU Native Gel Sample Buffer - 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, 0.1mM EDTA, pH 7.0, 100 mM NaCl, 50% v/v glycerol, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol.

3.2.3 Tris-Borate-EDTA (TBE) - 45 mM tris-borate, 1 mM EDTA, pH 8.0 with boric acid. To prepare one liter of solution, add 5.4 g Tris base, 2.75 g boric acid, 2.0 ml of 0.5 M EDTA, to 750 ml distilled, deionized water, and adjust pH to 8.0 with boric acid if necessary. Adjust volume one liter with ddH₂O.

3.2.4 Standard Phosphate Solute (SPS) - 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, 0.1mM EDTA, pH 7.0.

3.2.5 10X SPS (Stock solution used for sample preparation) - 1.0 mM EDTA, 50 mM NaH₂PO₄, 50 mM Na₂HPO₄, pH 7.0.

54
3.2.6 5M Sodium Chloride (Used for DNA sample prep and dialysis) — To prepare this solution, the calculated quantity of NaCl was added to ddH2O. After mixing for several hours, the pH should be adjusted to 7.0 using NaOH or HCl.

3.2.7 Acrylamide Stock Solution - 40% w/v acrylamide in water composed of 19.1 w/v of acrylamide and bis-acrylamide (to 60 ml of water add 38 g of acrylamide, 2 g of bis-acrylamide and dissolve. Adjust volume to 100 ml with water).

3.2.8 2.0 M Triethylammonium Acetate - Add 120-ml of glacial acetic acid to the water with vigorous stirring and then slowly add the triethylamine. Once the solution has been mixed and cooled, measure the pH and adjust, if necessary, to 7.0 to 7.5 with glacial acetic acid or triethylamine.

3.2.9 0.1 M Triethylamine Acetate (Used in Oligonucleotide purification protocols, HPLC, PPI) — To prepare this solution, the calculated quantity of 2.0 M triethylamine is added to water, and the pH is adjusted to 7.0 with acetic acid.

3.2.10 1 % Trifluoroacetic Acid — Add two volumes of trifluoroacetic acid to 98 volumes of HPLC Grade Water.

3.2.11 0.1 M TEA/A - 0.1 M triethylammonium acetate, (TEAA) pH 7.0. To prepare this solution, the calculated quantity of triethylamine is added to water, and the pH is adjusted to 7.0 with acetic acid.
3.2.12 **2.0 M TEA/TA** - 2.0 M triethyammonium acetate pH 7.0. To prepare this solution, the calculated quantity of triethylamine is added to water, and the pH is adjusted to 7.0 with acetic acid.

3.2.13 **0.1 % STAINS-ALL Stock Solution** - 1 g of STAINS-ALL in 100 ml of ddH2O.

3.2.14 **5 mM Trizma-HCl** - To prepare this solution add 4.728 g in 900 ml of ddH2O. Adjust the pH to 8.8 with HCl or NaOH and add ddH2O to a final volume of 1L.

3.2.15 **Gel Staining Solution (100-mL working solution)** - To prepare solution add 0.005% STAINS-ALL (5 ml of 0.1% Stock), 10% formamide (5 ml), 25% Isopropanol Alcohol (25 ml), 50 ml of the 15 mM Trizma-HCl, pH 8.0, and 15 ml of ddH2O.

3.3 Overview of Oligomer Synthesis

3.3.1.1 **Introduction to DNA Synthesis**

All DNA oligomers were synthesized on either an Applied Biosystems 380B DNA synthesizer or an Expedite Millipore 8909 Nucleic Acid synthesizer. Both instruments utilize the phosphoramidite method of Caruthers for the synthesis of each oligonucleotide, but each instrument performs the task differently. As in all chemical reactions, the reactant concentration, the stoichiometry, the length of the reaction time, and the reaction temperature play important roles in obtaining successful yields.
3.3.2 ABI 380B DNA Synthesizer

Synthesis on the ABI 380B was a solid-phase synthesis reaction. Solid phase synthesis depends on a number of variables, but mostly due to the quantity of reagent applied to the solid phase support. The amount of each reagent applied to the support will determine the quality of the reaction. Therefore if too little of the reagent is applied, the reaction will be halted, and the yields will be poor. In turn the poor yields will cause the failure sequences of the reaction to increase.

The quality of the synthesis is determined by the stoichiometry of the reagents, the flow rate, and the flow time on the column. If any of these variables are altered, the yields on the synthesis will decrease. An important note is that the flow rate, and the flow time on the column are controlled by a gas supply, typically anhydrous argon. Fortunately, the ABI 380B allows you to adjust the flow rate, and the flow time in order to increase your yields. The delivery time and flow are altered typically by "clogs" in the delivery lines. These "clogs will decrease the internal diameter causing the flow rate and time to decrease.

The flow rate specifications for the ABI 380B DNA synthesizer were generated by Tony Paiva, listing the flow rates for all the positions on the instrument. Each of the flow rates were measured at the column inlet.
Table 5: Flow rate specifications for the ABI model 380B DNA synthesizer.

The flow rate specifications listed are for acetonitrile in all positions. The rates listed below are for the 380B DNA synthesizer with version 1.34 software.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Position</th>
<th>CH₃CN flow gm / 60 sec</th>
<th>CH₃CN flow ml / 60 sec</th>
<th>CH₃CN flow sec / ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>0.57-0.60</td>
<td>1.45-1.55</td>
<td>0.64-0.69</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>0.57-0.60</td>
<td>1.45-1.55</td>
<td>0.64-0.69</td>
</tr>
<tr>
<td>G</td>
<td>3</td>
<td>0.57-0.60</td>
<td>1.45-1.55</td>
<td>0.64-0.69</td>
</tr>
<tr>
<td>T</td>
<td>4</td>
<td>0.57-0.60</td>
<td>1.45-1.55</td>
<td>0.64-0.69</td>
</tr>
<tr>
<td>open</td>
<td>5</td>
<td>0.57-0.60</td>
<td>1.45-1.55</td>
<td>0.64-0.69</td>
</tr>
<tr>
<td>open</td>
<td>6</td>
<td>0.57-0.60</td>
<td>1.45-1.55</td>
<td>0.64-0.69</td>
</tr>
<tr>
<td>open</td>
<td>7</td>
<td>0.57-0.60</td>
<td>1.45-1.55</td>
<td>0.64-0.59</td>
</tr>
<tr>
<td>open</td>
<td>8</td>
<td>0.57-0.60</td>
<td>1.45-1.55</td>
<td>0.64-0.69</td>
</tr>
<tr>
<td>Activator</td>
<td>9</td>
<td>0.57-0.60</td>
<td>1.45-1.55</td>
<td>0.64-0.69</td>
</tr>
<tr>
<td>NH₄OH</td>
<td>10</td>
<td>1.53-1.77</td>
<td>3.90-4.50</td>
<td>0.22-0.26</td>
</tr>
<tr>
<td>Cap A</td>
<td>11</td>
<td>0.63-0.79</td>
<td>1.60-2.00</td>
<td>0.5-0.63</td>
</tr>
<tr>
<td>Cap B</td>
<td>12</td>
<td>0.63-0.79</td>
<td>1.60-2.00</td>
<td>0.5-0.63</td>
</tr>
<tr>
<td>CH₃CN</td>
<td>13</td>
<td>0.65-0.71</td>
<td>1.65-1.8</td>
<td>0.55-0.61</td>
</tr>
<tr>
<td>Deblock</td>
<td>14</td>
<td>0.65-0.71</td>
<td>1.65-1.8</td>
<td>0.55-0.61</td>
</tr>
<tr>
<td>Oxidizer</td>
<td>15</td>
<td>0.65-0.71</td>
<td>1.65-1.8</td>
<td>0.55-0.61</td>
</tr>
<tr>
<td>CH₃CN</td>
<td>16</td>
<td>0.65-0.71</td>
<td>1.65-1.8</td>
<td>0.55-0.61</td>
</tr>
<tr>
<td>CH₃CN</td>
<td>18</td>
<td>0.65-0.71</td>
<td>1.65-1.8</td>
<td>0.55-0.61</td>
</tr>
</tbody>
</table>
3.3.2.1 Expedite Millipore 8900 Nucleic Acid Synthesizer

In the Expedite 8900 Nucleic Acid synthesizer, the fluid transport system consists of a microfluidic plate with fluid passages, valve actuators, and fluid injectors. Unlike the ABI 380B, that it is controlled by volume/time, the Millipore 8900 utilizes a pneumatic system that drives the fluid injectors and is controlled by volume. Each reagent is delivered as a fixed volume pulses by individual fluid injectors, and consistent reagent volumes are delivered to the reaction column. This allows accurate monitoring of reagents without calibrating the flow rates for each reagent, as done with the ABI 380B DNA synthesizer. The pneumatic control system contains a high pressure and a low-pressure system. The low-pressure system blankets the reagent bottles, while the high pressure system controls the pumping of reagents through the injectors by cycling gas pressure on a diaphragm. In Figure 14 is a fluidic diagram for the Millipore 8900 DNA synthesizer.

Still, with the advances made on the Millipore 8909 versus the ABI 380B model, clogs and problems can still arise. The only preventative measure to prevent clogging of the lines is to purge the system regularly, replace phosphoramidite reagents not in use with ACN, and determine a shelf life for all phosphoramidite and other reagents used for automated DNA synthesis.
With age, and the humidity experienced throughout the summer days in Northern New Jersey, shelf life of the reagents can become a serious problem. Therefore, shelf lives of each of the reagents were determined below. Monitoring the synthesis of oligonucleotides, checking for any insoluble particles in the reagent bottles and observing any spectroscopic changes in the reagents spectra's determined the reagent's shelf life. All unopened reagent bottles were kept in the dark under ambient conditions, in a dessicator.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Week(s) ABI - Open</th>
<th>Week(s) Expedite - Open</th>
<th>Week(s) Unopened</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3</td>
<td>4 - 5</td>
<td>12</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>4 - 5</td>
<td>12</td>
</tr>
<tr>
<td>G</td>
<td>2</td>
<td>3 - 4</td>
<td>12</td>
</tr>
<tr>
<td>T</td>
<td>3</td>
<td>4 - 5</td>
<td>12</td>
</tr>
<tr>
<td>Activator</td>
<td>2</td>
<td>2</td>
<td>12 - 24</td>
</tr>
<tr>
<td>NH4OH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cap A</td>
<td>4</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Cap B</td>
<td>4</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Deblock</td>
<td>8</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>Oxidizer</td>
<td>8</td>
<td>8</td>
<td>12 - 24</td>
</tr>
<tr>
<td>CH3CN</td>
<td>8</td>
<td>8</td>
<td>24</td>
</tr>
</tbody>
</table>

The reagents on the Milipore system have a longer shelf life than the reagents left on the ABI 380B instrument. This is due to the ABI 380B instrument's inability to prevent moisture from entering the system, faulty old o-rings/valves and pinhole leaks in the gas lines.
3.3.3 Overview of the Phosphoramidite Synthesis Procedure

All synthesis reactions occurred on a solid-phase support, specifically a centred pore glass (CPG). The synthesis protocol for most oligomers was with the 5'-terminal dimethoxytrityl group on (DMT-on). All synthesis began at the 3' terminus, which contained the first base of the sequence covalently linked to the CPG support and proceeded in the 3' to 5' direction in order to take advantage of the reactive primary alcohol in the 5' position. The phosphoramidite synthesis can be separated into six steps, detritylation, activation, coupling, acetylation, oxidation and cleavage.

3.3.3.1 Detritylation

A DMT protecting group located on the 5'-OH of the deoxyribose sugar prevents side reactions from occurring. Therefore, prior to the coupling reactions, the DMT group must be removed to provide a reactive site so the phosphoramidite can be linked. The DMT group is cleaved with 3% trichloroacetic acid (TCA) in dichloromethane (DCM). The reaction mechanism shown in Figure 15, in which an ether hydrolysis reaction occurs when the nucleophilic ether oxygen attacks the acid proton to form an oxonium ion. This oxonium ion undergoes an S_{N}1 cleavage reaction to form the desired oligonucleotides.

62
3.3.3.2 Activation

The activation step includes activating the new or incoming nucleoside with 0.45 M tetrazole in acetonitrile. The tetrazole protonates the diisopropylamine tertiary nitrogen, making the protonated form diisopropylamino-hydride, a good leaving group.
3.3.3.3 Coupling

The nucleophilic hydroxyl group of the support-bound mononucleotide displaced the protonated imidazole by nucleophilic attack of the phosphorus (3') resulting in cleavage at the phosphorus nitrogen bond.
3.3.3.4 Acetylation

The acetylation step also known as the end-capping step is done in order to prevent failure sequences to prevent further reactions from occurring at the 5'-hydroxyl site. The unreacted 5'-hydroxyl site of the failure sequence is acylated by acetic anhydride and 10% methylimidazole in THF. Therefore, any nucleoside that has a DMT protecting group attached will not undergo an acetylation reaction. The “end-capping” step in the
synthesis serves as two important features, first it provides an overall higher yield, and second it allows rapid purification at the end of the synthesis. The mechanism shown below, first illustrates the hydroxylation oxygen attacking the carbonyl of acetic anhydride, forming a tetrahedral intermediate.

Figure 18: Reaction mechanism for the hydroxylation oxygen attacking the carbonyl of acetic anhydride forming an intermediate.

The acetylation reagent proceeds through an addition/elimination mechanism to produce an acetate ion and an acetylated nucleobase. The next step in this mechanism involves the attack of the carbonyl acetic anhydride from the hydroxylation oxygen that causes the formation of a tetrahedral intermediate. An elimination reaction involving the tetrahedral intermediate produces the acetate ion and an acetylated nucleobase. The acetate ion is then reacted with the methylimidazole by an electrophilic addition producing 1-methyl-4-acetoimidazole.
3.3.3.5 Oxidation

The next step in the synthesis is the oxidation of the phosphite group in order to prevent degradation of the oligonucleotides chain. The phosphite group (3° oxidation state) is
reduced to a phosphate group (5' oxidation state) by reacting the oligonucleotides chain with 0.1 M iodine in H_2O, THF and pyridine. This oxidation-reduction mechanism shown below is necessary to provide the DNA backbone with the adequate positively charged backbone once deprotected.

Figure 20: Reaction mechanism for the oxidation of the base required for DNA synthesis

3.3.3.6 Cleavage

The last step performed on the solid phase support is the cleavage of the DNA from the CPG column. Cleavage is accomplished by breaking the succinate ester linkage and “freezing” the oligonucleotides from the resin. The process called for the addition of concentrated ammonium hydroxide, at room temperature to the column. Since the succinate ester is base labile, under basic conditions such as ammonium hydroxide, the

68
linkage can be broken. The linkage is broken via a hydrolysis mechanism as shown below (Figure 21).

Figure 21: Reaction mechanism for the cleavage of the DNA from the resin

The cleaved oligonucleotide was collected into a pyrex culture tube coated, on the outside, with a plastic safety layer. The coated collection tubes prevent catastrophic loss.
of the synthesized DNA should breakage occur. The cleaved oligonucleotides was collected in a pyrex coated culture tube, and placed in a 55°C water bath to begin the last and final step in the DNA synthesis process, deprotection.

3.3.3.3 Deprotection

In order to complete the synthesis of oligonucleotides a 5’-terminal DMT protecting group, various protecting groups, and the various groups on the exocyclic amine must be removed prior to any experimental use. Each nucleoside added in the reaction contains a different type of protecting group. The exocyclic amine protecting groups for guanine contain an isobutyl chloride protecting group, cytosine and adenine have a benzoyl chloride protecting group, and thymine requires no protecting group.

Since the exocyclic protecting groups are base-labile, at high temperatures the protecting groups undergo a base-hydrolysis reaction. To ensure that the oligonucleotides are fully deprotected, an additional 2-3 ml of fresh ammonium hydroxide is added, and placed in a 55°C water bath for 24 hours. Oligonucleotides with a high G-C rich content tend to need more time at 55°C water bath than other sequences. Each sample is stored at 5°C in ammonium hydroxide after deprotection is complete and before purification.
Figure 22: Reaction mechanism for the deprotection of the base required for DNA synthesis.
3.2.4 Monitoring the Synthesis and Determining Crude Oligomer Yield

Observing the trityl peaks from each of the synthesis steps in the reactions of the oligomer monitors the synthesis. After each DMT- group is removed, the absorbance of the solution containing the DMT group is measured, and at the completion of the reaction the number of peaks should be equal to \((n-1)\) where \(n\) = oligomer length. If the number of peaks were greater than \(n-1\), the synthesis was discarded and a new synthesis was performed. Another indication of a poorly synthesized oligomer is a gradual decline in
the trityl assay that does not recover, indicating a loss of efficiency at each synthetic step. An example of a successful synthesis and a synthesis with a loss of efficiency is shown in Figure 24.

Figure 24: Trityl Assay for DNA oligomers
Each peak represents a DMT group being removed or a base being added. Each peak can be quantitated and the overall yield can be calculated.

A decline in the trityl assay does not necessary denote that your synthesis should be discarded. The failure sequences of the synthesis are easily separated using RP-HPLC and the remaining synthesis can be salvaged. The overall yield can be calculated by taking the final cycle peak height divided by the cycle 1 peak height (Overall yield = \(\frac{PH_{\text{final}}}{PH_{\text{initial}}}\) * 100%). The step yield is approximated as the n1 peak height divided by the n peak height or by the overall synthesis yield (Step Yield \(= (\text{Overall Yield})^{\frac{1}{n+1}}\) where n = number of couplings).

Equation 3: \[
\text{Overall Yield} = \left(\frac{\text{Peak Height}_{\text{final}}}{\text{Peak Height}_{\text{initial}}}\right) \times (100%)
\]

Equation 4: \[
\text{Step Yield} = \left(\frac{\text{Peak Height}_{n+1}}{\text{Peak Height}_n}\right) \times (100%)
\]
where \(n = \) coupling number.

73
Equation 5: \[
\text{Step Yield} = (\text{Overall Yield})^{\frac{1}{n-1}}
\]
where \( n \) = number of couplings

After the completion of the synthesis, the DNA concentration is measured by taking a spectral scan from 320 nm to 210 nm. Initially the crude oligomer is diluted 1:50 with phosphate buffer solution. The crude DNA concentration was calculated with the dilution corrected absorbance value at 260 nm, using the relationship:

Equation 6: \[ A_{260} \text{ oligomer} = 27 \mu g \text{ DNA} \]

Equation 7: \[ \frac{(A_{260})(\text{dilution})}{0.027} = \mu g \text{ DNA/ml} \]

The value obtained for the total absorbance is contributed to the failure sequences, and the full-length DMT oligomers. Depending on the sequence and scale the total amount of DNA synthesized pre-purification can vary.

These numbers were generated with random sequences that did not contain any modification of the bases. Bases that contain modifications, such as methylation, gave different values, but not enough experiments were done to accurately determine the values for a range of modified sequence lengths.
Table 7: Typical synthesis yield observed for the ABI 380B and the Expedite 8900 DNA Synthesizers.

<table>
<thead>
<tr>
<th>Scale (µmol)</th>
<th>Sequence Length (bp)</th>
<th>Total Amount (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>8-35</td>
<td>25+</td>
</tr>
<tr>
<td></td>
<td>35-60</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>60+</td>
<td>ND</td>
</tr>
<tr>
<td>0.2</td>
<td>8-35</td>
<td>100 - 350</td>
</tr>
<tr>
<td></td>
<td>35-60</td>
<td>325+</td>
</tr>
<tr>
<td></td>
<td>60+</td>
<td>400+</td>
</tr>
<tr>
<td>1.0</td>
<td>8-35</td>
<td>500 - 800</td>
</tr>
<tr>
<td></td>
<td>35-60</td>
<td>800 - 1500</td>
</tr>
<tr>
<td></td>
<td>60+</td>
<td>1200+</td>
</tr>
</tbody>
</table>

3.4 Oligomer Purification Methods

3.4.1 Purification Overview

The methods our lab has adopted for purifying DNA oligomers are RP-HPLC, PolyPak II, and AX-HPLC. All have been proven to provide excellent results, with minimal loss of successfully synthesized strands. After either of the methods is performed, an analytical denaturing Urea-PAGE is done to check for purity. No matter which purification technique utilized the purification of the DNA oligomer is dependent on the
dimethoxytrityl (DMT) group attached or unattached to the 5' terminus of the synthetic oligonucleotides. Failure sequences that contain no DMT group are weakly bound to the column and easily separated from the product and those strands that are synthesized with the DMT-off will separate based on size of the DNA strand. The DMT-on procedure proved to be a better method of purification versus the DMT-off.

3.4.2 PolyPak II Purification

PolyPak II columns provide a wide array of benefits versus other chromatography methods. PP II limitations are that they only allow for oligomers synthesized up to 1 μmole, and with a length less than 75 bases. PP II allows for the removal of failure sequences via the DMT-on protocol. Buffer exchange and DNA concentration can be utilized due to the oligomers ability to be bound to the solid-support of the PP II cartridge. PP II also allows the DNA oligomers synthesized with the DMT-off to be salted, and purified by other methods such as RP-HPLC. PolyPak II is an excellent tool for purification of non-modified DNA oligomers but with oligomers that contained methylated sites, a different method such as RP-HPLC must be employed.

As previously mentioned the key to a purification of DNA oligomers lies in the DMT group. The synthesis is carried out in the 3' to 5' direction, and after each base is added a DMT group is added to the 5' terminus until the synthesis is complete and only one DMT group is found on the successful strand, while all other failure sequences have been "capped" and do not contain a DMT group. This difference is the basis for purification and separation used in RP-HPLC, and PolyPak II. Since the oligomers with the DMT-on
bind strongly to reverse-phase support, the full-length sequences are kept while the short failure sequences are left on the column. The polyPak II procedure goes as follows: First, the cartridge is prepared by first washing with acetonitrile to wash any organics off the column, and then condition the cartridge by wetting it with TFA, which acts as an ion pairing reagent to enhance the oligonucleotides binding to the resin; Second, is preparing the oligonucleotide sample by diluting it with three parts; Third is to load the crude oligomer solution in a 5-10 ml syringe connected to the PPII cartridge. Allow the DNA solution to pass through the cartridge at a speed of 1 drop/second to ensure the DNA will bind to the resin. This step is done a few times to ensure all of the DNA is bound to the resin; Fourth, remove the failure sequences by flushing the column with 6-mL of a 1:20 diluted solution of ammonium hydroxide followed by 4-mL of water; Fifth, detritylate the support-bound oligonucleotides by flushing the cartridge with 4-mL of a 2% solution of TFA followed with 4-mL of water; Sixth, elute the purified, detritylated oligonucleotides by flushing the cartridge with 20% acetonitrile. After the oligonucleotide is eluted from the cartridge, the purified oligonucleotide is concentrated by lyophilization with a Speed-Van lyophilizer and reconstituted in 1.0 mL of deionized water and the DNA concentration is determined. This procedure of purification can be done using a vacuum manifold or an automated Econo Pump as described in Tony Paiva’s Dissertation (2005) pgs 72-78. The makers of this product guarantee a 99% or greater purity should be expected. Unfortunately, oligonucleotide with modified bases tends to require different methods, and more delicate purification techniques such as RP-HPLC.
3.4.3 Preparative Reverse-Phase HPLC

3.4.3.1 Overview of RP-HPLC Purification

In RP-HPLC, the most polar components appear first. Increasing the mobile phase polarity increases the elution time. A highly polar mobile such as methanol, acetonitrile or tetrahydrofuran is used as the mobile phase. Another factor affecting the elution of the oligonucleotides is the hydrocarbon chain length. Usually C8 and C18 are utilized with siloxane R group. Longer chain hydrocarbons packed with the same particle size, using the same solvent, pH, temperature and flow rate will provide better separation than shorter hydrocarbon columns, Figure 25.83

Figure 25: The Effect of chain length on performance of RP HPLC

A siloxane column packed with 5-μmL particles. Mobile phase: 50:50 methanol/water. Flow rate: 1.0 ml/min.

Purification of oligonucleotides using RP-HPLC utilizes the same DMT characteristics as PP II columns use. The purification process using RP-HPLC requires a few more
lyophilizing steps than PP II producing lower yields, but higher purity. For modified strands, RP-HPLC has proven to produce high yields, and purity. RP-HPLC is the mode of purification used for all modified oligomers used in these experiments.

There are four steps in the purification of the oligonucleotides. First the tritylated DNA is separated from the failure sequences. Secondly, the full-length oligonucleotides are dehydrated using mildly acidic conditions. Third, the trityl alcohol is removed by an ether extraction. Lastly, the purified oligonucleotide is put through a trityl-off RP-HPLC process. Once the oligonucleotides are collected, the sample are lyophilized, and resuspended in deionized water to be dialyzed versus buffer. As each step is performed, HPLC allows you to monitor each of the steps using spectrophotometric methods, to ensure you that each step is done efficiently with a low amount of loss.

3.4.3.2 Trityl-On DNA, Prep-RP-HPLC Procedure

This gradient, developed by S. Marotti, is for the primary purification of trityl-on oligomers at the 1 µmol scale. This method was adjusted and used for each DNA oligomers synthesized. The DNA sample was taken from the oil bath and cooled to room temperature. To prevent the hydrolysis on the column, all ammonium hydroxide was removed from the sample. The solution was lyophilized to dryness and resuspended in 1.0 ml of 0.1 M triethylamine acetate pH 7.0 containing 2% acetonitrile. A 50 ml aliquot of the sample was injected onto the RP-HPLC column to determine the purity of the crude oligomers, and collect the eluting sample. This was performed till the DNA sample was clean of impurities and free from failure sequences. At times, a Waters Bondapak C-
Radial compression column was used to separate, table 8.

Table 8: Trityl-On DNA, Prep-RP-HPLC

Detector Setting: 2.0 AUFS, 280 nm
Injection Loop Size: 2.0 ml
Mobile Phase A: 0.1M triethylamine acetate pH 7.5 with 2% acetonitrile (filtered and degassed)
Mobile Phase B: acetonitrile (HPLC-grade, degassed)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (ml/min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>0.5</td>
<td>4.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>15.5</td>
<td>4.0</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>25.5</td>
<td>4.0</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>27.5</td>
<td>4.0</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

For oligomers such as Z8A, a gradient with less polarity in the final phase of the gradient was required. The gradient in Table 9 was used to purify this oligomer. The major peak would elute last in the chromatogram. Usually, any DNA peaks obtained between 20-30% acetonitrile were retained and lyophilized to dryness. Each sample was then dissolved in deionized water and analyzed by RP-HPLC or more commonly used technique, denaturing gel electrophoresis. The samples were then joined and deuterated.
Table 9: Trityl-OH DNA — Used for high CG rich sequences and methylated sequences.

Detector Setting: 2.0 AUFS, 295 nm Detector Setting, 0 AUFS, 280 nm
Mobile Phase A: 0.1 M tris(hydroxymethyl)acetate pH 7.5 with 2% acetonitrile (filtered and degassed)
Mobile Phase B: acetonitrile (HPLC-grade, degassed)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL/min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>0.5</td>
<td>4.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>20.5</td>
<td>4.0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>39.5</td>
<td>4.0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>32.5</td>
<td>4.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>40</td>
<td>4.6</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

The major peak would elute last in the chromatogram. DNA peaks obtained between 20-30% acetonitrile were retained and lyophilized to dryness. Each sample was dissolved in deionized water and analyzed by RP-HPLC or denaturing gel electrophoresis. The samples were then combined and a detritylation.

3.4.3.3 Detritylation

In the detritylation step, an equal volume of 0.4 N acetic acid is added to the DNA causing the hydrolysis of the acid labile DMT group from the oligomer. The detritylated reaction is monitored by RP-HPLC. A small aliquot is taken before the detritylation.
reaction, and an aliquot is taken after subsequent time to monitor the shift in the peak and
dissappearance of the trityl group peak over time. After the completion of the detritylation
step, ammonium hydroxide is added to the DNA solution in order to neutralize the
solution (pH > 8.0). The solution is then lyophilized to dryness and resuspended in
dionized water.

3.4.3.4 Ether Extraction

The ether wash or ether extraction is the most overlooked step in purification. It is one of
the most important to perform when working with synthetic DNA and performing
biophysical studies. The free floating DMT groups in solution will cause many variable
in thermographs using DSC, and UV melts, due to the DMT group. The ether extraction
requires the addition of 2-3 volumes of diethyl ether, vigorously shaken and allowed to
sit for at least 2-3 minutes. The ether layer is removed with a pipette and the process is
repeated two more times. The resulting ether extracted DNA solution is lyophilized and
prepared for analysis using gel electrophoresis. If any faint bands are present on the gel, a
second purification is performed.

3.4.3.5 Trityl-Off RP-HPLC Procedure

In cases in which the trityl group is synthesized with the trityl off, or the need for a
second purification is needed after detritylation has occurred, the trityl off RP-HPLC
procedure is used. In each case, an aliquot of DNA is taken and injected onto the RP-
HPLC column, using the gradient Analytical RP-HPLC shown below:
Table 10: Trityl-Off DNA – RP-HPLC Protocol

Detector Setting: 2.0 AUFS, 280 nm  Detector Setting: 2.0 AUFS, 280 nm
Mobile Phase A: 0.1M triethylamine acetate pH 7.5 with 2% acetonitrile (filtered and degassed)
Mobile Phase B: acetonitrile (HPLC-grade, degassed)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (ml/min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.0</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>0.5</td>
<td>4.0</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>5.5</td>
<td>4.0</td>
<td>83</td>
<td>17</td>
</tr>
<tr>
<td>6.0</td>
<td>4.0</td>
<td>83</td>
<td>17</td>
</tr>
<tr>
<td>7.0</td>
<td>4.0</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>15.0</td>
<td>4.0</td>
<td>97</td>
<td>3</td>
</tr>
</tbody>
</table>

Once the nature of the preparatory chromatograph is determined, a preparatory injection can be made and the remaining DNA can be purified by modified gradient created by Tony Pava called GC DNA-Prep-01 for high GC content strands. The major peak seen at approximately 10-15% acetonitrile for the Analytical RP-HPLC gradient or 20-25% acetonitrile for the GCDNA-Prep-01 gradient and collected as several fractions. Each fraction was lyophilized and reconstituted in deionized distilled water and then subsequently analyzed by RP-HPLC using the Analytical RP-HPLC gradient or analytical gel electrophoresis.
3.4.4 Dialysis

3.4.4.1 Overview of Dialysis

The last step in the purification process is dialysis. After the synthesized strands are either purified via PolyPak II or preparatory RP-HPLC, and then checked for purity by analytical RP-HPLC or gel electrophoresis, dialysis is performed. Dialysis is the process of separating colloids and colloids in solution by the difference in their rates of diffusion through a semipermeable membrane. The technique's objective is to exchange small ions or molecules across a semipermeable membrane, while retaining the macromolecule. In order to achieve this, it is necessary to select dialysis tubing with the pore size small enough that your macromolecule does not exchange but large enough that the ions, and small complexes are exchanged. The smallest oligomer, the 8-mer Z8A and Z8M with a molecular size of around 3200 Daltons was dialyzed using a cellulose acetate membrane of 1000 MWCO. The dialysis was treated first by cutting approximately a 100 cm segment and immersed in deionized distilled water. Sodium bicarbonate was added to the water in excess and EDTA was added to remove any metals present in the packaging of the dialysis tubing. The solution was then heated to 90°C for 10-15 minutes and then cooled to room temperature. This treatment removed any trace metal ions, destroyed any microbial growth and denatured any DNAses or RNAses. The dialysis tubing was then soaked in deionized distilled water for 2 hours with 2 separate buffer changes over that period. The dialysis bags were then ready to be used. Each 100 cm bag was then cut into 10 cm segments that would be used for dialysis. The 10 cm piece is sealed approximately 2 cm from the bottom of the tube with a dialysis clip and dental floss. Approximately 1 ml of the DNA solution was placed in the dialysis tubing and then sealed on the top with
dialysis clips and dental floss. The sample bag were then placed into a 1 liter vessel of buffer with a magnetic stirrer. The samples were dialyzed with a gentle stir, and multiple buffer changer were performed over a period of 24-48 hours to ensure the complete removal of unwanted ions.

Dialysis poses quite a few advantages over other buffer exchange techniques. Dialysis allows the system to reach a true equilibrium. In dialysis the exchange of ions goes from high concentration to low concentrations. In some cases competing ions can be added to the external solution in order to remove other ions electrostatically linked to the macromolecules. Such as the case of cobalt (III) hexamine, its electrostatic interaction with DNA molecules has proved to be an excellent reversible example. Cobalt (III) hexamine is removed from solution of DNA by introducing a different ion such as NaCl at high concentrations, followed by water to remove the NaCl ions. This proved to be an excellent technique used to reuse DNA strands and prevent re-synthesis of the same oligonucleotides. The only downside to dialysis is that any complexes bound irreversible are unable to be dissociated from the macromolecule. Another drawback to dialysis includes sample loss and sample dilution. Molecules such as DNA that possess highly charged or hydrophobic molecules can adhere to the membrane of the dialysis tubing, reducing the yield. After several buffer exchanges, the solution is transferred to small eppendorf tubes so they can be lyophilized and combined to determine the overall loss due to dialysis. The overall yield of the dialysis is calculated by using equation 5.

Equation 8: \[ \% \text{ yield} = \left( \frac{[\text{DNA}_{\text{post dialyze}}]}{[\text{DNA}_{\text{pre dialyze}}]} \right) \times (100\%) \]
The DNA concentration pre-dialysis and post-dialysis was determined by measuring the absorbance at 260 nm.

3.4.5 Lyophilization

3.4.5.1 Overview of Lyophilization

Lyophilization is the technique in which samples are removed of water or other solvents. Lyophilization does not remove salts, or other ionic interaction associated with salts, such as dialysis does. When using this method to evaporate solvents a few factors must be taken into account such as the strength of the vacuum and the temperature difference between the chamber temperature and the vapor trap. Each sample was placed in an eppendorf tube being careful not to fill the tubes more than ¾ of the tube volume with the tops of the tubes open. The temperature in the chamber is usually set between 25 – 45°C to decrease the amount of time necessary to lyophilize the solvent. The vapor chamber is set to 50°C to ensure the gases from the solvent are distilled and trapped, and the vacuum is set to 10-20 atmosphere (atm).

3.5 Oligomer Characterization

3.5.1 Analytical RP-HPLC

3.5.1.1 Analytical Overview

The purity analysis of oligomers was performed with three different analytical techniques, RP-HPLC, AXHPLC, or UREA-PAGE either singly or orthogonally in various combinations. The definitive assay used for the characterization of purity, size,
and consent was denaturing gel electrophoresis (UREA-PAGE). Unlike RP-HPLC and AX-HPLC, UREA-PAGE can separate N from N-1 oligomers, and was not subject to the limitations of secondary structure. In no instance were the results from a RP-HPLC or AX-HPLC chromatogram relied upon as the sole determinant of oligomer purity. Rather, these data were used as supplementary information to support the UREA-PAGE purity analysis results.

3.5.1.2 Analytical RP-HPLC

In reverse-phased HPLC, the stationary phase is nonpolar, often a hydrocarbon, and the mobile phase relatively polar (such as water, methanol or acetonitrile). In RP-HPLC the most polar component comes out first, and increasing the mobile phase polarity increase the elution time. For our particular DNA systems, this type of HPLC works best. It provides an excellent separation technique that offers selectivity and good resolution in the analysis of the full-length DNA, and its failure sequences. It provided a method to determine the purity of our sample. DNA sequences that are more susceptible to forming secondary structures sometimes elute as multiple peaks due to the structures hydrophobicity. This set back in determining the purity of the sample was very relevant in sequence containing >50% GC. RP-HPLC provides a relatively fast and effective technique to determine if secondary structures are forming, purity of the sample, and the presence of failure sequences. Each sample was dissolved in 100 μl of 0.1M triethylammoniumacetate, pH 7.0 containing approx. 30 μg of DNA. Approximately 50 μl of the reconstituted sample was injected onto the RP-HPLC column using the RP-HPLC gradient listed in tables 8-10. Samples that contained secondary structures were treated
with a denaturing agent and run again.

3.5.1.3 Data Analysis

Each synthesis was scrutinized for secondary structures and impurities. The purity of the sample was determined by a single peak in the chromatogram, along with a correct retention time. Chromatograms that contained more than one peak were isolated and repurified using a RP-HPLC. Samples that continuously contained secondary structure were analyzed by denaturing gel electrophoresis.

3.5.2 Analytical Gel Electrophoresis: Urea-PAGE Electrophoresis

3.5.2.1 Denaturing Gel Electrophoresis

Many different analytical techniques were used to determine purity and size, denaturing gel electrophoresis was the definitive tool used. Each sample was denatured prior to analysis till the completion of the experiment. In RP-HPLC, sample that eluted as multiple peaks can only be re-purified and sent through the column to separate, but denaturing gel electrophoresis eliminates the suggestion of secondary structures forming. The sensitivity of this tool allows for low sample load, very high resolution. In fact, DNA oligomers less than 10 bases provide 1 base pair resolution in most cases.

Denaturing gel electrophoresis separates by size or relative mass (V), charge of molecule (q), and the voltage gradient (E). DNAs are separated in an electrical field based on their mobility. The mobility of a macromolecule (R) is the distance the macromolecule
migrated from the top of the gel relative to a gel marker or molecular weight marker.

Equation 9: \[ R_e = \frac{qE}{f} \]

The theory is that the larger molecules become entrapped within the matrix of the gel, while the smaller elute through the gel first. This sieving process is the basis of separation in nucleic acid purification, allowing the failure sequences to travel faster than the sample DNA molecule. In all denaturation experiments, urea is used as the denaturant. They denature the DNA by competing for the hydrogen bond interactions, which stabilize the structure of DNA molecules. With this in mind, if each DNA sample is denatured, each molecule should have a similar size to mass ratio. Since the charge of the DNA molecule is from the phosphate backbone, larger molecules will have a greater charge to size ratio than smaller molecules, causing separation based on charge and size.

The ability of the gel to hold back the larger DNA molecules is due to the pore size of the gel. The pore size of the gel is related to the % acrylamide concentration. All of the DNA oligomers were analyzed using a 20% polyacrylamide gel. Table 11 can be used as a guide for determining the % acrylamide required to get optimum separation and resolution depending on the size of the sequence.
Table 1: A guide to the optimal acrylamide concentration for PAGE.
A guide to the optimal DNA size ranges that can be analyzed as a function of percent acrylamide concentration is shown below. The Urea-PAGE gels are defined as 8M Urea in buffer relevant to experiment with the polycrylamide concentration listed. Native Gels buffers will vary, and only the percent acrylamide is given. Note that the upper limits of the size ranges specified are optimistic. Good resolution (as opposed to visualization of a band) between the N and N+1 failure of these higher molecular weight species will require polycrylamide gels of lower percentages than those listed.

<table>
<thead>
<tr>
<th>Acrylamide (%)</th>
<th>Fragment Sizes</th>
<th>Migration of Bromophenol</th>
<th>Migration of Xylene Cyanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2 to 8</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>8 to 25</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>25 to 35</td>
<td>12</td>
<td>55</td>
</tr>
<tr>
<td>8</td>
<td>35 to 45</td>
<td>19</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>45 to 70</td>
<td>26</td>
<td>105</td>
</tr>
<tr>
<td>5</td>
<td>70 to 300</td>
<td>35</td>
<td>130</td>
</tr>
</tbody>
</table>

In order to visualize the DNA on the gel, some type of DNA-specific fluorescent must be used. For all gel electrophoresis experiments DNA-specific fluorescent staining dyes, Sybr-Green I (Molecular Probes Inc. Eugene Oregon) and stain-all solution were used.

Each sample corresponded to approximately 3 μg of DNA. The samples were placed into the bottom of a 250 μl microcentrifuge tubes and lyophilized to dryness. During lyophilization, a 20% acrylamide (19:1 acrylamide: bisacrylamide) gel solution containing 8.0 M urea, 40 mM Tris-acetate, 1 mM EDTA, pH 8.0 (1X TAE) was prepared. To 25 ml of gel solution 100 μl of 18% ammonium persulfate, and 50 μl of TEMED was added and immediately mixed. The mixture was immediately poured into a Novex II casting
stand (1 mm x 80 mm x 80 mm) and a 10-well comb was placed. In most cases, a pre-casted gel was used and the acrylamide solution was not needed and the comb was already set in the polymerized gel. During the experiment, the entire electrophoresis set-up was maintained at a temperature of 60°C. After samples were lyophilized and resuspended into loading buffer solution (1X TAE, 90% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol), the samples were then heated to 90°C for 10 minutes to ensure complete denaturation. A molecular size marker (10 bp ladder) was used to determine size of DNA oligomers. Each sample was loaded hot and was electrophoresed at 50 - 60 °C, 80 – 125 V constant voltage, 1X TAE pH 8.0, with gentle stirring, for approximately 1.0 – 1.5 hours or until the lower dye front was within 0.5 cm of the bottom of the gel cassette. Once the gel was run to completion, the gel was removed from the casting shell, and placed into plastic staining trays; a small amount of the staining dye was used, covered with aluminum foil then gently rotated at room temperature. All visualisation was done using an UV-transilluminator and photographs were taken by either an AlphaImager digital camera system or a Sony Cybershot Digital Camera with UV filter.

3.5.2.2 Urea-PAGE Data Analysis

Each sample was inspected for the correct oligomers size relative to the molecular weight marker. Any gel that contained unsatisfactory results such as irregular patterns in the gel, faint bands or poor resolution was repeated. Each sample was also inspected for failure sequences which travel below the main oligomers band.
Figure 26 shows a typical denaturing UREA-polyacrylamide gel for sequences Z8A and Z8M. Both oligomers are an 8 bp strand. Each lane contains either the sequence Z8A or Z8M under denaturing conditions. The gel provides us with useful information pertaining to the size of the DNA molecule and the purity or lack of failure sequences available in your sample.

**Figure 26: Denaturing UREA-polyacrylamide gel stained with Stains-All**

All samples separated on a denaturing polyacrylamide gels are inspected for purity, as indicated by the absence of failure sequences within the linear range of the assay, overall size, as indicated by the relative migration with respect to the molecular size markers, detection response, as indicated by the relative staining intensity of the DNA band, and linear range.
3.5.3 DNA Concentration Determination – The A260 Assay

3.5.3.1 Essential Principles

Spectrophotometry provides a non-destructive technique that provides specific information pertaining to quantitating the concentration of DNA in a sample. Absorbance difference can be related to structural changes in the molecule, such as denaturation in the case of DNA, or going from one state (single-stranded) to another state (double-stranded). This means of measuring the absorbance at a molecular level is why spectrophotometry is so widely used.

The electromagnetic theory of radiation states that a light wave consists of electric and magnetic fields that are perpendicular to each other but are in phase. Equation 10 states, the length of the wave, \( \lambda \), is related to the velocity of light in a vacuum, \( c \), and the frequency, \( \nu \).

\[
\text{Equation 10:} \quad \lambda = \frac{c}{\nu}
\]

The oscillation of the electrical field causes the molecules to interact with the light. By using Planck’s constant, \( h \), and the frequency you are able to determine the amount of energy in a photon.

\[
\text{Equation 11:} \quad E = h\nu
\]

When the energy of the photon corresponds to the difference between a ground state and
excited state of a molecular orbital electron, light energy is absorbed. Biological macromolecules such as DNA absorb light within the visible to ultraviolet range due to the π to π* molecular orbital transition. This phenomenon is caused by the π-electrons of the aromatic rings undergoing transitions towards higher energy states. Each ring system will absorb energy that corresponds to a specific wavelength.

The absorption of light is related primarily through the Beer-Lambert/Bouguer law. According to Bouguer, and later by Lambert, the intensity of transmitted light is related to the intensity of the incident light according to the following relationship (Equation 12),

\[ \log \left( \frac{I}{I_0} \right) = -\alpha b \text{ or } I = I_0 e^{-\alpha b} \]

where \( I \) is the intensity of the transmitted light, \( I_0 \) is the intensity of the incident light, \( \alpha \) is the absorptivity, and \( b \) is the thickness of the medium. According to Beer the absorptivity is equal to the molar absorptivity \( \varepsilon \) times the molar concentration \( c \), therefore;

\[ \alpha b = \varepsilon c b \]

where \( \varepsilon \) is the molar absorptivity (units \( \text{L}/(\text{mol} \cdot \text{cm}) \)), \( c \) is the concentration and \( b \) is the path length. The Beer-Lambert relationship can be redefined with;

\[ \log \left( \frac{I}{I_0} \right) = \varepsilon c b \]
The negative logarithm of the transmittance term is defined as the absorbance giving as the final form, Beer’s law.

\[ A = \varepsilon cb \]

3.5.3.2 Extinction Coefficients

An important factor in determining the concentration of DNA is the extinction coefficient. In Beer’s law, the extinction coefficient relates to the absorptivity of a solution to the concentration (assuming a constant path length of 1 cm). Since the extinction coefficient related to the DNA, which is related to the structure, changes in the solution will alter the extinction coefficient value. The extinction coefficients were calculated on a theoretical basis from the sum of individual molar absorptivities for all nucleotides in the sequence (Summed Extinction Coefficient or SEC). For each equation listed, C is DNA concentration in pmol/μl, Nx where X = A, C, G or T, is the number of nucleotides present in the sequence under consideration, \( \Lambda_{260} \) is the absorption at 260 nm, and \( \varepsilon \) is the molar extinction coefficient.

\[ \varepsilon = (15,200N_A) + (7050N_C) + (12010N_G) + (8400N_T) \]

\[ C (\text{pmol/μl}) = \Lambda_{260} \times \{100([1.5N_A] + [0.71N_C] + [1.2N_G] + [0.84N_T])\} \]

This approach assumes that all nucleotides in a polynucleotide structure have the same or similar molar absorptivities as the free nucleotides in solution. This approximation is only useful if the DNA structure does not assume any secondary structures. The SEC was

95
used to calculate the DNA concentration for most oligonucleotides due to the relative accuracy and universality of this method.

3.5.3.3 Sample Preparation and Spectral Scan Procedures

Prior to each experiment, the cuvettes and buffer were scanned from 400 nm to 200 nm due to possible sources of error arising from mismatched cuvettes. Each sample was prepared with 490 μl of 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, 0.1 mM EDTA, pH 7.0. The samples were mixed using a vortex mixer and cooled to centrifuge for 2-3 minutes. A 1:50 dilution consisting of 10 μl of DNA and 490 μl of phosphate buffer and the absorbance of the DNA sample was measured at 25°C with air in the reference side from 400 nm to 200 nm in 1 nm increments with either 1.0 nm bandwidth (for 2 nm wide cells) or 2.0 nm bandwidth (for 4 nm wide cells), and the data were overlaid on top of the blank buffer scan. The maximum wavelength, the absorbance at 260 nm and the absorbance at 280 nm were recorded for the sample and blank spectra. The concentration of DNA was calculated from the blank-corrected absorbance value at 260 nm, multiplied by the dilution factor divided by the extinction coefficient determined by either the SEC or SEC methods (Equation 17).

\[
[D\text{NA}] = \frac{(A_{260}) \text{ (dilution factor)}}{\epsilon}
\]

Those samples with absorbance values below 0.1 or above 1.5 at 260 nm were repeated with lower or higher dilutions, respectively, than described.
3.6 Cobalt (III) Complex Synthesis

3.6.1 Overview of Synthetic Approach

The five cobalt (III) complexes used to study interactions between DNA oligomers and simple cobalt (III) complexes are shown in figure 27.

Figure 27: The five Cobalt (III) molecules studied

Cobalt (III) Pentaammine Chloride  Cobalt (III) Tetraammine Dichloro

Cobalt (III) Hexaammine

Cobalt (III) Pentaammine Aquo  Cobalt (III) Tetraammine Diaquo

Each complex has a different ligand capable of dissociating and forming another bond between the metal core and another acceptor site. These cobalt (III) molecules have the
ability to interact with various complexes such as other cobalt (III) complexes and DNA molecules. The mode of binding involved in these reactions between DNA molecules and cobalt (III) complexes can range from non-covalent hydrogen bonding to covalent linkages. These complexes also have the ability to form dimers and other high order structures depending on the environment the complex is in and the lability of the ligands.

Taking all of these things into consideration, these five cobalt (III) complexes together allow us to study the interactions associated with cobalt (II) complexes that interact via an electrostatic interaction, and a covalent interaction. We are also able to vary the ligands attached to the core metal and determine if the lability of the ligand plays a part in the binding motif between simple cobalt (II) molecules and DNA. Lastly, these complexes can answer questions relating to conformational changes seen in DNA molecules and how these changes affect the biophysical attributes of DNA oligomers.

3.6.2 Procedure for the Synthesis of Simple Cobalt (II) Complexes

3.6.2.1 Cobalt (III) Pentammine Aquo

Cobalt (III) Pentammine Aquo was synthesized according to a procedure by Schlessinger and Williams in which the carbonato intermediate was formed from the oxidation of cobalt nitrate in the presence of a fourfold excess of ammonia.

Figure 28: Reaction Scheme carbonatopentamminecobalt (III) nitrate

\[ 4\text{Co(NO}_3\text{)}_3(s) + 4\text{NH}_3\text{CO}_2\text{(aq)} + 16\text{NH}_3\text{(l)} + \text{O}_2(g) \rightarrow 4[\text{Co(NH}_3\text{)}_6\text{NO}_3\text{(s)} + 4\text{NH}_3\text{NO}_2\text{(aq)} + 2\text{H}_2\text{O} \]

98
Once the intermediate carbonopentamminecobalt (III) nitrate reaction has come to its completion, the mixture is cooled on a filter, washed with up to 50 ml of ice-cold water, followed by alcohol and ether and dried at 50°C. (~64% yield) The crude material is recrystallized with water, filtered and washed with alcohol, and either and dried (~42% yield). The cobalt (II) derivative, cobalt (III) pentamminesquo is synthesized using the carbonopentamminecobalt (III) nitrate as the starting material. The carbonate intermediate was reacted with a 1:1 concentrated acid and water mixture, usually perchloric acid/water. Other acids such as nitric acid and hydriodic acid can be used for the reaction with the same ratio. The addition of the acid removes the carbonate ligand creating CO₂ gas.

Figure 2b: Reaction Scheme Cobalt (III) pentamminesquo

\[
\text{[Co(NH₃)₆NO₃]NO₃(s) + 3HClO₄ (aq) } \rightarrow \text{[Co(NH₃)₆Cl₃]}(\text{ClO₄})₃ (s) + \text{CO}_2 (g) + \text{HNO}_3 (aq)
\]

The reaction is complete as the carbon dioxide dissipates, then methanol is added to the slurry, filtered, washed with methanol and ether and dried at room temperature. (~88% yield)

3.6.2.2 Cobalt (III) Pentammine Chloro

The pentammineschlorocobalt(III) ion is synthesized by taking 1 gram of ammonium chloride and dissolving it in 9 ml of concentrated ammonia solution in a 100 ml Erlenmeyer flask. The mixture is shaken while 2 grams of finely powdered cobalt (II)
chloride hexa-hydrate is added in portions. A yellow-pink precipitation should form the hexaamminecobalt(II) chloride while the reddish cobalt chloride dissolves and reacts exothermically. Then 2 ml of 30% hydrogen peroxide is added to the warm ammnon chloride slurry in a thin stream from a burette, while continuously stirring in order for the oxidation to be efficient. All the cobalt (II) ammine dissolves to form a deep red liquid with foaming and further evolution of heat, which is corresponding to the production of the pentaaammineaquocobalt(III) ion. When the reaction seems to have come to a half, 6 ml of concentrated hydrochloric acid is added to the heated well-stirred solution. A purple product should precipitate from the almost boiling reaction mixture leaving a pale green-blue supernatant liquid. Heat the solution for 15 minutes on a steam bath, then cool to room temperature and filter off the product in a glass crucible. The mother liquor is discarded. The complex salt is then drained well, washed with 4 ml of ice-cold water in portions, followed by an equal volume of 6M HCl previously chilled to 0°C or below. The product is dried in a 100°C oven for one hour to complete the conversion of any remaining pentaaammineaquocobalt(III) complexes. (~60-75% yield)

Figure 30: Reaction Scheme Cobalt (II) pentaaamminechloro

\[
\text{NH}_3\text{Cl} (s) + \text{NH}_3 (aq) + \text{Co(Cl}_2\text{)} 6\text{H}_2\text{O (s) + 30 % H}_2\text{O}_2 (l) } \rightarrow \\
[\text{Co(NH}_3\text{Cl}_2 (s) + \text{H}_2\text{O (aq) + NH}_4\text{Cl (aq)]]
\]

3.6.2.3 Cis-Cobalt (II) Tetraammine Diaquo (cis-[Co(NH}_3\text{)}_4(H}_2\text{O}_2]^{2+})

The cis-[Co(NH}_3\text{)}_4(H}_2\text{O}_2]^{2+} ion is synthesized by taking 2 grams of the carbonatepentaaamminecobalt (III) Nitrate complex and dissolving it in 55 ml of 0.3 M
sulfuric acid. Once the solution is clear of bubbles from the release of carbon dioxide, 20 – 25 ml of ethanol is added in small portions. The precipitate is filtered off, and washed with 50% ethanol/water until pH 7.0. The final product is air-dried. (~80% yield)

Figure 31: Reaction Scheme cis-Cobalt (III)tetramminediaquo

\[ \text{[Co(NH}_3)_6\text{CO}_3\text{NO}_3\text{](s) + 0.3 M H}_2\text{SO}_4\text{(aq)} \rightarrow \text{cis-[Co(NH}_3)_6\text{(OH)}_2\text{]s} \text{(s) + CO}_2\text{(g)}} \]

3.6.2.4 Cis-Cobalt (III) Tetrammine Dichloro (cis-[Co(NH)_3Cl]^{2+})

A sand bath is heated to >80°C in order to heat the solution to 80°C. One gram (4mmol) of carbonatopentamminecobalt (III) nitrate is dissolved in 5 ml of water in a 50-ml flask, the solution is stirred and heated to 50-60°C for 3 minutes. Concentrated hydrochloric acid (3.3 – ml) is added quickly to prevent the solution from frothing over. The solution is then heated to 80°C for 5 minutes, and a dark green precipitate of the trans-dichlorotetramminecobalt (III) chloride is produced.

Figure 32: Reaction Scheme Cobalt (III) Tetrammine Dichloro

\[ \text{[Co(NH}_3)_6\text{CO}_3\text{NO}_3\text{](s) + HCl (aq)} \rightarrow \text{cis-[Co(NH}_3)_3\text{Cl}]\text{s} \text{(s) + CO}_2\text{(g)}} \]

The solution is cooled to room temperature in an ice bath, and the unpurified product is filtered. The product is washed with 30 ml of ice cold water to dissolve the cis isomer and 101
filtered immediately. Now that the trans isomer has been isolated from the solution, the cis isomer can crystallize by reducing the temperature of the aqueous methanol filtrate solution, filtered and the purple crystals of the cis isomer are collected. (45% - 55% yield)

3.7 Cobalt (III) Complex Characterization

3.7.1 Complex Characterization by UV/Vis Spectroscopy

Each of the cobalt complexes has its own specific spectra depending on the type of ligands attached to the cobalt (III) center, coordination of the ligands, type of isomer synthesized and the oxidation state of the complex. Table 12 lists the expected absorbance max for each of the cobalt (III) complexes.

Table 12: UV/Vis values for cobalt (III) complexes.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Exp. $\lambda_{max}$ (nm)</th>
<th>Exp. $\lambda_{max2}$ (nm)</th>
<th>Litt. $\lambda_{max}$ (nm)</th>
<th>Litt. $\lambda_{max2}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-[Co(NH$_3$)$_4$(CH$_3$)$_2$]</td>
<td>506</td>
<td>CT band</td>
<td>508</td>
<td>-</td>
</tr>
<tr>
<td>cis-[Co(NH$_3$)$_4$(OH)$_2$]</td>
<td>503</td>
<td>CT band</td>
<td>500</td>
<td>357</td>
</tr>
<tr>
<td>[Co(NH$_3$)$_2$OH$_2$]</td>
<td>491</td>
<td>346</td>
<td>487</td>
<td>343</td>
</tr>
<tr>
<td>[Co(NH$_3$)$_2$Cl]</td>
<td></td>
<td></td>
<td>508</td>
<td></td>
</tr>
<tr>
<td>[Co(NH$_3$)$_2$CO$_3$]</td>
<td></td>
<td></td>
<td>507</td>
<td></td>
</tr>
<tr>
<td>[Co(NH$_3$)$_4$]</td>
<td>475</td>
<td>347</td>
<td>477</td>
<td>340</td>
</tr>
</tbody>
</table>

The free cobalt (III) ion is d$^8$ and has relatively the same energy level diagram as does Fe
(II). The $^1A_g$ state of cobalt (III) originates from one of the high-energy singlet states of the free ion and drops rapidly and crosses the $^1T_{2g}$ state at a low $\Delta$ value. Therefore, molecules such as cobalt (III) hexamine, or octahedral cobalt (III) molecules have diamagnetic ground states.

The visible absorption spectra of cobalt (III) complexes are a sum of the transition from the $^1A_g$ ground state to other singlet states. Typically in octahedral cobalt (III) structures will give you two absorption bands found in the visible spectra which represent transitions to the upper states $^1T_{1g}$ and $^1T_{2g}$. In other cobalt (III) complexes that can exist in a cis or trans configuration, certain spectral features are indicative of cis or trans configuration. The reason spectral differences are seen between the cis and trans configuration is due to the splitting of the $^1T_{1g}$ state.

**Figure 33: Electronic Splitting in Cobalt (III) Complexes**

The levels for a regular octahedral and the splitting caused by the replacement of two ligands.
Splitting of the $^1T_{1g}$ will always occur, but the $^1T_{1g}$ state will only split in the trans isomer due to the positions of the ligands. Alternatively, the cis isomer lacks a symmetrical center and would expect a more intense spectrum than a trans isomer. Visible spectroscopy is a useful technique in monitoring and characterizing each of the cobalt (II) complexes.

3.7.2 Characterization by Infrared Spectroscopy

The formation of the cobalt complex can be monitored by IR spectroscopy and can be characterized by the ligand frequencies attached to the metal core.

Table 13: Table of Major Infrared Peaks for Cobalt (III) Complexes Studied.

<table>
<thead>
<tr>
<th>Complex</th>
<th>3163</th>
<th>2360</th>
<th>1558</th>
<th>1320</th>
<th>841</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-[Co(NH$_3$)$_4$(Cl)$_2$]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-[Co(NH$_3$)$_4$(OH)$_2$]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Co(NH$_3$)$_2$OH$_2$]</td>
<td>3276</td>
<td>2360</td>
<td>1558</td>
<td>1308</td>
<td>844</td>
</tr>
<tr>
<td>[Co(NH$_3$)$_3$Cl]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Co(NH$_3$)$_4$]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each of the IR spectra contain a group frequency region and a fingerprint region. First the fingerprint regions of most cobalt (III) complexes are relatively similar, having sharp peaks at ~800, ~1300 and ~1500 cm$^{-1}$. The ligands attached to the cobalt center will alter the group frequency region. For instance the IR spectra for pentaamine(chloro)cobalt(III)
chloride contains a fingerprint region similar to other cobalt (III) complexes, but has a pronounced amine peak around 3276 cm$^{-1}$. The frequency revealed at 2360 cm$^{-1}$ is typical for primary, secondary, or tertiary amines. Each cobalt (III) complex varies depending on the functional group associated with the metal core.

Another example, shown in Figure 32, is an IR spectra of cobalt (III)hexamine. This cobalt (III) molecule has six ammine groups attached to a cobalt metal core. The IR spectra reveal a strong band at around 3120 cm$^{-1}$, typically the group frequency used for amine groups.

Figure 34: IR Spectra of Pentaammine(Chloro)cobalt (III) Chloride

![IR Spectra of Pentaammine(Chloro)cobalt (III) Chloride](image)

The IR spectra for cobalt(III)pentammineaquo shown in figure 33, has a similar IR spectra to the previous two cobalt (III) complexes, but has a slight change in the group frequency. This change in the frequency is caused by the aquo molecule bound to the cobalt core. The water molecule is in equilibrium with a hydroxal group which will
provide a separate peak in the IR spectra. The peak associated with the aquo group comes around 3160 cm\(^{-1}\).

**Figure 35: IR Spectra of Hexamminecobalt(III) Chloride**

![IR Spectra of Hexamminecobalt(III) Chloride](image)

**Figure 36: IR Spectra of Pentaamine(aquo)Cobalt(III) Chloride**

Since IR depends largely on the molecular species that have small energy difference between
various vibrational and rotational states, any substitution of the ligands will alter the IR spectra. IR provided us with a method to monitor a reaction. Only elemental analysis, along with UV/VIS spectroscopy and IR spectroscopy allowed accurate characterization of each cobalt (III) complex.

3.8 DNA Structural Characterization

3.8.1 Circular Dichroism Spectra

3.8.1.1 Overview of Circular Dichroism Spectroscopy

Circular Dichroism is the differential absorption of left- and right-handed circularly polarized light. It provides information on the optical isomerism and secondary structure of molecules. The difference in the absorption (ΔA) between the left- (ΔA_L) and right-handed (ΔA_R) circularly polarized light gives us the equation:

Equation 18: \[ ΔA = (A_L - A_R) \]

This equation can also be expressed as:

Equation 19: \[ ΔA = (ε_L - ε_R) C l \]

Where \( ε_L \) and \( ε_R \) are the molar extinction coefficients for right-handed circularly polarized light and left-handed circularly polarized light, where \( C \) is the molar concentration and \( l \) is the path length. A combination of equation 18 and 19 defines the molar circular dichroism:

Equation 20: \[ Δε = (ε_L - ε_R) \]
Most CD measurements are reported in degrees of ellipticity ($\theta$). The molar ellipticity ($[\theta]$) is equal to $[\theta] = 3298 \Delta \epsilon$.

**Figure 37:** Schematic of the differences in the absorption of left and right handed circular polarized light.

A periodic variation in the polarization of the light beam is induced by the polarization modulator through all ellipticities from left circular through elliptical, unchanged linear and elliptical to right circular. This polarized light passes through the sample to a photomultiplier detector. If the sample is not optically active, the light beam does not vary through this cycle. With the introduction of an optically active sample, a preferential absorption is seen during one of the polarization periods and the intensity of the transmitted light now varies during the modulation cycle. The variation is directly related to the circular dichroism signature of the sample at that wavelength. Successive detection is performed at various wavelengths leads to the generation of the full CD spectrum. For CD to be exhibited for a sample, the sample must be optically active and not superimposable on its mirror image.
3.8.1.2 Sample Preparation and Procedures for CD Spectrophotometry

The DNA stock solution, in either water or low-salt PBS (5 mM NaH₂PO₄, 5 mM Na₂HPO₄, 0.1 mM EDTA, pH 7.0), is diluted to a final strand concentration between 1 to 9 μM in 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, 0.1 mM EDTA, pH 7.0 from concentrated buffer stock solutions. In all cases the optical densities of the DNA solutions were between 0.75 and 1.25 absorbance units at 260 nm. Each sample was treated with the appropriate NaCl or cobalt (III) complex solution by heating to 90-100 °C for 20 minutes, then slowly cooled to 25 °C over 60 minutes. The samples were equilibrated at 2-5 °C for 48 hours prior to measurement of the CD spectra. For measurement of the CD spectra, the samples were scanned from 320 nm to 210 nm, with variable bandwidth, in 1 nm steps, at 25 °C, with a 3-second averaging time. Temperature dependent CD experiments
were also performed to provide information pertaining to how the structure varies with temperature. The temperature ranged from 15°C - 65°C ensuring that the temperature is well below the melting point of the DNA oligomers. Triplicate scans were averaged and then manually background corrected, adjusted for offset and smoothed with the DOS-based Aviv version 3.2 CD software. The corrected data were imported into MS Excel for conversion to molar ellipticity, and then plotted with SigmaPlot, version 5.0, Origin, or Prism version 2.61.

3.8.2 Circular Dichroism Titrations

CD titrations allow a macromolecular transition to be observed as a function of salt concentration. As a drug or molecule binds to a particular part of macromolecule, the structure of that molecule will alter. This binding event is a stoichiometric event, meaning as the concentration of the drug or molecule increases, or the number of binding sites become occupied, the larger the transitional change that occurs. DNA molecules are capable of altering their structure due to sequence and environmental effects. CD titrations allow us to alter the environment by changing the salinity and observing conformational changes in DNA oligomers.

3.8.2.1 Sample Preparation and Procedure

Assuming an extinction coefficient of $8.5 \times 10^5$ for 18-base pair oligomers, the concentration of DNA used in the CD titrations was $1 \times 10^{-5}$ M per strand. Two solutions of each oligomer were prepared in standard phosphate buffer solution, pH 7.0, and in high and low salt concentrations. Both the high and low salt stock solutions were
reconstituted in buffer, heated to 90°C, slowly cooled, and equilibrated for 24 hours at 4°C prior to titration experiment. Aliquots of the high salt concentration are titrated into the low salt concentrated solution containing the same DNA concentration. The solution was allowed to equilibrate for 15 minutes and a CD spectrum was recorded after each addition to monitor any transitional changes. The transitional change is monitored by the wavelength with the greatest difference between the pre-transition and the post-transition by calculating the percent transition:

**Equation 21:**  
\[ \% \text{ transition} = \frac{\Delta \theta_{295} \text{ (salt)}}{\Delta \theta_{295} \text{ (total)}} \times 100 \]

The value $\Delta \theta_{295} \text{ (salt)}$ is the change in the ellipticity at 295 nm at a given salt concentration. The value $\Delta \theta_{295}$ (salt) is equal to the $\theta_{295}$ value at low salt concentration minus the $\theta_{295}$ value at that given salt concentration.

**Equation 22:**  
\[ \Delta \theta_{295} \text{ (salt)} = \Delta \theta_{295} \text{ (low salt)} - \Delta \theta_{295} \text{ (salt)} \]

The value $\Delta \theta_{295} \text{ (total)}$ is the total change in ellipticity and is the difference in the ellipticity values between the low salt and the high salt.

**Equation 23:**  
\[ \Delta \theta_{295} \text{ (total)} = \Delta \theta_{295} \text{ (low salt)} - \Delta \theta_{295} \text{ (high salt)} \]

The salt concentration, [salt], at the transition midpoint is the salt concentration where the percent transition is equal to 50%.
3.8.3 UV-Thermal Melts as a Function of [Salt] (Cobalt (III) Complex)

3.8.3.1 Essential Principles – DNA Structure, $T_m$ and the Differential Ion Binding Term

The shielding of the repulsive negative charged DNA phosphate backbone causes the spacing and rise of the DNA helix to occur. DNA helices can assume a more compacted structure when the counterion condensation between the positively charged ion in solution and the negatively charged phosphate backbone. Instability in DNA is caused by repulsive forces, and the reduction of this force contribute to the overall stability of the DNA molecules. Base stacking is another factor that stabilizes the DNA molecule and determines the overall stability. An indirect mode of determining the stability of a DNA structure is by altering the salt. Salt effects can also indirectly measure the water activity, and the phosphate-spacing between different structural forms through determination of the differential ion binding term. The term, $\Delta n$, known as the differential ion binding term determines the number of ions released due to denaturation. The number of ions released also gives you an insight into the spacing of the phosphate groups along the DNA backbone.

The theoretical basis for this measurement was derived first by Manning and Record who developed the polyelectrolyte theories, and derived an equation describing the relationship between [salt] and $T_m$:

\[
\frac{dT_m}{d\log[M]} = 2.303RT_{m}^{2}/\Delta H_{m} \cdot 1\Delta n
\]

\textbf{Equation 24:}

where $T_m$ is the midpoint of the melting transition, $[M]$ is the molar salt concentration, $R$ is the universal gas constant, $\Delta H$ is the transition enthalpy, and $n$ is the differential ion
binding term. The differential ion binding term allows calculation of the number of sodium ions released upon strand denaturation.

3.8.3.2 Sample Preparation and Procedures for UV-Thermal Melts as a Function of [Sodium Ion]

Samples of DNA were prepared from concentrated stock solutions to achieve a starting DNA concentration between 27 - 37 μg/ml in 110 mM Standard Phosphate Buffer Solution, pH 7.0 with cobalt concentrations spanning the range of 50 μM to 1 mM and sodium ranging from 15 mM to 4.5 M NaCl. The DNA samples were heated from 90 – 100 °C for 10 minutes, cooled to room temperature over 30 minutes, then degassed with a Savant Speed Vac under vacuum for 10 minutes. The samples were placed in quartz cuvettes with silicone caps to prevent evaporation of solvent while samples are heated. A Cary model 100E Bio spectrophotometer with Cary Thermal software was programmed to heat the samples from 10 °C to 90 °C at 0.5 °C/minute, then cool the samples from 90 °C to 10 °C both at 0.5 °C/minute with a 10-minute equilibration between ramps. The temperature “ramps” were repeated for a total of 5 - 8 cycles while absorbance was monitored at the wavelength of maximum absorption difference for each strand. Most thermal melt scans were performed under continuous nitrogen purge of the sample chamber to prevent water condensation on the cuvette surfaces. Thermal melts with significant hysteresis indicating nonequilibrium conditions or noisy melt profiles were repeated.

The Varian "Thermal" software version 2.0 was used to fit the data for $T_m$, enthalpy and the equilibrium constant. The free energy and entropy were automatically derived using

113
the same software from the equilibrium constant and the Gibbs equation. The data were imported into Origin graphical analysis software, and a plot of $T_m$ versus $\log[\text{sodium}]$ or $\log[\text{cobalt]}$ was constructed. The slope of the line was determined from a fit with a least squares linear regression model. The differential ion binding term, was calculated from the slopes of these lines with:

\[ T_m = \left(2.303RT_m^2/\Delta I\Delta n\right)\log[M] \]

where the X-axis is $\log[\text{sodium}]$, $Y$ is the melting temperature $T_m$, $R$ is the universal gas constant, and $n$ is the differential ion binding term.

3.8.4 Singular Value Decomposition

The method of singular value decomposition (SVD) was used to establish the number of significant spectral species contributing to the experimental CD spectra. For each DNA sequence, it covered the range 220 - 320 nm with data spaced at 2 nm intervals. The data was obtained for at least 16 different concentrations. These experimental spectra were combined to create a data matrix as follows. The rows of $A$ contain the molar ellipticity values at each salt concentration at a particular wavelength. Thus, for each wavelength where measurements were taken, there is a row in $A$. Since data were collected at 16 different salt concentrations and 46 wavelengths, $A$ has 46 rows and 16 columns, and is thus a $46 \times 16$ matrix. SVD decomposes $A$ as the product $A = USV$, $U$ is a $46 \times 16$ matrix whose columns are orthonormal basis vectors. $V^t$ is the transpose of a $16 \times 16$ orthonormal matrix $V$. $S$ is a diagonal $16 \times 16$ matrix and contains the so-called singular
values. The coefficients of $V^s$ combine the basis spectra $U$ to form $A$. As a representative result, the five largest singular values obtained from the CD data measured are critiqued. The number of linearly independent orthogonal components contain the number of significant singular values.

Three criteria were used to establish the minimum number of singular values; (1) the magnitude of the singular values; (2) the randomness of corresponding columns of the $U$ and $V$ matrices; and (3) the ability to selectively excluded singular values beyond the minimum number of supposed significant ones. Each singular value is a weighted factor corresponding to basis vector $U$. In order to determine which component obtained by SVD are significantly above the noise, the first-order autocorrelation function must be performed;

**Equation 26:**

$$C(X_i) = \Sigma X_{ij}X_{j+1,i}$$

$X_{ij}$ and $X_{j+1,i}$ are the $j$th and the $j + 1$ row elements of column $i$. $C(X_i)$, is in effect, a measurement of smoothness between adjacent row and elements. Since the columns vectors of $U$ and $V$ are normalized to unity, $C(X_i)$ varies between 1 and -1. Values near -1 indicate rapid row to row variations, or "noise" and suggest randomness. Significant singular values have corresponding non-random columns in the $U$ and $V$ matrices, and the largest positive values of $C(X_i)$. To ensure accuracy, the CD spectra were reconstructed using an $S$ matrix that contained only the highest singular values. The reconstructed data matrix $A$, and the resultant residual plotted and inspected for randomness.
3.9 Thermodynamic Characterisation

3.9.1 UV-Thermal Melts (Optical Melts)

3.9.1.1 Wavelength Optimization

The key is to obtaining precise and accurate DNA melts by monitoring the DNA transition at the correct wavelength. Monitoring of a DNA transition as the incorrect wavelength does not necessarily denote that denaturation is not occurring. Without the correct wavelength, the transition from double stranded to single stranded cannot be observed spectroscopically. As mentioned in the previous sections, the extinction coefficient of DNA is dependent on the DNA sequence, and also, the extent of denaturation of the DNA molecule. DNA molecules that have longer sequences, and more base stacking interaction between bases absorb more light than nucleotides that do not have base stacking interactions between bases. In order to accurately perform a DNA melt, absorbance spectra of a fully denatured and native must be taken. The largest difference at a particular wavelength is the optimum wavelength for the experiment.

3.9.2 Sample Preparation and Procedures for Wavelength Optimization

Each DNA sample was taken from a concentrated stock, diluted to the appropriate concentration using the standard phosphate buffer solution or the appropriate salt concentrations, heated from 90 - 100 °C for 20 minutes, and cooled to room temperature over 45 minutes. A Cary model 100E Bio spectrophotometer with Cary "Scan" software was programmed to equilibrate the DNA solutions at 10 °C (or other start temperature) for 15 minutes then scan the sample from 400 nm to 210 nm with 1 nm bandpass at 200 nm/minute. Upon successful data collection the spectrophotometer was programmed to
equilibrate the DNA solution at 90°C (or other final temperature) for 15 minutes then scan the sample from 400 nm to 200 nm with 1 nm bandpass at 200 nm/minute. The two spectra at different temperature were overlaid and the largest difference at a particular wavelength is used to monitor the DNA thermal denaturation experiment.

3.9.2.1 Theory of UV-Thermal Melt

The extinction coefficient of DNA is important for understanding UV-thermal melts. Solution environment, temperature, sequence content are all factors that affect the extinction coefficient of DNA. Any of these changes alter the base stacking interactions that ultimately stabilize the DNA helices and allow for more light to be absorbed. When a UV-thermal melt is performed, the DNA molecules goes from a double stranded species to a single stranded species. When this transition occurs, an increase in the absorbance occurs due to the loss of base-stacking of the DNA structure. This phenomenon is known as hyperchromism.

The melting curve can be used to determine the melting temperature, the equilibrium populations, the enthalpy of dissociation, the equilibrium constant and lastly through other methods, the free energy and entropy terms for the transition. Melting of DNA can be thought of as a thermodynamically-driven process with two end-states consisting of either the fully folded native form or the single-stranded denatured form. This model is the basis for determining thermodynamic parameters of DNA molecules. For instance, if the denaturant is temperature, then the distribution as reflected in the equilibrium
constant, $K$, of each species is described by the van’t Hoff relationship.

**Equation 27:** \[ \frac{d(\ln K)}{dT} = \frac{\Delta H}{RT^2} \]

Marky and Breslauer redefined this equation by incorporating experimental measurable variables, such as the extent of transition, $\alpha$, the molecularity of the association, $n$, and the strand concentration, $C_s$.

**Equation 23:** $K = \frac{\alpha}{[(C/n)^{n-1} (1-\alpha)^n]}$ (nonself-complementary sequences)

**Equation 29:** $K = \frac{\alpha}{[n(C_s)^{n-1} (1-\alpha)^n]}$ (self-complementary sequences)

From this equation the van’t Hoff enthalpy can be calculated by substituting for $K$ into the van’t Hoff equation, differentiating with respect to $T$ then solving for enthalpy to yield:

**Equation 30:** \[ H_v = \frac{(2 + 2n)RT_n}{6\alpha(\Delta S/\Delta T)} \]

Equation 30 is valid for both self-complementary and non self-complementary sequences. Another method in determining the transition enthalpy is by the shape of the alpha plot. The equilibrium constant, $K$, is calculated from $\alpha$ at each temperature. A plot of $\ln K$ vs $1/T$, along with equation 19, where $K$ is the $\alpha$-derived equilibrium constant, $R$ is the universal gas constant, $\Delta H$ is the van’t Hoff transition enthalpy, and $\Delta S$ is the transition entropy.

118
Equation 31: \[ \ln K = -(\Delta H/RT) + (\Delta S/R) \]

The slope of the plot gives \( \Delta H/R \) and the intercept gives \( \Delta S/R \). As with all model dependent methods, assumptions must be made. First, the information from the denaturation experiments are from a DNA transition that is a two-state transition. Any deviation from this assumption, such as the formation of an intermediate structure, invalidates the use of this model. The second assumption is that the DNA denaturation does not account for any change in heat capacity between the native and denatured forms.

Equation 32: \[ \Delta C_p = 0 \]

Using UV-melting techniques, model-dependent thermodynamics are obtained and used to compare versus calorimetrically derived data to determine if the transition is indeed a two-state. UV-melting techniques also offer a means to establish stability ranking of sequences and is more sensitive than calorimetry and require much lower DNA concentrations.

3.9.2.2 Sample Preparation and Procedures

Each DNA sample was taken from a concentrated stock, diluted to the appropriate concentration using the standard phosphate buffer solution or the appropriate salt concentrations, heated from 90 – 100 °C for 10 minutes, and cooled to room temperature over 45 minutes. A Cary model 100E Bio spectrophotometer with Cary, “Thermal” software was programmed to heat the samples from 10 °C to 90° C at 0.5 °C/minute, then
cool the samples from 90 °C to 10 °C at 0.5 °C/minute with a 10-minute equilibration between ramps. The ramp repeats totaled 6-10 cycles, and the absorbance at the wavelength of the maximum absorption difference for each strand. Thermal monitoring of the sample temperature was accomplished by using the Peltier block probe-set. All thermal melt scans were performed under a nitrogen purge in order to prevent water condensation on the cuvettes. If hysteresis does occur due to a nonequilibrium melt, the experiment was repeated and corrections were made for that particular experiment.

All data was first analyzed by the Varian “Thermal” software version 2.0 using the total strand concentration, the molecularity \( n = 1, 2 \) Duplex \( \rightarrow \) SS and a self-complementary model to fit the data for \( T_m \), enthalpy and the equilibrium constant. The data was then checked manually using Origin and plotted with either Sigma Plot or Origin and entered into a spreadsheet for collation and further data processing.

3.9.3 Differential Scanning Calorimetry (DSC)

3.9.3.2 Principles of DSC in DNA Research

Calorimetry consists of three different types of instrumentation, (1) the batch calorimeter (2) the flow calorimeter and (3) the titration calorimeter. Flow calorimeters measure heat as a function of reaction time, and titration calorimeters measure heat as function of added reactant at some concentration. The batch calorimeter measures heat as a function of some variable. A differential scanning calorimeter measures the heat associated with a particular transition, specifically a DNA molecules going from a double stranded duplex to a single stranded molecule.
Differential scanning calorimeters are of two basic designs – the heat flux or the power compensation calorimeter. The Nano-II DSC is a power compensation calorimeter. Heat flux calorimeters raise the temperature of the reaction cell and measure the heat transferred to the surroundings, or transferred from the surroundings. Power compensation calorimeters measure the heat of a transition as a function of temperature. It does this by measuring the amount of power required to maintain a constant temperature between a sample cell containing the molecule and a blank cell containing the buffer solution alone. Endothermic type reactions will cause the temperature of the sample cell to lag behind, and exothermic reactions will cause the sample cell temperature to run hotter than the reference cell. The amount of heat, in the form of electrical power supplied from or subtracted by the external Peltier device to maintain a constant temperature between the sample cell and the blank cell is measured and reported as the μW of power required as function of temperature. The raw heat capacity data is then converted to the molar heat capacity using the molecular weight, cell volume and the concentration of the sample within the sample cell.

3.9.3.3 Theoretical Basis of DSC

The enthalpy relationship is the theoretical framework that provides thermodynamic data from calorimetric measurements, where the enthalpy, H, is related to the energy of the system, E, and the work done by the system, PV.

Equation 33: \[ H = E + PV \]

Since the enthalpy is not known, only the changes in enthalpy are measured, reducing
Equation 34: \[ \Delta H = \Delta E + \Delta (PV) \]

If this equation 23 is differentiated, we obtain the following relationship defined in terms of energy \((E)\), pressure \((P)\), and volume \((V)\) shown in equation 24:

Equation 35: \[ d\Delta H = d\Delta E + PdV + VdP \]

The energy term, “\(d\Delta E\)”, is related to the heat and work with the following relationship.

Equation 36: \[ d\Delta E = dQ + dW \]

By substituting equation 25 into the differentiated definition for enthalpy, it produces the following relationship between heat \((Q)\), work \((W)\), pressure \((P)\) and volume \((V)\).

Equation 37: \[ d\Delta H = dQ + dW + PdV + VdP \]

Equation 37 is then simplified with some restrictions or assumptions. First, the process is carried out at constant pressure.

Equation 38: \[ dP = 0 \]

Secondly, the only work allowed is mechanical work against the ambient pressure and since the work from the system to the surroundings is negative, equation 27 can be derived.

Equation 39: \[ dW = -PdV \]

Then by substituting both the constant pressure and work relationships into the enthalpy definition, it produces the following relationship with respect to enthalpy and heat capacity.

Equation 40: \[ d\Delta H = dQ - PdV + PdV + VdP \]

122
\[ \frac{dQ_p}{dT} = \frac{dQ_T}{0} + 0 = 0 \]

\[ \frac{dH_p}{dQ} = \frac{dQ}{dQ_T} \]

To convert the heat term, \( Q \), to experimentally and calorimetrically relevant parameters, requires another set of manipulations. Using the relationship between the heat (\( Q \)), temperature and the heat capacity (\( C_p \)) at constant pressure,

**Equation 41:**

\[ C_p = \frac{dQ}{dT} \]

and solving this equation for \( dQ \) produces a relationship that can be substituted into the enthalpy/heat relationship (Equation 42) to produce the following

**Equation 42:**

\[ dH = C_p dT \]

Integration of this equation gives equation 30, which is the equation that makes calorimetry possible.

**Equation 43:**

\[ \Delta H = \int C_p dT \]

Power data from a typical DSC experiment is converted to the molar excess heat capacity using the following relationship where \( Q_e \) is the excess heat at constant pressure, \( T \) is the temperature, and \( M \) is the number of moles of sample.

**Equation 44:**

\[ (dQ_e/dT)(1/N) \approx C_p^\infty \]

The applied excess power to the sample cell is converted to heat using the following relationship where the heat (\( W \)) in \( \mu \)Joules, is related to the current (\( i \)) in \( \mu \)amperes, the resistance (\( R \)), and the time (\( t \))

**Equation 45:**

\[ W = i^2 R t \]
3.9.3.4 Sample Preparation and Procedures

The buffer solution used for the DSC samples, also served as the “blank” scan for the subtraction of the residual heat capacity of the buffer. Buffer solutions with any peaks or baseline drifts larger than 5 µW were discarded. Because the nature of the buffer solution could change upon storage (bacterial growth, shifts in pH etc.) buffer versus buffer measurements were conducted, at a minimum, every fifth sample on the DSC. Samples for DSC were exhaustively dialyzed into the experimentally relevant buffer solution. The DNA samples in this project were dissolved in concentration ranging from 0 µM up to 400 µM. Each of the DNA samples had to be highly concentrated in order to obtain the best resolution. The DNA samples for DSC were between 400 and 555 µg/ml (minimum concentration) in a volume from 750 to 1000 µl. The DNA sample solution was heated from 90 °C to 109 °C for 10 –15 minutes, mixed with a vortex mixer, then rapidly cooled to room temperature by centrifugation in a microcentrifuge for 5 minutes at 14,000 rpm. The DNA solution was filtered through a 0.45 µm nylon syringe filter then degassed in a glass dessicator (without dessicant) for 10 – 15 minutes at room temperature under a vacuum at 25 in Hg. The DSC capillary cells were thoroughly rinsed with duplicate 1 liter washes of deionized distilled water between samples, and then given a final rinse with triplicate 1 ml washes of buffer solution in each cell prior to sample loading. Bubbles within the cells were dislodged by slowly “pumping” the solution within the cell several times. The solutions were allowed to equilibrate inside the DSC until the applied power between the cells reached a constant value over 15 minutes. The cells were pressurized to 3 atmospheres of pressure to prevent evaporation and boiling of the sample. The Nano-II DSC was programmed to heat the samples (“ramp up”) from 5 °C
to 100°C at 0.5 °C/minute, then cool the samples ("ramp down") from 100 °C to 5 °C at 0.5 °C/minute, for 8 to 10 ramps with a 10-minute equilibration between ramps. All data were collected with "RunDSC" software and initially processed with "CpCalc" software from CSC Inc. Post-measurement all thermographic raw data were initially inspected for completeness of the transition, overall shape of the transition, noise level, the number of peaks, the nature of the peaks, the amount of heat required for the transition (peak height), scan-to-scan reproducibility, heating/cooling symmetry, and vertical position of the DSC trace. Acceptable thermographic data were imported into "CpCalc" software for blank subtraction and calculation of the molar heat capacities. If thermograms of a buffer blank were super-imposable then a representative blank scan was selected and subtracted from the heat capacity measurements of all scans of a particular sample. If the thermograms of the blank were not super-imposable across all scans then the individual thermograms were subtracted in a one-to-one correspondence with the sample scans - buffer blank 1 scan was subtracted from sample scan 1 etc.

Thermodynamic information was calculated from the integrated area of the transition curve. The baselines of these transitions were drawn to match, as closely as possible, the slope and/or curvature of the pre/post transition baselines. Selection of the start and stop points for baseline fitting was consistently applied across all scans of a sample, and across all scans of a triplet repeat series. The molar heat capacity, the enthalpy, and the entropy were calculated from the integrated area under the heat capacity curve, the molecular weight in kD, the cell volume and the DNA concentration in mg/ml. The calorimetrically-derived van't Hoff modeling of the data was achieved with either Origin
3.19 Algorithmic Determination of Free Energy from CD Data

3.10.2 Overview

In order to quantitatively analyze a transition, whether it be a three-state or two state, a model must be assumed. A program called FitAll™ provided by Jonathan Chaires allowed for the calculation of the free energy of conformational changes in molecules by entering a few variables and assuming a model for the system. For instance, the B-to-Z transition is a three-state transition. Let us consider this reaction:

Figure 39: Three-state model proposed for the B-to-Z transition

\[ B \xleftrightarrow{K_1} I \xleftrightarrow{K_2} BZ \]

B represents the initial conformation, I is the intermediate, and BZ is the final conformation. Each transition is assumed to be non-cooperative. \( K_1 \) and \( K_2 \) define the salt dependent equilibrium constants for the first and second transition steps. These equilibrium constants are defined as:

Equation 46: \( K_1 = [I] / [B] \times \left( \frac{a_1}{a_1} \right)^{-1} \) \( K_2 = [BZ] / [I] \times \left( \frac{a_1}{a_1} \right)^{-1} \)

In the equation, \( a_1 \) refers to the salt activity. \( a_1 \) refers to the sodium activity at the transition midpoint (i = 1 or 2), and \( \eta \) is a phenomenological coefficient that includes contributions from differential ion and water uptake and release. The parameter is not to
be taken as, or confused with, the parameter $\Delta n$ utilized in polyelectrolyte theories based on counterion condensation. The condensation relation for the three-state model is:

**Equation 47:**

$$C_r = [B] + [I] + [BZ]$$

By substitution of the relations in Equation 47 into equation 48, followed by rearrangement, it is easily expressed as:

**Equation 48:**

$$[B] = C_r / (1 + K_1 + K_2 K_3)$$

### 3.10.3 Analysis of Experiments

Equations 15, 16, and 17 were incorporated into an algorithm suitable for the analysis of the experimental transitions curves for our systems by nonlinear least-square methods. The procedure is as follows. The parameters to be fit are $a_1$, $r_1$, $r_2$, and $n_2$. $C_r$ is known.

At any wavelength and any salt concentration, the apparent molar ellipticity $\theta_{app}$ is assumed to be additive.

**Equation 49:**

$$\theta_{app} = \theta_a ([B]) + \theta_a ([I]) + \theta_{2e} ([BZ]) / C_r$$

The $\theta_a$ refers to the molar ellipticity for the species $x = b$, $I$, $BZ$. The normalized spectral response is shown as
Equation 10: \[ \text{NSR} = (\theta_{\text{end}} - \theta_{\text{init}}) / (\theta_i - \theta_{\text{end}}) \]

The variables \( \theta_i \) and \( \theta_{\text{end}} \) are obtained from data at the beginning and end points of the iterations. The parameters \( \theta_i \) may be treated as either a fugacity parameter or a constant. The K values obtained for each transition can then be used to calculate the Gibbs Free Energy (\( \Delta G \))

Equation 51: \[ \Delta G = -RT \ln K \]

at each concentration along the transition, ultimately attaining the total free energy of the transition.

Equation 52: \[ \Delta G_{\text{total}} = \Delta G_i + \Delta G_k \]

3.11 Isothermal Titration Calorimetry

3.11.2 Overview of ITC

Of all the techniques that are currently available to measure binding, isothermal titration calorimetry is the only one capable of measuring not only the magnitude of the binding affinity but also the magnitude of the two thermodynamic terms that define the binding affinity: the enthalpy (\( \Delta H \)) and entropy (\( \Delta S \)) changes. Recent advances in instrumentation have facilitated the development of experimental designs that permit the direct measurement of arbitrarily high binding affinities, the coupling of binding to protonation/deprotonation processes and the analysis of binding thermodynamics in terms of structural parameters. Because isothermal titration calorimetry has the capability to
measure different energetic contributions to the binding affinity, it provides a unique bridge between computational and experimental analysis. As such, it is increasingly becoming an essential tool in molecular design.

3.11.3 The ITC experiment

The experiment is performed at a constant temperature by titrating one binding partner (called the ‘titrant’, e.g. $L$) into a solution containing the other binding partner (called the ‘tirand’, e.g. $M$) in the sample cell of the calorimeter. After each addition of a small aliquot of $L$, the heat released or absorbed in the sample cell is measured with respect to a reference cell filled with buffer. The heat change is expressed as the electrical power ($W$ s$^{-1}$) required to maintain a constant small temperature difference between the sample cell and the reference cell, which are both placed in an adiabatic jacket. Addition of $L$ is automated and occurs from a precision syringe driven by a computer-controlled stepper motor. The contents of the sample cell are stirred to effect rapid mixing of the reactants.

In commercially available instruments, volumes of sample cells are in the range 0.2–1.4 mL. The amount of titrant required per experiment depends on the magnitude of the heat change; 10–100 nmoles of protein are typical.

Figure 40 shows the raw data of an ITC experiment. Each peak corresponds to the heat released on addition of an aliquot of ligand to the receptor. Integration of the differential power signal with respect to time yields the apparent heat change, $\Delta Q_{app}$, between additions $i$ to $i-1$.
Equation 53: \[ \Delta q_{\text{app}} = \Delta q_i - \Delta q_{i-1} \]

\( \Delta q_{\text{app}} \) corresponds to the area of the \( i \)th peak in Fig. 36A. If \( K_A \) is large and the molar ratio of \( L \) to \( M \) at the beginning of the titration is low, then virtually all the ligand is bound to the receptor and the peak areas are similar. As the fractional saturation increases, \( \Delta q_{\text{app}} \) gradually decreases. Eventually all receptor sites are saturated. Small heat changes registered after full saturation are caused by the heat of ligand dilution, \( q_{\text{dil}} \), and by other nonspecific effects, \( q_{\text{nsp}} \). \( \Delta q_{\text{app}} \) is proportional to the volume of the calorimetric cell, \( V_{\text{cell}} \), to the change in concentration of the bound ligand, \( \Delta [L]^\text{bound} = [L]^\text{bound} - [L]^\text{bound} \), and to the apparent molar enthalpy of association, \( \Delta H_{\text{app}} \).

Equation 54: \[ q_{\text{app}} = \Delta q_i + \Delta q_{i-1} + \Delta q_{i-2} \]
\[ = \Delta [L]^\text{bound} \times V_{\text{cell}} \times H_{\text{app}} \]

\( \Delta q_i + \Delta q_{i-1} \) is obtained from a blank titration of ligand into buffer. \( V_{\text{cell}} \) is known, and \( \Delta H_{\text{app}} \) is constant at fixed pressure, temperature and solvent conditions. \( \Delta H_{\text{app}} \) and \( K_A \) are calculated from:

Equation 55: \[ \Delta q_i = \Delta q_{\text{app}} - \Delta q_{\text{dil}} - \Delta q_{\text{nsp}} - n[M]_{\text{tot}} V_{\text{cell}} \Delta H_{\text{app}} \times R \]

\( [M]_{\text{tot}} \) is the total concentration of \( M \) in the sample cell of the calorimeter. \( \Delta q_i \) is the effective heat change caused by the formation of complex \( ML \) at the \( i \)th step of the titration, and \( R \) is the root of the quadratic equation.
Equation 56:  \[ Y_i^2 - Y = \frac{1}{x} \left[ 1 + \left( \frac{1}{nK_a[M]_{tot}} \right) + \frac{n[L]_{tot}[M]_{tot}}{[L]_{tot}} \right] = 0 \]

\( Y_i \) is the degree of saturation defined by \( Y_i = A \frac{[L]_{tot}}{[M]_{tot}} \). [L]_{tot} is the total concentration of L added until injection i, and n is the number of identical and independent binding sites for the ligand L on the receptor M. A nonlinear regression procedure based on Equation 43 yields \( n, K_a \), and \( \Delta H_{norm} \) from a single titration experiment.

The experimental data can be plotted in two ways. In the differential mode, the total heat accumulated up to injection i is normalized to the total ligand concentration at step i and is plotted against the total ligand concentration at step i (or against the ratio of the total ligand concentration at step i to the total receptor concentration, \([L]_{tot}/[M]_{tot}\)). This yields the familiar sigmoidal titration curve shown in Figure 40B from which the total colorimetric heat change per mol of complex, \( \Delta H_{norm} \), can be calculated. In the integral mode, the total cumulative heat is plotted against the total ligand concentration to yield a hyperbolic saturation curve (Figure 40C). The same parameters are obtained from either plot. Comparative statistical analysis of the two plots can give information about the accumulation of systematic errors. The number of binding sites, n, and \( \Delta H_{norm} \) are strongly correlated, and the successful deconvolution of the binding isotherm often depends on additional independent information about the number of binding sites of M.
Figure 40: A Typical ITC experimental Data including raw and integrated Data

ITC titration data describing the formation of a 16 bp DNA duplex by mixing the complementary strands at 30°C. Panel A shows the differential power signal recorded in the experiment. After integration with respect to time and normalisation per mol of added ligand (which in this case is single-stranded DNA), $\Delta H_{\text{app}}$, $K_a$ and $n$ can be calculated from Eq. 43 by nonlinear least squares analysis, as detailed in the text. The integrated data can be plotted in two ways: as a sigmoidal plot (panel B) or as a hyperbolic saturation curve (panel C). The $\Delta H_{\text{app}}$ obtained from this analysis is a global property of the system corrected for nonspecific effects. The solid lines in panels B and C correspond to $\Delta H_{\text{app}} = 43 kJ / mol$, $K_a = 3.1$, and $n = 1$. 

132
The case discussed applies only to the binding of L to a receptor M with n identical and independent binding sites. In the case of a receptor with multiple binding sites of different affinity, statistical thermodynamic treatment of the data is required. The partition function Q gives a general description of a binding reaction (Wyman and Gill, 1990). Q relates the sum of concentrations of all species to the concentration of an arbitrarily chosen reference species, conventionally the unligated form of a receptor:

\[
Q = \frac{[M] + \sum_{j=1}^{n} [M|j]}{[M]} = 1 + \frac{\sum_{j=1}^{n} [M|j]}{[M]} = 1 + \sum_{j=1}^{n} \beta_j [L]^j
\]

In this equation, \([L]\) and \([M|j]\) are the concentrations of free ligand and ligated receptor, respectively, \(n\) is the number of binding sites on the receptor, and \(\beta_j\) is the overall binding constant relative to the unligated receptor. It can be written in terms of the overall step-wise (macroscopic) binding constants, \(K\), as

\[
\beta_j = \prod_{i=1}^{n} K_i
\]

or in terms of the intrinsic (microscopic) site binding constants, \(k\), as

\[
\beta_j = \frac{n!}{j!(n-j)!} \prod_{i=1}^{n} K_i
\]

Different binding models can be discriminated depending on the definition of \(\beta_j\). In the case of a single binding site, equation 46 reduces to:

133
Equation 60: \[ Q = 1 + K_A[L] \]

where \( K_A \) is the intrinsic (microscopic) site binding constant. If the receptor has one set of \( j \) identical and independent binding sites:

Equation 61: \[ Q = (1 + K_A[L])^j \]

For \( m \) sets, each containing \( j \) identical and independent binding sites:

Equation 62: \[ Q = \prod_{i=1}^{m} (1 + K_{A,i}[L])^j \]

In the case of cooperativity between \( a \) priori identical binding sites, the first ligand binds with \( K_{A,1} = K_A \), the second with \( K_{A,2} = a \times K_A \), the third with \( K_{A,3} = a \times b \times K_A \), and so on. The cooperativity factors \( a, b, \ldots \) account for the change of intrinsic binding affinity of the unoccupied sites when the degree of saturation increases. Equation 49 shows that the deconvolution of the binding isotherm from an ITC experiment requires calculation of the change in the degree of saturation, \( Y_i \), with ligand concentration. In terms of the binding partition function, \( Y_i \) is expressed by \( Y_i \)

Equation 63: \[ Y_i = \frac{\partial \ln Q}{\partial \ln [L]} = \frac{\sum_{j=1}^{\infty} \beta_j [L]^j}{Q} \]

Since \([L]_0 = [M]_0 \times Y_0\), the total heat change after completion of injection \( i \) is:

134
Equation 64: \[ \Delta q_i = M_i \times V_{cell} \times \sum_{j=1}^{n} \frac{\Delta H_{exp,i} \beta[i,L_j]}{Q} \]

With the help of this statistical thermodynamic treatment it is possible to deconvolute a heat binding isotherm of a complex system involving non-equivalents and/or interacting binding sites. There are instructive examples demonstrating the strength of this approach by Eisenstein\(^9\), Ferrari and Lohman\(^8\), Hyne and Spicer\(^1\), and Bruzzone and Connelly\(^9\). The deconvolution has a firm thermodynamic foundation and avoids additional assumptions as is necessary in the analysis of spectroscopic binding data. In practice, however, the success much depends on the quality of the experimental data and on the number of coupled fitting parameters.

3.11.3.2 Free Energy Changes

As in other binding experiments, to obtain reliable binding constants the concentrations of the interacting species have to be in a proper range so that both the free ligands and the complex are populated. If the concentration of binding sites is very much higher than \(1/K_a\), all the ligand added will be bound until saturation, and the binding isotherm as displayed in Fig. 36B has a rectangular shape with a slope approaching infinity. In the opposite case in which the binding site concentration is much below \(1/K_a\), the binding isotherm is very shallow and full saturation is difficult to approach. For accurate values of \(K_a\), the concentration of receptor binding sites should not be very much higher than \(1/K_a\). The dimensionless number obtained by multiplying \(K_a\) with the total binding site
concentration is called the \( \varepsilon \)-value.\(^{93}\) As a rule of thumb, \( \varepsilon \)-values between 10 and 100 give good \( K_A \)-values. Often, however, optimal concentrations are not accessible. For very tight binding reactions, optimal concentrations are too small to yield measurable heat changes. It is for this reason that even with the most sensitive instruments presently available, \( K_A \) higher than about \( 10^7 \text{ M}^{-1} \) (\( \Delta G \approx -50 \text{ kJ mol}^{-1} \) at 25°C) can not be very accurately measured. At the other extreme when \( K_A \) is very low, the concentrations required for \( \varepsilon \)-values between 10 and 100 may be so high that aggregation of macromolecules can obscure the binding reaction.

If \( \Delta G \) of binding is temperature dependent, it is sometimes possible to choose a temperature at which \( K_A \) can be measured by ITC. Unfortunately, often \( K_A \) is practically independent of temperature because of strong enthalpy/entropy compensation. In this case, the thermodynamic linkage theory provides a general framework for calculating high binding constants from an appropriate thermodynamic cycle. For example, \( K_A \) can change with pH and one may choose a pH where \( K_A \) can be obtained by ITC. If the \( pK_a \) values of the ligated and the ligand-free form of the receptor is known, \( \Delta G \) at the tight binding pH conditions can be calculated.\(^{94,95}\) Alternatively, ITC titrations can be conducted by titrating a strongly binding ligand into a solution containing the receptor already saturated with a weaker ligand. Free binding energies are obtained from such a displacement experiment if the strong and weak ligands exhibit suitable differences in binding enthalpy.\(^{96}\)
3.11.3.3 Enthalpy Changes

The molar binding enthalpy $\Delta H_{mb}$ is a fitting parameter according to Eqs. 47 and 52 and is obtained together with $K_a$ from data collected in the optimal concentration range. A better practice is to measure $\Delta H_{mb}$ at concentrations where the binding partners are fully associated when the degree of saturation is still low. In such experiments the accuracy of $\Delta H_{mb}$ is better, yet the $c$-value is too high for accurate determination of $K_a$. Thus, in practice $K_a$ and $\Delta H_{mb}$ are best obtained from experiments performed at different concentration ratios. The enthalpy change measured by ITC is a global property of the whole system. It is the total heat released or absorbed in the calorimetric cell on each addition of the ligand. The total heat contains contributions arising from nonspecific effects. These are the heat of dilution of the ligand into buffer, heat caused by the incomplete match of the temperatures of the solutions in the cell and at the injection syringe tip, or heat effects from mixing of buffers of slightly different chemical composition. The nonspecific heat effects are accounted for by $\Delta H_{dil}$ and $\Delta H_{mix}$ in Equation 47 and 48. But even the corrected heat change $\Delta H_{c}$ of Equation 48 is itself composed of different contributions. This is the reason why the molar enthalpy change is an apparent quantity ($\Delta H_{app}$). It only depends on the initial and final state of the binding reaction, the solvated free molecules and the final solvated complex.

3.11.3.4 Contribution of reorganization of solvent to $\Delta H_{mb}$ and $\Delta G$

From the comparison of the calorimetric enthalpy change in H$_2$O and D$_2$O it was concluded that solvent reorganization accounts for a large portion of $\Delta H_{mb}$ in macromolecules.$^{97,98}$ Indeed, in high-resolution crystal structures, water molecules can be
seen at the complex interface where they may improve the complementarity of the
interacting surfaces, and bound water is sometimes visible at the empty contact surface of
the free molecules.99,100,103 Extended H-bond networks at the complex interface can make
the enthalpy change more favorable, often counterbalanced by an entropic penalty.102,103
That addition or removal of interfacial water contributes to the free energy of binding was
directly shown by lowering the water activity through addition of glycerol or another
eosmolyte.104,105,109 Complexes with a low degree of surface complementarity and with no
net change of hydration are tolerant to osmotic pressure.107

3.11.3.5 Contribution of direct noncovalent bonds to ΔH\text{int}
Apart from the bulk hydration effects, direct noncovalent bonds at the interface contribute to ΔH\text{int}. These contributions may be considered to represent the binding
enthalpy in a strict sense. Yet it is very difficult to sort out the enthalpy of formation of
each specific noncovalent interaction. The net enthalpy effect of a particular noncovalent
bond X-Y at the interface can result from the balance between the interaction enthalpy of
the bond X-Y in the complex and the enthalpies from bonds between the solvent or
solutes and X and Y in the isolated molecules. Further, subtle rearrangements of the
packing interactions at the binding site compared to the free molecule may add to ΔH\text{int}.

Mutational approaches have been tried to analyze the contributions of individual bonds to
the enthalpy change. Examples are alanine scanning mutagenesis 108, removal of a
particular H-bond at the binding site or the construction of double mutant cycles.109,110

Calorimetric analysis of mutants suffers from a major problem: Can one attribute a
change of ΔH\text{int} to the removal of a specific contact? Or is the enthalpic effect of an
indirect nature and decomposition of $\Delta H_{\text{ad}}$ in terms of individual residue-residue contacts or even atom-atom interactions is not possible? It has been argued on theoretical grounds that such decomposition of $\Delta H_{\text{ad}}$ (as well as of $\Delta G$ and $\Delta S$) is not possible (Mark and van Gunsteren, 1994). But others have argued in favor of decomposition of $\Delta H_{\text{ad}}$ (Boreš and Carplus, 1995; Brady and Sharp, 1995). In general, changes of $\Delta H_{\text{ad}}$ are not, or only weakly correlated to changes in $\Delta G$. Recent careful analysis of three protein systems indeed suggests the correlation can not be made.\textsuperscript{112} One reason for this unfortunate situation is enthalpy/entropy compensation. The overall change in binding enthalpy due to a particular mutation can be partly compensated by an entropy change. In general, the only small change in the free energy of binding. This common thermodynamic behavior is thought to reflect a major role of compensating enthalpy and entropy contributions of water to the binding process.\textsuperscript{112,113}

3.11.3.6 *Calorimetric and van't Hoff enthalpy changes.*

The van’t Hoff enthalpy change ($\Delta H_{\text{ad}}$) is calculated from the temperature dependence of $K_a$ obtained either by ITC or calculated from spectroscopic or other data [Eq (46)]. Hence, $\Delta H_{\text{ad}}$ reflects the enthalpy intimately associated with the binding event that causes the signal change, for example the quenching of the fluorescence of a tryptophan residue in the complex $ML$. Therefore, $\Delta H_{\text{ad}}$ equals $\Delta H_{\text{cal}}$ only if the binding reaction follows a two-state transition between free and bound molecules and if the signal change used to calculate $K_a$ reflects the entire population of free and bound molecules. Otherwise, $\Delta H_{\text{ad}}$ and $\Delta H_{\text{cal}}$ are different. Indeed, systematic discrepancies between $\Delta H_{\text{ad}}$ and $\Delta H_{\text{cal}}$ have been reported\textsuperscript{114,115} and there have been attempts to rationalize the differences.
On the other hand, a ratio of $\Delta H_{\text{av}}/\Delta H_{\text{II}} = 1$ can be taken to indicate that a binding event conforms to a two-state transition from free molecules to the complex with no detectable intermediates and with at most very minor contributions from water reorganization, conformational rearrangements, or changes in the state of protonation.

3.11.3.7 Hydration Effects

Water is an important component of DNA and protein structure and therefore it is necessary to account for solvation effects when examining macromolecular stability and biomolecule–ligand interactions. However, the interaction of water and ions with biological molecules is complicated and the thermodynamic consequences of hydration effects can be difficult to rationalize. Currently, there is a lack of detailed thermodynamic studies that are designed to quantify the role of water in binding interactions. Therefore it can be problematic to include a consideration of hydration effects in drug design strategies. One possibility for assessing the role of water in binding interactions as well as nucleic acid/protein stability is to combine the use of calorimetry with osmotic stress. The application of osmotic stress to a solution using neutral solutes is useful for studying water uptake/release since there is a thermodynamic linkage between osmotic pressure dependence and functionally significant changes in hydration of a macromolecular system. By adding neutral solutes such as PEG or dextran to a solution containing the biological molecule osmotic stress is created and movement of water away from the substrate (i.e., into the higher osmotic pressure of the bulk solvent) is favored. If the macromolecule undergoes a conformational change where the molecules expand and water must be taken up to cover the additional surface area, then osmotic stress will
inhibit the conformation change. Conversely, if there is a net loss of solvent exposed surface area, then water must be removed into bulk solvent and an increased osmotic stress will favor the process. Most reactions of interest that involve changes in the hydration of the molecule can be monitored via osmotic stress. ITC is a convenient method for obtaining this type of datum since the variation of the binding constant with osmotic pressure (concentration of osmolyte) can be evaluated. To date osmotic stress has been used to measure the changes in macromolecular hydration for several different reactions involving biological molecules. The effects of altering water activity by the addition of co-solutes on melting of duplex and triplex DNA has been investigated. Here changes in the number of bound waters as DNA unfolds were evaluated and the concomitant effect of the free energy of DNA melting was determined. The release/uptake of water and counterions has also been addressed using osmotic stress methods for drug-DNA interactions as well as TATA binding protein-DNA interactions. Here ITC was used to differentiate the effects of water release and cation binding. An important addition to the repertoire of techniques that can be used to interrogate protein and nucleic acid systems in order to gain insights into hydration effects is pressure perturbation calorimetry (PPC). This novel technique, developed at MicroCal, is designed to be used in conjunction with the ultra-sensitive VP-DSC. PPC works by evaluating the thermal coefficient of expansion of the partial volume of a solute. This is achieved by changing the applied pressure above the solution of protein or DNA under study. The measured heat of the sample with respect to buffer can be used to calculate. These data are useful because they can be used to quantify accessible surfaces where biomolecules and biomolecule-ligand complexes interact with solvent. PPC directly measures volume

141
changes that result from heat induced conformational changes. Therefore, the relationship between volumetric changes, structure and solvation can be conveniently evaluated. It has recently been successfully applied to lipid micellar systems.

### 3.11.3.8 Heat Capacity Changes

Modern ITC instruments allow to precise measurement of $\Delta H_{\text{app}}$ between about 5 and 70°C, and $\Delta C_p$ can be calculated from a plot of $\Delta H_{\text{app}}$ versus $T$ (Eq. 3). In many cases such plots are linear within the experimental error in a narrow temperature range, suggesting that $\Delta C_p$ itself does not depend on temperature. What is the origin of the change in heat capacity when a complex is formed? $\Delta C_p$ is almost always negative if the complex is taken as the reference state. This means that the complex has a smaller heat capacity than the sum of its free components. This and other observations as well as theoretical considerations indicate that $\Delta C_p$ originates from changes in the degree of surface hydration in the free and the complexed molecules, and to a lesser extent also from changes in molecular vibrations.\[11,12,21\] The often seen temperature-independence of $\Delta C_p$ infers that neither the area of contact surface nor the difference of the vibrational content between the complex and its components change within the observed temperature range.

### 3.11.3.9 Calculated $\Delta C_p$

The association of two proteins in a complex can be compared to the folding of a single protein. In both reactions a substantial fraction of polar and nonpolar surface is buried and the degree of surface hydration is likely to change. There are semiempirical methods
to calculate ΔC_p from the change of the water-accessible polar and nonpolar surface area in protein folding and in protein association. Several communications report good agreement between the experimentally determined ΔC_p and that calculated from the molecular surface buried in the complex. However, a good correlation seems to hold only for those interactions that conform to a lock and-key or rigid-body binding model. Very often there models are inadequate. Significant discrepancies between calculated and measured ΔC_p were reported for small ions and binding for protein-protein complexes, and most notably for protein-DNA complexes. It is accepted that a lack of correlation between measured and calculated values of ΔC_p is a consequence of folding transitions coupled to the association event, and also to significant dynamic restriction of vibrational modes at the complex interface. Interestingly, weaker complexes tend to show larger ΔC_p values per unit of surface area of contact. The likely reason is enhanced enthalpic and entropic fluctuation at a less tight complex interface. Large conformational rearrangements during binding and the preexistence of temperature-dependent conformational equilibria can cause deviations from linearity in the plots of ΔH_res against T, that is, temperature-dependent ΔC_p. No doubt, the methods to calculate ΔC_p from the parametrization of structural data need much improvement before becoming reliable predictors of heat capacity changes. In the meantime one has to rely on experiments. Here, a very careful examination of the conformational states of the molecules in isolation and of the complexes they form is required to account for the observed energetic values in structural terms.
3.11.3.10 Entropy

The entropy of association can be calculated from measured values of $\Delta G$, $\Delta H$ and $\Delta C_p$. The change in entropy of a complex relative to the unligated molecules is largely caused by hydration effects because the entropy of hydration of polar and apolar groups is large and there is significant reduction of water accessible surface on binding. Therefore, when a complex is formed, the overall entropy change is often large and often positive. Occasionally, however, ordering of water at the complex interface occurs, which contributes unfavorably to $\Delta S$ and favorably to $\Delta H$. Another important, unfavorable contribution to the entropy change originates from the reduction of side chain mobility at the binding site. Furthermore, a statistical term has to be added to the entropy change to account for the reduction in the number of particles and their degrees of freedom in the complex. Different estimates of the mixing entropy have been discussed, yet it appears that the ‘cratic’ correction accounts adequately for the loss of translation and rotation. Obviously, a negative entropy change can have different origins and, most importantly, does not necessarily indicate that hydration of the interface remains unchanged or increases with respect to the free complex partners. On the other hand, a positive $\Delta S$ is a strong indication that water molecules have been expelled from the complex interface.

The beauty of isothermal calorimetry is in its simplicity, which allows one to obtain the entire set of thermodynamic parameters from performing only a few experiments at a series of different temperatures. The difficulty, however, lies in the fact that the observed heat change, which is the immediate outcome of ITC, is a global property. Only in those cases where the binding reaction follows a lock-and-key or rigidbody mechanism that can
be described by a two-state transition between free and complexed molecules, and in
which there is no change in the protonation state of $L$ and/or $M$ nor in the hydration state
of the interface, $\Delta H_{ap}$ is equal to the 'true' binding enthalpy attributable to noncovalent
bonds in the complex. A good indicator — though no proof — for this simple mechanism is
the equality $\Delta H_{eq} = \Delta H_{ap}$. In the majority of cases $\Delta H_{ap}$ has different origins, which
often are difficult to distinguish. The contributions to $\Delta H_{ap}$ sometimes can be separated.

For example, a positive entropy change is a good indication for the extrusion of water
from the complex interface. A change of $\Delta H_{ap}$ with the chemical nature of the buffer
points to a protonation/deprotonation event concurrent with the binding reaction, and
careful analysis of $\Delta H_{ap}$ with buffer and pH can reveal the number and sometimes even
the nature of the group(s) whose ionization state changes. Finally, calculation of $\Delta C_p$
from the area of the surface buried in the complex (possible only if the necessary three-
dimensional structures are available) and comparison with $\Delta C_p$ obtained by ITC can give
a clue as to changes in conformational states and vibrational contents between the
complex and its free components.
4 RESULTS

4.1 Structural Studies

4.1.1 Cobalt (III) -DNA Complexes
The DNA oligomer's Z8A and Z8M consist of alternating purine pyrimidine sequence.

4.1.2 UV/VIS Spectroscopy
The oligomer Z8A has a typical UV/ VIS spectrum from 320 to 210 nm in physiological conditions (115 nM Na+, pH 7.0). The UV/ VIS spectrum has a peak at 260 nm and a trough at -230 nm. The Z8M has a slightly different UV/ VIS spectrum, possibly due to the sequence. For the Z8M oligomers, there is a trough at -230 nm, and a peak at 266 nm, along with an apparent shoulder around 272 nm. The Z8M sequence was analyzed by gel electrophoresis under denaturing conditions and scrutinized to determine purity of the sample. The sample was also run under native conditions to determine if this change in the spectra is due to secondary structures. Both experiments reveal a sample high in purity and free of major secondary structures. This variation in the spectra between the Z8A and Z8M oligomers can only be due to the disruption of the electronic absorbance from the large bulky methyl substituent at each cytosine.

4.1.3 Circular Dichroism
The structural differences in the two DNA oligomers and any of the Cobalt-DNA oligomers have been observed by Circular Dichroism (CD). The structures of Z8A and Z8M are slightly different at physiological conditions (115 nM Na+, pH 7.0) as seen in Figure 41. Both Z8A and Z8M have typical B-DNA like CD spectra: a peak at 280 nm and a trough at 255 nm, but overall the shape of the spectra vary.

146
Figure 41: The CD spectra of the Z8A and Z8M oligonucleotides

The CD spectra for the Z8A and Z8M sequences at 25 °C in 5 mM monobasic sodium phosphate, 1 mM dibasic sodium phosphate, 0.1 mM EDTA, 100 mM sodium chloride, pH 7.0. The CD spectra are similar to that of B-form DNA structure. All CD spectra have been adjusted for oligomer concentration.

Since CD measures the difference in the left circular polarized light versus the right-handed circular polarized light, the difference in the shape of the spectrum is due to either a great average value for the left versus the right-handed circular light or vice versa. The methylated Z8M oligomers has a slightly different structure than the Z8A oligomers.

4.1.4 Effects of [Na⁺] by CD Spectroscopy

The effects of sodium concentration on the structure of the Z8A and Z8M oligomers were studied by Circular Dichroism. For each of the measurements, the oligomers were prepared in 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, pH 7.0 with sodium chloride added to achieve a total sodium ion concentrations of 0.05, 0.1, 1, 1.5, 2, 2.5, 3, 3.5, or 4 M. For each of the measurements, the DNA concentrations were the same with in each Na⁺ titration, but varied between each experiment. The effects of Na⁺ concentration shows a
response to the dehydration and gives some insight into how these oligomers hydrate.

These particular sequences have been known to alter their conformations under certain [Na\(^+\)] concentrations. The Z8M oligomer has a typical right-handed B-DNA conformation under normal physiological conditions. As we increase the concentration of the NaCl, a change in the CD spectrum begins to occur. This spectral change begins at a concentration of 0.25 M NaCl and continues to change till a concentration of approximately 4.0 M NaCl. The most noticeable change is in the peak and trough of the CD spectrum. For normal B-DNA the peak is formed at 280 nm and a trough at around 255 nm. As we increased the concentration of NaCl, the spectra begins to invert, and the peak is formed at 255 nm and a trough is formed at ~290 nm. The spectrum found at 4.0 M NaCl is typical for a left-handed Z-DNA conformation. We find that the conformational change begins at around 0.25 M NaCl and is completely converted at ~2.0 M NaCl. Figure 42 shows a typical CD titration of NaCl at 25°C for concentrations ranging from 0–2.25 M NaCl.
Figure 42: The CD spectra of the Z8M oligonucleotide at various NaCl Concentrations

The CD spectra for the Z8M sequence at 25 °C at various concentrations of NaCl. The CD data reveals a B-to-Z conformational transition as the NaCl concentration increases. Samples of each strand were prepared in 15 mM NaCl buffer and titrated with the same concentration of that strand in 5.0 M NaCl to monitor the B-to-Z transition. Both the low salt and high salt stock solution were reconstituted in buffer, heated to 95°C, slowly cooled, and equilibrated for 48 hours at 4°C prior to titration experiment. The CD spectrum of Z-ENa is characterized by a trough at 293 nm. The conformational change is complete at around ~3.0 M. All CD spectra have been adjusted for oligomer concentration.

For the analogous sequence, Z8A, the same type of experiment was performed to observe any structural changes due to [Na⁺] effects. The CD spectrum observes small changes in the spectra as we increase the concentration to 2M NaCl shown in Figure 43.
Figure 43: The CD spectra of the ZRA oligonucleotide at various NaCl Concentrations

The CD spectra for the ZRA sequence at 25 °C at various concentrations of NaCl. The CD data does not reveal a B-to-Z conformational transition as the NaCl concentration increases. Samples of each strand were prepared in 15 mM NaCl buffer and titrated with the same concentration of that strand in 5.0 M NaCl to monitor the B-to-Z transition. Both the low salt and high salt stock solutions were reconstituted in buffer, heated to 95°C, slowly cooled, and equilibrated for 48 hours at 4°C prior to titration experiment. At all concentrations of NaCl, the CD spectra have a B-form DNA structure. CD spectra have been adjusted for oligomer concentration.

As we increase the concentration of NaCl, the peak of the spectra for each scan changes with each titration. The peak at 115 mM SPB, pH 7.0 is at 280 nm, but as the concentration increases, the peak shows a slight blue shift. This is seen up to 2.0 M NaCl concentrations. The trough of each of the scans remains at 255 nm at each subsequent titration. The ZRA oligomer is not involved in an apparent B-to-Z conformational change, but due to the blue shift, a structural change is indicated. Typically in drug-DNA interactions, the CD spectrum shows an apparent blue shift and an increase in the
absorption due to some form of intercalation or binding between the bases. The phenomena reported here is quite different. The blue shift could be involved in some other binding event or simply just a dehydration process that alters the structure slightly but not at the magnitude seen with the Z8M oligomer.

A number of techniques are used to monitor conformational changes and determine midpoints of these transitions. The technique used by Shomoly [134] (1991) determines the percent transition by monitoring the ellipticity at a specific wavelength at a given salt concentration using the equation 65 below:

**Equation 65:** \[ \% \text{ transition} = \frac{\theta_{225} (\text{NaCl})}{\theta_{225}(\text{total})} \times 100 \]

The value \( \theta_{225} (\text{NaCl}) \) is the change in the ellipticity at 225 nm at a given salt concentration. The value \( \theta_{225}(\text{total}) \) is equal to the \( \theta_{225} \) value in 115 mM SPB minus the \( \theta_{225} \) value at the given salt concentration, thus generating equation 66:

**Equation 66:** \[ \delta \theta_{225}(\text{NaCl}) = \theta_{225}(115 \text{ mM SPB}) - \theta_{225}([\text{NaCl}]) \]

The value \( \delta \theta_{225}(\text{total}) \) is the total change in ellipticity and is the difference in the ellipticity values between 115 mM NaCl and 2.5 M NaCl.

**Equation 67:** \[ \delta \theta_{225}(\text{total}) = \theta_{225}(115 \text{ mM SPB}) - \theta_{225}(2.5 \text{ M NaCl}) \]
From equation 67, a graph is generated monitoring the fraction of transition for the Z8M oligomer as it goes from a right-handed B-DNA conformation to a left-handed Z-DNA conformation (Figure 44).

**Figure 44: The Normalized Spectra Response as a function of NaCl for the Z8M oligomer**

The fraction of transition for the B-to-Z transition as a function of NaCl concentration for the Z8M oligomer. Experimental data were obtained from the molar ellipticities at 295 nm. The fraction of transition is utilized to observe the number of transitions, and calculate the Gibbs free energy of the B-to-Z transition.

The fraction of transition observes conformational changes at specific concentration of inducer. For our particular system, the B-to-Z transition begins at ~25 mM of NaCl and is fully Z form at ~2.25 M NaCl.

### 4.1.5 Effects of Cobalt (II) Complexes by CD Spectroscopy

To observe the effects of simple Cobalt (II) complexes on Z8A and Z8M, cobalt (II) hexamine was used. The effects of [Co(NH₃)₆]²⁺ concentration on the structure of the Z8A and Z8M oligomers were studied by circular dichroism. For each of the measurements, the oligomers were prepared in 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, pH 7.0.
with cobalt (III) hexammine added to achieve a total cobalt concentration of 50, 100, 150, 200, 250, 300, 350, or 400 μM. For each of the measurements, the DNA concentrations were the same with in each cobalt (III) titration, but varied between each experiment. The effects of cobalt (III) concentration corresponds to a number of different types of binding events possible, such as electrostatic interactions, covalent interactions or simply Van der Waals interactions.

It has been shown that under certain concentrations of [Co(NH₃)₆]³⁺, poly (G-C) oligomers can undergo the B-to-Z conformational change. The 8 base pair DNA duplex is far smaller than any other previously published work investigating conformational changes due to [Co(NH₃)₆]³⁺. Figure 45 is the CD spectrum of Z8M under various [Co(NH₃)₆]³⁺ concentrations. At 115 mM SPB, the Z8M oligomers assumes a right-handed B-DNA conformation, but as the concentration increases a noticeable change in the peak height and trough begin to arise. With increasing concentration of [Co(NH₃)₆]³⁺, an inversion in the CD spectra begins to occur. The spectra change begins at 50 μM [Co(NH₃)₆]³⁺ and the CD spectra are completely inverted at a concentration of 400 μM.

This inversion in the CD spectra is typical for a B-to-Z conformational change. The same experiment was done using the Z8B oligomers and at each concentration the same CD spectra was generated. Unfortunately, there is a limitation on these types of experiments using cobalt. At concentration above 500 μM, aggregation begins to occur, and the CD spectrum is distorted.
The CD spectra for the Z8M sequences at 25 °C at various concentrations of \([\text{Co(NH}_3\text{)}_6]^2+\). The CD data reveals a B-to-Z conformational transition as the \([\text{Co(NH}_3\text{)}_6]^2+\) concentration increases. Samples of each strand were prepared in standard phosphate buffer and titrated with the same concentration of \(\text{Co(NH}_3\text{)}_6^3+\) to monitor the B-to-Z transition. Both the low salt and high salt stock solutions were reconstituted in buffer, heated to 95°C, slowly cooled, and equilibrated for 48 hours at 4°C prior to titration experiment. The conformational change is complete at around 200 μM. All CD spectra have been adjusted for oligomer concentration.

The highest concentration used in this experiment involving the Z8A oligomer was 400 μM. Possibly at higher concentration or under different environmental conditions, the Z8A oligomers may be involved in some type of conformational change similar to its analogue, Z8M, but no structural changes were observed for this particular sequence involving \([\text{Co(NH}_3\text{)}_6]^2+\).

The fraction of transition (equation 54) can also be used for the Z inducer cobalt (III) hexammine. Figure 46 is a plot of the fraction of transition at 295 nm versus the concentration of the \([\text{Co(NH}_3\text{)}_6]^2+\) for the Z8M oligomer.
The graph reveals that the B-to-Z transition using [Co(NH₃)₆]³⁺ begins at a concentration of ~15 µM. The interaction between [Co(NH₃)₆]³⁺ and the Z8M oligomers induces the Z form DNA at much less concentration than NaCl. The Z8A oligomers did not provide enough structural change via CD to account for a fraction of transition for either Z inducer.

4.1.6 Single Value Decomposition

While performing the CD titration for both NaCl and [Co(NH₃)₆]³⁺, an interesting discovery was found. It was thought that the B-to-Z transition would be a simple two step transition, but after reviewing the fraction of transition graphs, an additional transition is observed for both NaCl and [Co(NH₃)₆]³⁺. In order to fully characterize this transition as
a two step transition, singular value decomposition must be performed to determine the number of transitions mathematically.

To perform singular value decomposition, a wavelength interval is chosen every 1 nm and the ellipticity at each wavelength is entered into a matrix. In Cartesian coordinates a vector is a list of three numbers that represent its value as projected on the x, y, and z axes. A digitalized CD spectra follows the same theory, in that it is a vector which corresponds to each wavelength. The vector is equal to the Δε at a particular wavelength.

The raw data measured in Figures 43 and 44 have 64 digitized entries, one at every other wavelength ranging from 210 to 320 nm. When a titration experiment is performed, the vector can then form into a matrix consisting of 16 different titrations and 64 rows. This matrix is known as Matrix A, which contains the 16 different titrations (each row represents a different indicator concentration) and the 64 rows representing the 2-nm increments between 210-320, as shown in Figure 47.

A series representing a set of CD values allows a mathematical equation to be applied to this data set. The theorem used to analyze the data is the singular value decomposition (SVD) theorem. The SVD theorem states that any matrix can be decomposed into a product of three matrices.

Equation 68: \[ A = U S V^T \]

156
### Figure 47: Matrix A of the measured CD spectra for the NaCl titrations of 28M

Matrix A consists of the ellipticities at each wavelength for the set of 16 titrations. The CD data was corrected for con-traction, and the values were put into Mathcad for analysis.

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</table>

The matrix U is a unitary column matrix of orthogonal basis vectors, which has the same dimensions as matrix A. The matrix S has entries called singular values on the main diagonal, and zeros elsewhere. Thus each singular value in S corresponds to and multiplies a particular column basis vector in U.
Figure 48: Matrix $S$ of the measured CD spectra for the NaCl solutions of ZBM

Matrix $S$ is the product of the singular value decomposition of matrix $A$. Matrix $S$ has singular values on the main diagonal and zeros elsewhere.

\[
\begin{bmatrix}
 40.7 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 110.2 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 12.071 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0.0489 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 4.1405 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 2.9321 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\end{bmatrix}
\]

The product matrix $US$ has orthogonal columns terms of the wavelength basis. The matrix $V^T$ is a unitary row matrix of coefficients whose entries fit the basis CD spectra of US to the original data of $A$ in the least-square sense.

Figure 49: Matrix $V^T$ of the measured CD spectra for the NaCl solutions of ZBM

Matrix $V^T$ is the product of the singular value decomposition of matrix $A$. Matrix $V^T$ is a unitary row matrix of coefficients whose entries fit the basis CD spectra of $US$ to the original data of $A$ in the least-square sense.

\[
\begin{bmatrix}
 -0.354 & 0.1598 & 0.2047 & 0.1275 & 0.0194 & 0.1720 & 0.5300 & 0.0052 & 0.0582 & 0.01176 \\
 -0.355 & -0.1994 & -0.02317 & -0.00107 & 0.003325 & -0.38845 & -0.00566 & -0.18449 & 0.23449 & 0.21115 \\
 -0.359 & -0.1718 & -0.09501 & -0.11848 & -0.2031 & -0.08281 & -0.10032 & -0.23835 & -0.07437 & 0.31139 \\
 -0.364 & 0.1455 & -0.12355 & -0.10118 & -0.00110 & 0.04845 & 0.02869 & -0.48089 & 0.037761 & 0.39891 \\
 -0.331 & -0.03322 & 0.17133 & 0.16861 & 0.28116 & -0.17672 & -0.30478 & 0.13364 & -0.04166 & 0.10049 \\
 -0.345 & -0.0456 & -0.19685 & -0.12565 & 0.11907 & -0.07477 & 0.17065 & 0.20797 & 0.2782 & 0.47022 \\
 -0.379 & -0.00755 & 0.43322 & 0.11268 & -0.25372 & 0.25842 & 0.29923 & -0.24808 & 0.501715 & 0.27559 \\
 -0.226 & 0.13445 & -0.12407 & 0.25365 & 0.28914 & 0.43291 & 0.15714 & -0.27335 & 0.50111 & 0.67727 \\
 -0.223 & 0.1149 & -0.07321 & 0.01154 & -0.29604 & -0.27976 & 0.21718 & 0.43939 & -0.10885 & -0.04268 \\
 -0.197 & 0.13935 & -0.16945 & -0.37253 & -0.3016 & 0.1417 & -0.1288 & -0.19665 & -0.37361 & 0.23224 \\
 -0.180 & 0.33996 & -0.4916 & -0.3913 & -0.02721 & 0.40748 & 0.14538 & -0.15955 & 0.12392 & -0.43791 \\
 -0.143 & -0.29904 & -0.035623 & 0.12346 & -0.19383 & 0.006955 & -0.55943 & 0.2472 & -0.00141 & -0.016653 \\
 0.140 & 0.29674 & -0.6332 & -0.46233 & 0.17637 & -0.15999 & -0.28981 & -0.11986 & -0.25585 & -0.02337 \\
 0.037 & 0.48863 & 0.2551 & -0.096885 & -0.39163 & 0.39246 & 0.25153 & -0.2082 & 0.31885 & 0.1769 \\
 -0.008 & -0.41905 & 0.21646 & -0.17004 & 0.3448 & -0.01419 & 0.076411 & -0.09372 & -0.14571 & -0.07256 \\
 -0.003 & -0.41905 & 0.21646 & 0.17004 & 0.3448 & -0.01419 & 0.076411 & -0.09372 & -0.14571 & -0.07256 \\
\end{bmatrix}
\]

The $V^T$ matrix contains the least-square coefficients that fit the basis CD spectra in US to
the original data. The most significant basis CD spectrum represents the common features in the series. Figure 50 is a plot of the three most significant species for the conformational change in the B-to-Z transition.

**Figure 50: Significant Basis Spectra**

The three most significant bases spectra for the B-to-Z transition, obtained by singular value decomposition. Spectra I is the most significant basis spectrum; II is the second and III is the third most significant species. The basis spectra are calculated from the product US, with S containing only the three highest singular values from matrix S.

![Diagram](image)

**Figure 51** plots the coefficient in \( V^T \) as a function of added [salt] for the three most important basis CD spectra.

The third most important basis CD spectrum (I, green) is highly scattered, another indication that this basis is not significant above the noise, and can be investigated analytically by using the first-order autocorrelation function, \( C \), as a measure of smoothness and the \( X_i \) are the elements of the vector.
Figure 51: Residual Plots

The amplitude vectors for the three most significant components as obtained by singular value decomposition from the data for ZSM. The residual plots of the data where obtained from the V matrix, in which the V Coefficient is plotted versus the Z-inducer concentration.

For the data in Figure 47, the significant basis vectors gave values of C greater than 0.3, whereas the meaningless vectors gave values less than 0.1 as shown in Table 14.

Equation 69:

$$ C = \sum_{i=1}^{n} X_i X_i' $$

A residual plot of the data was then generated to observe randomness. The residual plot is found by subtracting the A_{exp} from the A_{calc}.

The data obtained from the SVD analysis suggest that we have three major species involved in the B-to-Z transition for both Z inducers NaCl and Cobalt (II) hexamine.

The data provides a mathematical explanation for the non-second order transition seen in the fraction of transition CD data. This also allows us to propose a mechanism for the B-to-Z transition for the ZSM dimer using NaCl and \((Co(NH)_3)_2\) as the Z inducers.
Table 14: Results of SVD of the CD spectra for the B-to-Z transition

The five largest singular values obtained from the CD data of [Co(NH₃)₆]³⁺. Including the values for matrix U, matrix V and the residual plots. The non-random residual plots predict the major species in the transition.

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<th>Singular value</th>
<th>U matrix</th>
<th>V matrix</th>
<th>Residual plot</th>
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</tbody>
</table>

The data obtained from the SVD analysis suggest that we have three major species involved in the B-to-Z transition for both Z inducers NaCl and Cobalt (III) hexamine.

The data provides a mathematical explanation for the non-second order transition seen in the fraction of transition CD data. This also allows us to propose a mechanism for the B-to-Z transition for the Z8M oligomers using NaCl and [Co(NH₃)₆]³⁺ as the Z inducers.

4.1.7 CD Studies of Co(III) – DNA Complexes

Cobalt (III) complexes bearing labile ligands are of interest due to their ability to associate with the N7 and O6 of guanine. The N7 is a common covalent coordination site; and the O6 position is generally a very common hydrogen bond acceptor site for the hydrogen containing ligands of cobalt (III) complexes. Both DNA oligomers Z8A and Z8M contain potential GC binding regions. This is one of the reason’s Z8A and Z8M was used for these set of experiments.

161
4.1.7.1 Cobalt (III) Pentammine Aqua [(Co(NH₃)₅(H₂O))³⁺]
The second set of cobalt (III) – DNA complexes studied involved cobalt (III) pentammineaqua. Each sample was treated with either 50, 100, 150, 250, 300, 350, or 400 μM concentrations of [(Co(NH₃)₅(H₂O))³⁺]. The DNA concentration was within the range of 5 – 10 x 10⁻³ M in base pairs. The spectra are corrected for concentration variations by determining the molar ellipticity at each concentration. The CD spectra for the Z8A and Z8M oligomers are shown in Figure 52.

Figure 52: CD Spectra of Z8A and Z8M in the presence of [(Co(NH₃)₅(H₂O))³⁺]
The CD spectra of the Z8A and Z8M oligomers in 50 – 400 μM of [(Co(NH₃)₅(H₂O))³⁺]. The structural difference is computed at each concentration and the molar ellipticity is adjusted due to concentration variations of each DNA scan. Each of the DNA oligomers had a concentration of 4.2 x 10⁻³ M in base pairs.

The CD spectra for Z8A and Z8M show very slight variations up to a concentration of 400 μM of the cobalt (III) complex. Previous studies done by Hicks, show that by treating the Z8M with [(Co(NH₃)₅(H₂O))³⁺], followed by heating samples to 90°C and allowing to incubate for at least 48 hours and then dialysis, gives an altered B-DNA CD spectra. This experiment was done in a manner similar to Hick’s in that the DNA samples
were treated with the cobalt (III) complex, heated to 95°C and allowed to incubate for at least 48 hours. The sample had not been dialyzed prior to the cobalt (III) complex was added.

The slight change in the CD spectra is shown by a small isoelectric point located at ~265 nm for the Z8A oligomers and ~275 nm for the Z8M oligomers. Typically any isoelectric point is indicative of a structural change. Though the CD spectrum does not contain a drastic difference from one concentration to another, a structural change is apparent due to the presence of this isoelectric point. The conformation of this cobalt (III) – DNA complex is difficult to obtain but the theory is that the cobalt (III) complex initially electrostatically binds to negatively charged phosphate backbone, and over time buries its way with in the helix to covalently bond to the N7 of the guanine base. This cobalt (III) complex is in equilibrium with the hydroxyl ligand, and since an aquo group is a fairly labile ligand, it should be available to bond with the N7 of the guanine.

4.1.7.2 Cobalt(III)pentamminechloro [(Co)(NH₃)₅Cl]³⁺

The next set of cobalt (III) – DNA complexes studied involved [(Co)(NH₃)₅Cl]³⁺. Each sample was treated with either 50, 100, 150, 250, 300, 350, or 400 μM concentrations of [(Co)(NH₃)₅Cl]³⁺. The DNA concentration was within the range of 5 – 10 x 10⁻⁷ M in base pairs. The spectra are corrected for concentration variations by determining the molar ellipticity at each concentration. The CD spectra for the Z8A and Z8M oligomers are shown in Figure 53.
Figure 53: CD Spectra of Z8A and Z8M in the presence of [(Co)(NH3)6Cl]

The CD spectra of the Z8A and Z8M oligomers in 50 - 400 μM of [(Co)(NH3)6Cl]. The structural difference is compared at each concentration and the molar ellipticity is adjusted due to concentration variations of each DNA scan. Each of the DNA oligomers had a concentration of 5.1 x 10^5 M in base pairs.

Compared to [(Co(NH3)6OH)]+, [(Co)(NH3)6Cl]3+ contains a less effective leaving group (Chloro). The reasoning behind using various cobalt (III) complexes with different ligands is to observe any structural change in the DNA oligomers due to some type of binding event. Since the binding is related to not just the charge of the cobalt complex but also to the labiality of the ligands attached to the cobalt core. In the previous example, [(Co(NH3)6OH)]3+ revealed very little structural changes up to 400 μM. A similar trend is seen with the [(Co)(NH3)6Cl]3+ complex for both the Z8A and Z8M oligomers. As the concentration increases, the CD spectrums remain the same, indicative of no major structural change in the DNA oligomers therefore, no binding is observed by CD.

4.1.7.3 cis-cobalt(III)tetrathiafulvalene, cis-[Co(NH3)6(H2O)2]3+

The fourth set of cobalt (III) - DNA complexes studied involved cis-[Co(NH3)6(H2O)2]3+. Each sample was treated with either 50, 100, 150, 250, 300, 350, or 400 μM.
concentrations of cis-[Co(NH₃)₆(H₂O)₂]³⁺. The DNA concentration was within the range of 5 – 10 x 10⁻⁵ M in base pairs. The spectra are corrected for concentration variations by determining the molar ellipticity at each concentration. The CD spectra for the Z8A and Z8M oligomers are shown in Figure 54.

**Figure 54:** CD spectra of various concentrations of cis-[Co(NH₃)₆(H₂O)₂]³⁺ for Z8A and Z8M Oligomer

CD spectra at 25°C for the DNA oligomer (A) Z8A and (B) Z8M at various concentrations of the cobalt (III) complex, cis-[Co(NH₃)₆(H₂O)₂]³⁺. CD spectra were obtained at 25°C, and the DNA concentration was within the range of 5 – 10 x 10⁻⁵ M in base pairs with a pH of 7.0. Ellipticity is adjusted according to DNA concentration.

An interesting comparison is shown in figure 50. As we previously displayed in figure 38 and 41, the Z8M oligomer due to the methylation of the cytosine induces a structural change at much lower concentrations of the Z inducers. For the cis-[Co(NH₃)₆(H₂O)₂]³⁺ – DNA complexes, the CD spectra’s show the opposite. As the concentration of cis-[Co(NH₃)₆(H₂O)₂]³⁺ increase in the Z8A oligomer, a change in the peak height and a change in the peak location occur. As the concentration of the cobalt increases, a blue shift is revealed with the peak height decreasing revealing some structural change in the
DNA oligomers. On the other hand, the Z8M oligomer shows no apparent change in the CD spectra with increasing cobalt concentrations.

Cis-[Co(NH₃)₆(H₂O)₂]³⁺ is capable of bonding at two locations due to the two labile aquo groups located on the cobalt (III) core. The cis-[Co(NH₃)₆(H₂O)₂]³⁺ is also in a different structural conformation that the other cobalt (III) complexes (octahedral) making it difficult to interact with guanine bases, preventing the interaction with the N7 of the guanine in the Z8M oligomers. The Z8A oligomers does not contain a bulky methyl group that may interfere with the binding of this cobalt (III) complex and the DNA oligomers.

4.1.7.4 Structural Comparisons by CD Spectroscopy

Each of the cobalt – DNA complexes were treated similarly. Any changes in the CD spectra are due to changes in the DNA structure. When comparing each of the cobalt – DNA complexes to the native Z8M oligomers a dramatic change in the CD spectra is observed. For the oligomers Z8M, at lower salt concentrations of 2 M NaCl and 200 mM [Co(NH₃)₆Cl]³⁺, the CD spectra is inverted into a Z conformation, while the other cobalt (III) complexes have similar B-like CD spectra. The most obvious difference between the low and high salt complexes is the blue shift found in the CD spectra’s of cobalt(III)pentammineaquo/chloro/and diqua.

166
Figure 55: CD spectra of Cobalt (III) Complex's and NaCl at high and low concentration

CD spectra at 25°C for the DNA oligomer, Z8M, at (A) low salt concentrations and (B) high concentration. CD spectra were obtained at 25°C in SPB, pH 7.0. Ellipticity is adjusted according to DNA concentration.

4.1.8 Temperature Dependence CD Spectroscopy

Temperature dependent CD spectroscopy allows you to monitor structural changes. Typically, denaturation occurs due to hydrogen bonds breaking leading to destabilization of the helix. A few factors can cause denaturation. The first is a denaturant such as UREA, in which the chemical blocks H-bonding from occurring preventing the stabilization of the DNA duplex. The second is heat, which thermal energy breaks those H-bonds stabilizing the helix. Temperature is an important role in determining stability of the cobalt (III)–DNA complexes and provides insight into the interactions between the cobalt (III) complexes and the Z8A or Z8M oligomers.

Each sample was treated as shown in the previous CD Spectroscopy sections. The Z8A and Z8M oligomers were treated with a cobalt (III) complex at 50, 100, 150, 250, 300, 350, or 400 µM concentrations. The DNA concentration was within the range of 5 – 10 x 10^{-5} M in base pairs. The samples were then heated to 95°C for 10-15 minutes and
allowed to incubate at 5°C for 48 hours. Each experiment required the use of the Peltier system located on the CD instrument in order to heat the sample compartment. Each scan ranged from 320 nm to 210 nm, collecting data at every 1 nm, allowing for an equilibrium time of 10 minutes at each temperature ranging from 25 – 55°C. The buffer blank was then subtracted from the data, and smoothed in Origin, where the data was collected and plotted.

4.1.8.1 \([\text{Co(NH}_2\text{)}_3(\text{H}_2\text{O})]^+\)

The first set of results involved \([\text{Co(NH}_2\text{)}_3(\text{H}_2\text{O})]^+\). The temperature range of 25 – 55°C was utilized to investigate how the structure would react at various temperatures without denaturing the DNA. Prior to these experiments, UV melts were performed so that a melting temperature \((T_m)\) was observed and a temperature required to denature the DNA oligomers was obtained. The results for the \([\text{Co(NH}_2\text{)}_3(\text{H}_2\text{O})]^+ – Z8M DNA oligomer\) are shown in Figure 56.

The results for the \([\text{Co(NH}_2\text{)}_3(\text{H}_2\text{O})]_2\) – Z8A DNA oligomer are shown in Figure 57. The data obtained from the temperature dependent CD scans of both DNA oligomers provide useful information pertaining to the structure of each \([\text{Co(NH}_2\text{)}_3(\text{H}_2\text{O})]^+ – \text{DNA complex at various concentrations. For instance, we learned from these experiments that at each concentration of } [\text{Co(NH}_2\text{)}_3(\text{H}_2\text{O})]^+\), the ellipticity increases with temperature. Another interesting observation is a noticeable red line shift found in each sample. The red line shift is caused by the increase in temperature.

168
Figure 56: Temperature Dependent CD Spectrum of [Co(NH₃)₆(H₂O)]³⁺ at various concentrations

CD spectra's of [Co(NH₃)₆(H₂O)]³⁺ at concentration of 50, 100, 150, 250, 300, 350, or 400 μM for the Z8M oligomer. Each concentration is observed between a temperature of 15 – 55°C to determine if any structural changes may occur due to environmental effects. The DNA concentration was within the range of 5 – 10 x 10⁻⁷ M in base pairs. The samples were then heated to 95°C for 10-15 minutes and allowed to incubate at 5°C for 48 hours prior to CD experiments.
Figure 57: Temperature Dependent CD Spectrum of \(\text{[Co(NH}_3\text{)_6(H}_2\text{O})_2]^3^+\) at various concentrations

CD spectra's of \(\text{[Co(NH}_3\text{)_6(H}_2\text{O})_2]^3^+\) at concentration of 50, 100, 150, 250, 300, 350, or 400 µM for the ZnA oligomer. Each concentration is observed between a temperature of 15 – 55°C to determine if any structural changes may occur due to environmental effects. The DNA concentration was within the range of 5 – 10 x 10^7 M in base pairs. The samples were then heated to 95°C for 10-15 minutes and allowed to incubate at 5°C for 48 hours prior to CD experiments.

4.1.8.2 Cis-[Co(NH)_3(H_2O)_2]^3^+

The second set of experiments involves the cis-[Co(NH)_3(H_2O)_2]^3^+ complex. As previously mentioned, "aquo" groups are very labile groups that are capable of binding covalently to the N7 of the guanine base. This method of temperature dependence can
serve as a secondary technique in determining if temperature disrupts H-bonding more at low or high $[\text{Co}^{II}]$.

**Figure 58: Temperature Dependent CD Spectrum of cis-[Co(NH$_3$)$_6$(H$_2$O)$_2$]$^{3+}$ at various concentrations**

CD spectra's of cis-[Co(NH$_3$)$_6$(H$_2$O)$_2$]$^{3+}$ at concentration of 50, 100, 150, 250, 300, 350, or 400 µM for the Z8M oligomer. Each concentration is observed between a temperature of 15 – 55°C to determine if any structural changes may occur due to environmental effects. The DNA concentration was within the range of 5 – 10 x 10$^{-5}$ M in base pairs. The samples were then heated to 95°C for 10-15 minutes and allowed to incubate at 37°C for 48 hours prior to CD experiments.

4.1.8.3 cis-[Co(NH$_3$)$_6$(H$_2$O)$_2$]$^{3+}$ (Z8A)

171
The idea of the cis-[Co(NH$_3$)$_6$(H$_2$O)$_2$]$^{3+}$ is that due to the two labile aqua groups, it should be more prone to covalently bonding to the DNA bases.

Figure 59: Temperature Dependent CD Spectrum of cis-[Co(NH$_3$)$_6$(H$_2$O)$_2$]$^{3+}$ at various concentrations

CD spectra's of cis-[Co(NH$_3$)$_6$(H$_2$O)$_2$]$^{3+}$ at concentration of 50, 100, 150, 250, 300, 350, or 400 µM for the Z8A oligomer. Each concentration is observed between a temperature of 15 – 50ºC to determine if any structural changes may occur due to environmental effects. The DNA concentration was within the range of 5 – 10 x 10$^{-3}$ M in base pairs. The samples were then heated to 95ºC for 10-15 minutes and allowed to incubate at 5ºC for 48 hours prior to CD experiments.

As shown in Figure 59, at 50 µM of cis-[Co(NH$_3$)$_6$(H$_2$O)$_2$]$^{3+}$ the ellipticity changes

172
drastically as the temperature increases.

As the concentration of cis-[Co(NH$_3)_6$(H$_2$O)$_2$]$^{13+}$ is increased the DNA's stability relative to its structure begins to increase. This interaction between the DNA oligomer and the cis-[Co(NH$_3)_6$(H$_2$O)$_2$]$^{13+}$ may be just electrostatic interactions stabilizing or shielding the phosphate backbone, or it could in fact be related to covalent bonding with the DNA bases.

4.1.8.4 [Co(NH$_3)_6$Cl]$^{13+}$ (Z8A and Z8M)

The [Co(NH$_3)_6$Cl]$^{13+}$ complexes also contain a fairly labile ligand (-Cl) that too is capable of dissociating and the cobalt complex can then bond to a position on the DNA helix.

Unfortunately for the Z8A oligomer, the only slight variation in the DNA structure is seen at 200 μM [Co(NH$_3)_6$Cl]$^{13+}$ at 55 °C (Figure 60).

The Z8M oligomer produced CD spectra's typical of right-handed B-DNA (Figure 61). The variation in the CD spectra between concentrations is related to the temperature. The Z8A - Co(NH$_3)_6$Cl CD data represented a similar trend seen throughout these DNA - cobalt experiments, between the temperature range of 25 - 55°C, little to no structural change is evident. The Z8M oligomer follows the same trend as its analogue Z8A, in that temperature plays a small role in any type of structural change between the temperature range of 25 - 55°C.
Figure 60: Temperature Dependent CD Spectrum of [Cu(NH$_3$)$_2$Cl]$^{1+}$ at various concentration for the ZRA oligomer.

CD spectra's of [Cu(NH$_3$)$_2$Cl]$^{1+}$ at concentration of 50, 100, 150, 250, 300, 350, or 400 µM for the ZRA oligomer. Each concentration is observed between a temperature of 15 - 55°C to determine if any structural changes may occur due to environmental effects. The DNA concentration was within the range of 5 - 10 x 10$^6$ bps in base pairs. The samples were then heated to 95°C for 10-15 minutes and allowed to incubate at 5°C for 48 hours prior to CD experiments.
Figure 61: Temperature Dependent CD Spectrum of [Co(NH₃)₆]Cl²⁺ at various concentrations for the ZnM Oligomer

CD spectra's of [Co(NH₃)₆]Cl²⁺ at concentration of 50, 100, 150, 250, 300, 350, or 400 µM for the ZnM oligomer. Each concentration is observed between a temperature of 15 – 55°C to determine if any structural changes may occur due to environmental effects. The DNA concentration was within the range of 5 – 10 x 10⁻⁷ M in base pairs. The samples were then heated to 95°C for 10-15 minutes and allowed to incubate at 5°C for 48 hours prior to CD experiments.

175
4.1.9 Differential Ion Binding Term

The differential ion binding term was measured for the Z8A and Z8M oligomers in 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, pH 7.0 with sodium chloride or cobalt (III) solution added from a stock solution to produce a final concentration of 15, 25, 50, 100, 250, and 500 mM NaCl and.

The absorbance versus temperature data was collected and a melting temperature was obtained along with a van't Hoff enthalpy for each of the oligomers at each "salt" concentration. The differential ion binding term was calculated from the slope of the Tm versus log [Na⁺] plot and data was fitted by linear regression. The Δn value is obtained from equation 70.

\[ \text{Equation 70:} \quad \frac{dT_m}{d\log [M]} = \left( 2.363RT_m^2 / \Delta H_m \right) \Delta n \]

The data shows very interesting comparisons between the two salts of interest. As suspected the number of ions being released for NaCl would be higher than the number of ions being released due to cobalt (III) hexammine. This relationship is due to the ability for cobalt (III) hexammine to shield the negatively charged phosphate backbone due to its large bulky structure. The NaCl on the other hand is a smaller molecule and is involved in many more electrostatic binding events than a large bulky cobalt complex.
Figure 62: Differential Ion Binding Plots for NaCl and $[\text{Co(NH}_3\text{)}_6^{3+}]$.

The calculated values were obtained from equation 70 and shown in Table 15. The values for $[\text{Co(NH}_3\text{)}_6^{3+}]$ were obtained from concentration range of 50 - 400 µM. The blue line is the ZBA oligomer, while the black line is the ZBM oligomer. Linear correlation provided the slope of the line (>99.5%). The values obtained for NaCl were obtained from a concentration of 0 - 4 M. The blue line (red data points) are the values for the ZBA oligomer, while the black line are the values for the ZBM oligomer (>95%).

Table 15: Differential Ion Binding Values for NaCl and $[\text{Co(NH}_3\text{)}_6^{3+}]$.

The Δn values were obtained from equation 70, and calculated using Origin plotting software.

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Slope</th>
<th>Intercept</th>
<th>$r^2$</th>
<th>$\Delta n_{\text{ori}}$</th>
<th>$T_{\text{ori}}$ [K]</th>
<th>$\Delta n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\text{Co(NH}_3\text{)}_6^{3+}]$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZBA</td>
<td>13.8</td>
<td>371</td>
<td>0.98</td>
<td>40102</td>
<td>354</td>
<td>1.00</td>
</tr>
<tr>
<td>ZBM</td>
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<td>0.99</td>
<td>48102</td>
<td>335</td>
<td>0.60</td>
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<td>NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZBA</td>
<td>14.2</td>
<td>326</td>
<td>0.96</td>
<td>45201</td>
<td>333</td>
<td>1.32</td>
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<tr>
<td>ZBM</td>
<td>7.23</td>
<td>332</td>
<td>0.96</td>
<td>50102</td>
<td>331</td>
<td>0.76</td>
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</tbody>
</table>

The values of "n" for each of the oligomers give you numerical values near the number one, suggesting that one mole of the salt ion is released upon, melting one mole of DNA strand.

177
4.1.10 Native Gel Electrophoresis

The Z8A and Z8M oligomers in the presence of each of the cobalt (III) complexes, were examined by native polyacrylamide gradient-gel electrophoresis to determine the molecularity of the structures formed and investigate migratory difference on gel due to binding. All oligomers, at concentrations spanning the range of 100 to 200 μg/ml were previously equilibrated in 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, pH 7.0, in 200 μM of the cobalt (III) complex solution for a minimum of 48 hours prior and dialyzed versus buffer. Each Cobalt (III) – DNA complex was analyzed parallel to the native strands Z8A and Z8M.

Analysis of the Z8A and Z8M oligomers and their cobalt (III) complexes show duplexes, that migrate at different rates (Figure 63). The Cobalt (III) – DNA complexes migrate at a faster rate than the untreated Z8A and Z8M oligomers. Though a molecular weight marker was not run on this particular gel, each of the samples used were exhaustively dialyzed versus buffer and scrutinized on RP-HPLC prior to solutions being made and taken from the same DNA stock. This ensured that the differences in each lane is not due to changes in length due to failure sequences, or impurities but due to changes in mobility due to shape and possibly size.
Figure 63: Native Gel Electrophoresis of DNA–Cobalt (III) Complexes

Typically the native gels consisted of either a 15 or 20% Acrylamide. For shorter oligomers it provided the best resolution. Each sample was treated with the appropriate cobalt (III) complex, heat annealed, equilibrated at 8°C for 48 hours and exhaustively dialyzed to ensure non-bound cobalt is not present in gel run. The change in the mobility is due to the structural change related to the cobalt (III) complex binding to the DNA.

#1 - Z8A
#2 - Z8A / 400 μM Co(NH₃)₆(OH)₃
#3 - Z8A / 400 μM Co(NH₃)₆(OH)₂
#4 - Z8A / 400 μM Co(NH₃)₆(Cl)
#5 - Z8M
#6 - Z8M400 μM Co(NH₃)₆(OH)
#7 - Z8M400 μM Co(NH₃)₆(OH)₂
#8 - Z8M / 400 μM Co(NH₃)₆(Cl)

The migratory rate of the oligomers in Lane #1 and #5 are the same, showing very little change due to methylation in the Z8M oligomer. A common trend is seen for the oligomers for both Z8A and Z8M. Lane #2 and Lane #6 have the fast mobility, followed by Lane #4 and #8, and finally lanes #3 and #7 have the least mobility. The difference in the mobility between each of the cobalt-DNA complexes is associated with the change
4.2 Thermodynamic

4.2.1 Thermodynamic Parameters by UV-melting technique

The thermodynamic parameters for the Z8A and Z8M sequences were initially determined by optical melting techniques in 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, pH 7.0, 100 mM NaCl, with DNA concentrations between 1.0 μM and 9.5 μM. Each sample was incubated with the cobalt (III) complex or salt and allowed to equilibrate prior to running the samples. The absorbance versus temperature data (Figure 60 - 79) were collected and processed as previously described to obtain the transition melting temperature, the van't Hoff enthalpy, entropy, and Gibbs free energy for duplex to single-strand transition. The values obtained for the melting temperature, van't Hoff enthalpy, entropy and Gibbs free energy are recorded in tables 16 and 17. The derivative of each curve was then determined and plotted versus the temperature.

The Z8A and Z8M oligomers were examined with respect to cobalt (III) complex and concentration of [salt]. The enthalpy of the duplex to single-strand (SS) did not have any trend necessarily relating cobalt structure to enthalpy. The one trend was that as the concentration of the salt increases the enthalpy value became more favorable. A similar trend was found relating the free energy to the cobalt complex except for the data involving the cobalt(III)pentammineaquo and the Z8M oligomer. The data reveals that as the concentration of the cobalt increases the free energy becomes more favorable. This is the only data recorded from this series that showed a favorable free energy as the
concentration increases. The first set of experiments investigates the interactions between the ZSM oligomer and each of the cobalt (III) complexes at high and low salt concentrations. The enthalpy and entropy values were determined by equation 15. The free energy was calculated using the Gibbs Free Energy equation at 37°C (Table 16).

Each set of experiments contained at least 7 different concentrations of the DNA oligomer.

Table 16: Thermodynamic Parameters for the ZSM Oligomer.

The thermodynamic parameters of associated with the ZSM oligomers in the presence of NaCl and Cobalt (III) complex at high and low salt concentrations. The errors in determining the enthalpy and the entropy values from the van't Hoff plots are ± 5%.

<table>
<thead>
<tr>
<th>ZSM</th>
<th>ΔH_{f} (kcal/mol)</th>
<th>ΔS_{f} (kcal/mol K)</th>
<th>ΔG_{f} (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>-64.1 ± 0.2</td>
<td>-93.0 ± 0.5</td>
<td>-36.4 ± 0.3</td>
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<tr>
<td>2 M NaCl</td>
<td>-57.8 ± 0.4</td>
<td>-91.1 ± 0.2</td>
<td>-37.1 ± 0.1</td>
</tr>
<tr>
<td>4 M NaCl</td>
<td>-76.8 ± 0.2</td>
<td>-44.1 ± 0.1</td>
<td>-63.70 ± 0.3</td>
</tr>
<tr>
<td>[Co(NH3)6]Cl2</td>
<td>ΔH_{f} (kcal/mol)</td>
<td>ΔS_{f} (kcal/mol K)</td>
<td>ΔG_{f} (kcal/mol)</td>
</tr>
<tr>
<td>200 μM</td>
<td>-60.1 ± 0.2</td>
<td>-51.3 ± 0.7</td>
<td>-24.8 ± 0.5</td>
</tr>
<tr>
<td>400 μM</td>
<td>-66.5 ± 0.6</td>
<td>-67.6 ± 0.9</td>
<td>-46.3 ± 0.3</td>
</tr>
<tr>
<td>[Co(NH3)6]Cl2</td>
<td>ΔH_{f} (kcal/mol)</td>
<td>ΔS_{f} (kcal/mol K)</td>
<td>ΔG_{f} (kcal/mol)</td>
</tr>
<tr>
<td>200 μM</td>
<td>-67.4 ± 0.3</td>
<td>-110.7 ± 0.4</td>
<td>-34.6 ± 0.3</td>
</tr>
<tr>
<td>400 μM</td>
<td>-70.2 ± 0.3</td>
<td>-115.2 ± 0.2</td>
<td>-35.8 ± 0.5</td>
</tr>
<tr>
<td>[Co(NH3)6(H2O)]NO3</td>
<td>ΔH_{f} (kcal/mol)</td>
<td>ΔS_{f} (kcal/mol K)</td>
<td>ΔG_{f} (kcal/mol)</td>
</tr>
<tr>
<td>200 μM</td>
<td>-56.1 ± 0.5</td>
<td>-81.5 ± 0.8</td>
<td>-21.8 ± 0.3</td>
</tr>
<tr>
<td>400 μM</td>
<td>-62.1 ± 0.1</td>
<td>-145.6 ± 0.2</td>
<td>-18.7 ± 0.6</td>
</tr>
<tr>
<td>cis-[Co(NH3)6(H2O)]Cl2</td>
<td>ΔH_{f} (kcal/mol)</td>
<td>ΔS_{f} (kcal/mol K)</td>
<td>ΔG_{f} (kcal/mol)</td>
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<tr>
<td>200 μM</td>
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<td>-54.8 ± 0.5</td>
<td>-25.0 ± 0.4</td>
</tr>
<tr>
<td>400 μM</td>
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<td>-55.9 ± 0.3</td>
<td>-26.8 ± 0.2</td>
</tr>
<tr>
<td>cis-[Co(NH3)6Cl2]Cl2</td>
<td>ΔH_{f} (kcal/mol)</td>
<td>ΔS_{f} (kcal/mol K)</td>
<td>ΔG_{f} (kcal/mol)</td>
</tr>
<tr>
<td>200 μM</td>
<td>-64.3 ± 0.6</td>
<td>-95.9 ± 0.5</td>
<td>-35.9 ± 0.9</td>
</tr>
<tr>
<td>400 μM</td>
<td>-66.4 ± 0.4</td>
<td>-95.9 ± 0.5</td>
<td>-37.8 ± 0.9</td>
</tr>
</tbody>
</table>

181
The next set of experiments involves investigating the interaction between the Z8A and cobalt (III) complexes along with NaCl. Table 16 contains the enthalpy, entropy and free energy values calculated for each of the cobalt (III) complexes along with NaCl at high and low salt concentrations.

**Table 17: Thermodynamic Parameters for the Z8A Oligomer.**

The thermodynamic parameters associated with the Z8A oligomers in the presence of NaCl and Cobalt (III) complex at high and low salt concentrations. The errors in determining the enthalpy and the entropy values from the van’t Hoff plots are ± 5%.

<table>
<thead>
<tr>
<th></th>
<th>ΔH&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol)</th>
<th>ΔS&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol K)</th>
<th>*ΔG&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z8A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 M NaCl</td>
<td>-79.42 ± 0.2</td>
<td>-112.38 ± 0.1</td>
<td>-43.23 ± 0.1</td>
</tr>
<tr>
<td>4 M NaCl</td>
<td>-81.42 ± 0.2</td>
<td>-121.2 ± 0.2</td>
<td>-45.42 ± 0.2</td>
</tr>
<tr>
<td>[Co(NH₃)₂Cl]Cl₂</td>
<td>ΔH&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol)</td>
<td>ΔS&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol K)</td>
<td>*ΔG&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol)</td>
</tr>
<tr>
<td>200 μM</td>
<td>-75.2 ± 0.1</td>
<td>-111.3 ± 0.5</td>
<td>-42.5 ± 0.6</td>
</tr>
<tr>
<td>400 μM</td>
<td>-83.9 ± 0.2</td>
<td>-123.9 ± 0.3</td>
<td>-46.9 ± 0.6</td>
</tr>
<tr>
<td>[Co(NH₃)₄(H₂O)]NO₃</td>
<td>ΔH&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol)</td>
<td>ΔS&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol K)</td>
<td>*ΔG&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol)</td>
</tr>
<tr>
<td>200 μM</td>
<td>-71.5 ± 0.7</td>
<td>-103.8 ± 0.4</td>
<td>-40.5 ± 0.9</td>
</tr>
<tr>
<td>400 μM</td>
<td>-72.6 ± 0.2</td>
<td>-103.5 ± 0.5</td>
<td>-41.8 ± 0.3</td>
</tr>
<tr>
<td>cis-[Co(NH₃)₄(H₂O)]Cl₂</td>
<td>ΔH&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol)</td>
<td>ΔS&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol K)</td>
<td>*ΔG&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol)</td>
</tr>
<tr>
<td>200 μM</td>
<td>-63.5 ± 0.3</td>
<td>-86.3 ± 0.7</td>
<td>-37.8 ± 0.2</td>
</tr>
<tr>
<td>400 μM</td>
<td>-67.2 ± 0.7</td>
<td>-96.1 ± 0.8</td>
<td>-39.5 ± 0.5</td>
</tr>
<tr>
<td>cis-[Co(NH₃)₄(CH₃)₃]Cl₂</td>
<td>ΔH&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol)</td>
<td>ΔS&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol K)</td>
<td>*ΔG&lt;sub&gt;θ&lt;/sub&gt; (kcal/mol)</td>
</tr>
<tr>
<td>200 μM</td>
<td>-66.3 ± 0.5</td>
<td>-102.3 ± 0.3</td>
<td>-35.8 ± 0.8</td>
</tr>
<tr>
<td>400 μM</td>
<td>-68.5 ± 0.5</td>
<td>-107.3 ± 0.5</td>
<td>-36.5 ± 0.4</td>
</tr>
</tbody>
</table>

The first set of UV melts investigates the two DNA oligomers in the presence of the SPB buffer solution. These values provide a standard to be used to compare versus the treated NaCl and Cobalt (III) – DNA complexes.

182
Figure 64: Absorbance vs Temperature for the Z8A and Z8M in SPB Conditions

Absorbance (273 nm) versus temperature curves for solutions of the Z8M and Z8A oligomer in pH 7.0 SPB conditions. The temperature was increased from 20°C to 95°C at a rate of 0.3°C per minute. An absorbance reading was taken every 0.1°C resulting in 750 absorbance vs temperature points per curve. DNA solutions were melted over a concentration range of 1.0 x 10^-3 to 2.0 x 10^-3 M in base pairs using cuvettes with path lengths of 0.1 cm, 0.5 cm and 1.0 cm. The determination of the melting temperature was determined by obtaining the derivative of the melting curve and plotting ΔA/ΔT versus temperature to obtain the plots to the right.

![Graphs showing absorbance vs temperature for Z8A and Z8M](image)

The values obtained for the melting temperature (T_m) were determined by the peak of the transition in the ΔA/ΔT versus temperature plots. As the concentration of the oligomer increases, the T_m value increases (Figure 60). Many of the scans were omitted due to insufficient upper baselines, and a direct curve analysis was not obtained. Hence the T_m values were obtained from the inflection points of the first derivative (ΔA/ΔT) of the melting plots. For the Z8A and Z8m oligomers at SPB conditions, all of the melting
transitions have monophasic type transitions as shown in figure 60. The linear relationship between the melting temperature and the concentration is shown in figure 65 which resulted in high correlation factors.

**Figure 65: Plot of Tm versus In CT for the Z8A and Z8M oligomer in SPB Solution**

The Z8A and Z8M oligomers were reconstituted in SPB solution, heated to 55°C, slowly cooled and equilibrated for 48 hours at 4°C prior to melting experiment. The solutions of each oligomer were tested over a concentration range of 1.0 x 10^-7 to 2.0 x 10^-5 M per base pair. Each set of plots were analyzed by Origin plotting program using a best fit linear equation to obtain the slope of the line.

The slopes and y intercept calculated the Van't Hoff values. The Van't Hoff values for the Z8A oligomer in standard phosphate buffer conditions yields a -69.34 kcal/mol (ΔH), a -161.83 kcal/mol K (ΔS) and a -39.1 kcal/mol for the free energy (ΔG). The Van't Hoff values for the Z8M oligomer in standard phosphate buffer conditions yields a -64.1 kcal/mol (ΔH), a -93.6 kcal/mol K (ΔS) and a -36.4 kcal/mol for the free energy (ΔG).

184
Figure 66: Absorbance vs Temperature for the Z8A in 2M and 4M NaCl Conditions

Absorbance (273 nm) versus temperature curves for solutions of the Z8M and Z8A oligomers in pH 7.0 SPB conditions. The temperature was increased from 20°C to 95°C at a rate of 0.5°C per minute. An absorbance reading was taken every 0.1°C resulting in 750 absorbance vs temperature points per curve. DNA solution was melted over a concentration range of 1.0 x 10^{-5} to 2.0 x 10^{-5} M in base pairs using cuvettes with path lengths of 0.1 cm, 0.5 cm and 1.8 cm. The determination of the melting temperature was determined by obtaining the derivative of the melting curve and plotting ΔA/ΔT versus temperature to obtain the plots to the right.

The optical melting studies performed for the Z8A oligomers at 2 M NaCl and 4 M NaCl concentrations are shown in figure 61. As previously shown by CD titrations, the Z8A oligomer does not induce a structural change even at high salt concentrations. Therefore, the thermodynamic values obtained for the optical melting studies are related to salt effects and DNA concentration and not conformational changes. As shown in table 16, the free energy relationship reveals that as the NaCl concentration increases, the free
energy value becomes more favorable. The stabilization effect is compensated for by enthalpic stabilization resulting in the more favorable ΔG values. The data for the Z8M oligomer in low and high NaCl concentration is shown in figure 67.

Figure 67: Absorbance vs Temperature for the Z8M in 2 M and 4 M NaCl
Absorbance (273 nm) versus temperature curves for solutions of the Z8M and Z8Δ oligomers in pH 7.0 SPB conditions. The temperature was increased from 20°C to 95°C at a rate of 0.3°C per minute. An absorbance reading was taken every 0.1°C resulting in 75°C absorbance vs temperature points per curve. DNA solution were melted over a concentration range of 1.0 x 10^{-5} to 2.9 x 10^{-5} M in base pairs using cuvettes with path lengths of 0.1 cm, 0.5 cm and 1.6 cm. The determination of the melting temperature was determined by obtaining the derivative of the melting curve and plotting dA/dT versus temperature to obtain the peak to the right.

The duplexes Z8M, under low salt concentrations is in a right-handed, B-DNA
conformation, and the Z8M oligomers under high salt concentration is in a left-handed Z-DNA conformation as previously described by CD. The optical melting studies will provide insight into the thermodynamics related to the left-handed Z conformation stability versus the right-handed B conformation at low salt concentrations.

Figure 68: Plot of $T_m$ versus $M_C$ for the Z8A and Z8M oligomer in 2 M and 4 M NaCl
The Z8A and Z8M oligomers were reconstituted in 2 M and 4 M NaCl, heated to 95°C, slowly cooled and equilibrated for 48 hours at 4°C prior to melting experiment. The solutions of each oligomer were melted over a concentration range of $1.0 \times 10^{-5}$ to $2.0 \times 10^{-5}$ M per base pair. Each set of plots were analyzed by Origin plotting program using a best fit linear equation to obtain the slope of the line.
The next set of optical melting experiments portrays the results of cobalt (III) complexes that interact with either the Z8A or Z8M oligomer. The CD data reveals slight conformational changes in some of the cobalt (III) – DNA complexes. The temperature dependent CD experiments reveal stability increases depending on the cobalt (III) complex ligands. These optical melting studies provide Van't Hoff Analysis on all five cobalt (III) – DNA complexes at low and high concentrations.

Cobalt (III) pentamminechloro is a cobalt (III) complex that contains a chloride ligand, that is capable of interacting with certain bases on the DNA helix, forming a stronger covalent linkage than the H-bond interaction previously established. In turn, the Van't Hoff values should give some indication on whether the stability is due to a binding even or due to some type of electrostatic interaction associated with the cobalt (III) complexes positive charge and the negatively charged phosphate backbone of the DNA molecule.

Cobalt (III) pentamminechloro reveals that as the concentration of the Cobalt (III) complex is introduced, the enthalpy value increases for both Z8A and Z8M oligomer. Also, the entropic contributions to the system in both the Z8A and Z8M become more disordered as the concentration of the cobalt (III) complex increases. This in turn, compensated for the free energy value making it more favorable as the concentration increased.
Figure 65: Absorbance vs Temperature for the ZMA in 200 µM [Co(NH$_3$)$_6$Cl$_4$]$.^4^5$
Absorbance (273 nm) versus temperature curves for solutions of the ZSM and ZMA oligomer in pH 7.0 SPB conditions. The temperature was increased at a rate of 0.5°C per minute. An absorbance reading was taken every 0.1°C resulting in 750 absorbance vs temperature points for each curve. DNA solution was melted over a concentration range of 1.0 x 10$^{-5}$ to 2.0 x 10$^{-5}$ M in base pairs using cuvettes with path lengths of 0.1 cm, 0.5 cm, and 1.0 cm. The determination of the melting temperature was determined by obtaining the derivative of the melting curve and plotting dA/dT versus temperature to obtain the plot to the right.

Figure 70: Absorbance vs Temperature for the ZSM in 200 µM and 400 µM [Co(NH$_3$)$_6$Cl$_4$]$.^4^5$
Absorbance (273 nm) versus temperature curves for solutions of the ZSM and ZMA oligomer in pH 7.0 SPB conditions. The temperature was increased from 20°C to 95°C at a rate of 0.5°C per minute. An absorbance reading was taken every 0.1°C resulting in 750 absorbance vs temperature points per curve. DNA solution was melted over a concentration range of 1.0 x 10$^{-5}$ to 2.0 x 10$^{-5}$ M in base pairs using cuvettes with path lengths of 0.1 cm, 0.5 cm, and 1.0 cm. The determination of the melting temperature was determined by obtaining the derivative of the melting curve and plotting dA/dT versus temperature to obtain the plot to the right.
An example of the typical melting curves for both the Z8A and Z8M in the presence of the cobalt (III) pentamminechloro salt is shown in figure 63. The derivative plots of dA/dT versus temperature provide a comparison of each DNA concentration, and allow the $T_m$ values to be obtained.

Previously published work from Hicks, and Shearow investigate the interaction between cobalt (III) pentammineququa and the oligomer Z8M. Their results showed that the cobalt(III)pentammineququa was involved in some type of binding to the guanine bases of the DNA oligomers. They determined this by Atomic absorption, by incubating the samples in the various concentrations of the cobalt(III)pentammineququa and then dialyzing the samples to remove any of the unbound cobalt (III)pentammine.
Figure 71: Plot of T_m versus ln C_p for the Z3A and ZSM oligomer in 200 μM and 400 μM [Co(NH)₆]Cl₃.

The Z3A and ZSM oligomers were reconstituted in 200 μM and 400 μM [Co(NH)₆]Cl₃, heated to 95°C, slowly cooled and equilibrated for 48 hours at 4°C prior to melting experiment. The solutions of each oligomer were melted over a concentration range of 1.0 x 10⁻⁶ to 2.0 x 10⁻⁵ M per base pair. Each set of plots were analyzed by Origin plotting program using a best fit linear equation to obtain the slope of the line.

Then the samples were put through an AA that detected any cobalt bound to the sample. This insight allowed us to investigate other oligomers, such as the Z3A oligomer and determine if in fact it followed a similar trend as the ZSM oligomer.
Figure 72: Absorbance vs Temperature for the Z2M in 200 μM and 400 μM [Co(NH₃)₆Cl₂(4)²⁺]

Absorbance (273 nm) versus temperature curves for solutions of the Z2M and Z8A oligomer in pH 7.6 SPB conditions. The temperature was increased from 20°C to 95°C at a rate of 0.3°C per minute. An absorbance reading was taken every 0.1°C resulting in 750 absorbance vs temperature points per curve. DNA solution were melted over a concentration range of 1.0 x 10⁻⁷ to 2.0 x 10⁻⁵ M in base pairs using cuvettes with path lengths of 0.1 cm, 0.5 cm and 1.0 cm. The determination of the melting temperature was determined by obtaining the derivative of the melting curve and plotting dA/dT versus temperature to obtain the plots to the right.

The results for the Z2M oligomer are shown in figure 65, which has the typical optical melting scans for the Z2M oligomer at low and high cobalt (III) pentamminespiro concentrations and the dA/dT versus temperature plots. The Tm versus [DNA] plots (figure 71) provided high correlation values that produced the Van’t Hoff Values listed in table 15 and 16. For the Z8A oligomer, the introduction of the
cobalt(III)pentammineaquo increased the decreased the enthalpic, entropic and free energy value at the concentration of the cobalt complex increased. Compared to the Z8A oligomer in SPB solution, the cobalt (III) pentammineaquo – DNA complex are slightly more stable than the pentamer oligomer (Z8A in SPB solution). As for the Z8M oligomer, it follows a similar trend as the Z8A oligomers does.

Figure 73: Plot of $T_m$ versus in $C_i$ for the Z8A and Z8M oligomer in 200 μM and 400 μM Cu(NH$_3$)$_6$(H$_2$O)$_2$$^{2+}$

The Z8A and Z8M oligomers were reconstituted in 200 μM and 400 μM [Cu(NH$_3$)$_6$(H$_2$O)$_2$$^{2+}$], heated to 95°C, slowly cooled and equilibrated for 48 hours at 4°C prior to melting experiment. The solutions of each oligomer were melted over a concentration range of 1.0 x 10$^{-5}$ to 2.0 x 10$^{-5}$ M per base pair. Each set of plots were analyzed by Origin plotting program using a best fit linear equation to obtain the slope of the line.

The Z8M oligomer increases its stability as the concentration of the cobalt (III) pentammineaquo is introduced. The Z8M oligomer also has a slightly greater enthalpic,
The interaction between the Z8A and Z8M oligomer with cis- cobalt (III) tetramminediaquap is expected to interact with the DNA molecules more favorably than if the cobalt core had just one aquo as in the case with cobalt (III) pentammine aqu. The optical melting scans shown in figure 74, reveal a two-state transition, with upper and lower baselines present for both Z8A and Z8M oligomers. The data is then plotted ΔA/ΔT versus temperature and the Tm values where obtained. As the concentration of the DNA increases the Tm values increase.

Figure 74: Absorbance vs Temperature for the Z8M in 200 μM \( \text{Co(NH}_3\text{)}_6\text{(H}_2\text{O})_2\text{Cl}^+ \)
Absorbance (275 nm) versus temperature curves for solutions of the Z8M oligomer in pH 7.0 SPB conditions. The temperature was increased from 20°C to 95°C at a rate of 0.3°C per minute. An absorbance reading was taken every 0.1°C resulting in 750 absorbance vs temperature points per curve. DNA solution were melted over a concentration range of 1.0 x 10^{-5} to 2.0 x 10^{-3} M in base pairs using cuvettes with path lengths of 0.1 cm, 0.5 cm and 1.0 cm. The determination of the melting temperature was determined by obtaining the derivative of the melting curve and plotting ΔA/ΔT versus temperature to obtain the plot to the right.

The results of the optical melting studies where obtained again by plotting Tm versus in Cl, and obtaining a slope of the best fit line. As you can see, the data are quite linear and van't Hoff analysis is easily obtained.

194
Figure 75: Plot of $T_m$ versus $C_T$ for the Z8A and Z8M oligomer in 200 μM and 400 μM \( \text{Fe}(\text{H}_{2} \text{O})_{6}^{3+} \)

The Z8A and Z8M oligomers were reconstituted in 200 μM and 400 μM \( \text{[Co(Ni)}_{3}\text{H}_{6} \text{O)}_{6}^{2+} \)
heated to 95°C, slowly cooled and equilibrated for 48 hours at 5°C prior to melting experiment. The solutions of each oligomer were melted over a concentration range of 1.0 x 10^{-3} to 2.0 x 10^{-3} M per base pair. Each set of plots were analyzed by Origin plotting program using a best fit linear equation to obtain the slope of the line.

The least-square linear regression analysis of these data resulted in high correlation factors, and the slopes and y intercept calculated from these plots were used to determine the thermodynamic parameters listed in table 15 and 16.

The cis-cobalt(III)tetraminedichloro series investigated how the addition of another while ligand would interfere with the stability of the Z8A and Z8M oligomers. The data
below shows a typical optical melting scan for Z8M oligomer in the presence of 200 and 400 µM of cis-cobalt(III)tetroamine dichloro.

Figure 7c: Absorbance vs Temperature for the Z8M Oligomer in 200 µM and 400 µM [Co(NH₃)₆Cl₂]³⁺
Absorbance (273 nm) versus temperature curves for solutions of the Z8M oligomer in pH 7.0 [Co(NH₃)₆Cl₂]³⁺ conditions. The temperature was increased from 20°C to 95°C at a rate of 9.3°C per minute. An absorbance reading was taken every 0.1°C resulting in 750 absorbance vs temperature points per curve. DNA solutions were melted over a concentration range of 1.0 x 10⁻⁷ to 2.0 x 10⁻⁷ M in base pairs using cuvettes with path lengths of 0.1 cm, 0.5 cm and 1.0 cm. The determination of the melting temperature was determined by obtaining the derivative of the melting curve and plotting dA/dT versus temperature to obtain the plots to the right.

The van't Hoff values determined by the figures below provide interesting data revealing that the cis-cobalt(III)tetroamine dichloro is enthalpically less favorable for both DNA
oligomers Z8A and Z8M compared to the parent oligomer. In fact, the entropic values obtained and the free energy values are relatively the same as the parent strand.

Figure 77: Plot of $T_m$ versus $C_1$ for the Z8M oligomer in 200 µM and 400 µM [Co(NH$_3$)$_6$(Cl)$_2$]$^{3-}$

The Z8A and Z8M oligomers were reconstituted in 200 µM and 400 µM [Co(NH$_3$)$_6$(Cl)$_2$]$^{3-}$, heated to 95°C, slowly cooled and equilibrated for 48 hours at 4°C prior to melting experiment. The solutions of each oligomer were melted over a concentration range of $1.0 \times 10^{-3}$ to $2.0 \times 10^{-2}$ M per base pair. Each set of plots were analyzed by Origin plotting program using a best fit linear equation to obtain the slope of the line.

Each of the cobalt (III) series optical melting experiments produced data that can no be compared to DSC values. All optical melting experiments yielded two-state transitions, that were reversible transitions. In fact, at high concentrations, the DNA oligomers proved to have fast equilibration times than at lower concentration of cobalt (III) complex. This relationship between the concentration of the cobalt (III) complex and the equilibration time was unfortunately not studies, but was simply an observation made from experimental studies.
4.2.2 Thermodynamic Parameters by Differential Scanning Calorimetry (DSC)

The thermodynamic parameters for the Z8A and Z8M cobalt (III) complexes were determined by using differential scanning calorimetry in 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, pH 7.0, 100 mM NaCl, with DNA concentrations between 20 µM and 128 µM. The excess heat capacity versus the temperature data was collected and processed using the CpCalc software as previously described to obtain the Tₘ, the molar enthalpy, entropy, and the Gibbs free energy value for the double-stranded to single-stranded transition.

The calorimetric values obtained tend to be considerably more precise than the derived Van't Hoff analysis parameters for a number of reasons. The DSC values obtained are through a model independent method, and therefore no assumptions are made about the state of the system. Fortunately, the van't Hoff values for each of the cobalt (III)–DNA complexes and the salt dependent studies were conveniently derived due to its two-state nature, but oligomers that assume a different state transition are unable to use the Van’t Hoff analysis. With DSC, the enthalpies are obtained through a model independent method that eliminates the problems associated with van’t Hoff variation analysis. The DSC data also contains less noise or measurement artifacts, such as biphasic melt profiles that were common to the transitions for the duplex melts and 75-mers. Finally, the enthalpy values observed follow a trend that is observed structurally by circular dichroism.

Due to idiosyncrasies of the calorimetric software (“CpCalc” by Calorimetric Science
Corporaion), the reported thermodynamic parameters were expressed as the enthalpy, entropy and free energy for the duplex (D/P) to single-strand (SS) transition. The indirect direction meant that, assuming the SS-state was isoenergetic across all cobalt (III) - DNA oligomer series, the more favorable enthalpy was achieved with a larger (more positive) ΔH values, and that favorable entropy was achieved with smaller (less positive) ΔS values. Likewise, the free energy or stability relationships were reversed in a similar manner. The stability of the oligomers became favorable with increased positive magnitudes. This direction is opposite to that reported for the UV-thermal melts, where a more negative enthalpy term indicated increased interactions, and a more positive entropy term indicated increased disorder.

The Z8A and Z8M oligomers were studied by differential scanning calorimetry within the concentration range of 128 μM, produced the following trends as a function of cobalt (III) complex and its concentration (Table 18, Figure 73).

First, the enthalpy term became less favorable from 60.7 kcal/mol for the Z8A oligomer in SPB to 51.2 kcal/mol in the presence of cobalt (III) hexamine. The melting temperature of the Z8A oligomer is higher for the cobalt (III) hexamine – DNA complexes than the parent Z8A oligomer. When the Z8A oligomer is introduced to cobalt (III) pentamine-chloro, at low concentrations of the cobalt complex, the DNA complex is less favorable than the parent strand, but as the concentration increases, the enthalpy value becomes more favorable. This trend is seen for each of the Z8A - cobalt (III) complexes. This trend is consistent with the UV-melt data that indicated that the enthalpy
became more favorable as a function of cobalt (III) concentration.

Table 18: DSC Values Obtained for the Z8A - Cobalt (III) Molecules.
The thermodynamic parameters of the Z8A oligomer is derived from differential scanning calorimetry measurements, as shown below as a function of the Cobalt (III) molecule concentration. For all thermodynamic parameters listed below the assumed direction is the duplex to single-strand transition. Each sample was reconstituted in the appropriate cobalt (III) solution, treated to 55°C, equilibrated for 48 hours at 5°C prior to each experiment.

<table>
<thead>
<tr>
<th>Z8A</th>
<th>Tm  (°C)</th>
<th>ΔHm (kcal/mol)</th>
<th>ΔS_m (kcal/mol K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Phosphate Buffer</td>
<td>71.1</td>
<td>60.7 ± 0.5</td>
<td>0.184 ± 0.1</td>
</tr>
<tr>
<td>[Co(NH3)6]3(C2)</td>
<td>Tm  (°C)</td>
<td>ΔHm (kcal/mol)</td>
<td>ΔS_m (kcal/mol K)</td>
</tr>
<tr>
<td>700 μM</td>
<td>72.3</td>
<td>51.2 ± 0.7</td>
<td>0.152 ± 0.1</td>
</tr>
<tr>
<td>400 μM</td>
<td>78.2</td>
<td>55.4 ± 0.7</td>
<td>0.196 ± 0.1</td>
</tr>
<tr>
<td>[Co(NH3)6(CN)3]2</td>
<td>Tm  (°C)</td>
<td>ΔHm (kcal/mol)</td>
<td>ΔS_m (kcal/mol K)</td>
</tr>
<tr>
<td>200 μM</td>
<td>69.8</td>
<td>52.0 ± 0.6</td>
<td>0.152 ± 0.1</td>
</tr>
<tr>
<td>400 μM</td>
<td>76.4</td>
<td>66.5 ± 0.7</td>
<td>0.196 ± 0.1</td>
</tr>
<tr>
<td>[Co(NH3)6(H2O)3]NO3</td>
<td>Tm  (°C)</td>
<td>ΔHm (kcal/mol)</td>
<td>ΔS_m (kcal/mol K)</td>
</tr>
<tr>
<td>200 μM</td>
<td>80.4</td>
<td>64.4 ± 0.3</td>
<td>0.182 ± 0.3</td>
</tr>
<tr>
<td>400 μM</td>
<td>86.1</td>
<td>71.3 ± 0.3</td>
<td>0.198 ± 0.3</td>
</tr>
<tr>
<td>cis-[Co(NH3)6(H2O)3]C2</td>
<td>Tm  (°C)</td>
<td>ΔHm (kcal/mol)</td>
<td>ΔS_m (kcal/mol K)</td>
</tr>
<tr>
<td>200 μM</td>
<td>73.2</td>
<td>62.1 ± 0.8</td>
<td>0.177 ± 0.3</td>
</tr>
<tr>
<td>400 μM</td>
<td>71.5</td>
<td>64.5 ± 0.1</td>
<td>0.185 ± 0.3</td>
</tr>
<tr>
<td>cis-[Co(NH3)6(Cl)3]C2</td>
<td>Tm  (°C)</td>
<td>ΔHm (kcal/mol)</td>
<td>ΔS_m (kcal/mol K)</td>
</tr>
<tr>
<td>200 μM</td>
<td>72.1</td>
<td>53.6 ± 0.9</td>
<td>0.179 ± 0.3</td>
</tr>
<tr>
<td>400 μM</td>
<td>74.3</td>
<td>63.2 ± 0.6</td>
<td>0.175 ± 0.3</td>
</tr>
</tbody>
</table>
Table 19: DSC Values Obtained for the Z8M - Cobalt (III) Molecules.

The thermodynamic parameters of the Z8M oligomer is derived from differential scanning calorimetry measurements, as shown below as a function of the Cobalt (III) molecule concentration. For all thermodynamic parameters listed below the assumed direction is the duplex to single-strand transition. Each sample was reconstituted in the appropriate cobalt (III) solution, heated to 95°C, equilibrated for 48 hours at 4°C prior to each experiment.

<table>
<thead>
<tr>
<th>Z8M</th>
<th>T_m (°C)</th>
<th>ΔH_m (kcal/mol)</th>
<th>ΔS_m (kcal/mol K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Phosphate Buffer</td>
<td>72.5</td>
<td>62 ± 0.2</td>
<td>0.175 ± 0.3</td>
</tr>
<tr>
<td>[Co(NH3)6]Cl2</td>
<td>74.1</td>
<td>42.1 ± 0.2</td>
<td>0.130 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>81.8</td>
<td>64.3 ± 0.1</td>
<td>0.181 ± 0.8</td>
</tr>
<tr>
<td>[Co(NH3)6]Cl2Cl</td>
<td>73.1</td>
<td>51.6 ± 3.5</td>
<td>0.155 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>80.7</td>
<td>73.5 ± 0.6</td>
<td>0.208 ± 0.3</td>
</tr>
<tr>
<td>[Co(NH3)3(NO3)Cl]</td>
<td>73.0</td>
<td>57.7 ± 0.8</td>
<td>0.107 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>81.6</td>
<td>65.3 ± 0.5</td>
<td>0.184 ± 0.6</td>
</tr>
<tr>
<td>cis-[Co(NH3)3(NO3)Cl2]</td>
<td>73.4</td>
<td>45.2 ± 0.5</td>
<td>0.105 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>77.2</td>
<td>47.9 ± 0.5</td>
<td>0.111 ± 0.3</td>
</tr>
<tr>
<td>cis-[Co(NH3)3(CH3)2Cl2]</td>
<td>72.0</td>
<td>60.1 ± 0.3</td>
<td>0.170 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>76.9</td>
<td>62.5 ± 0.4</td>
<td>0.176 ± 0.6</td>
</tr>
</tbody>
</table>

The entropy became more favorable, increasing from 60.7 kcal/(mol K), for the ZBA oligomer, to 51.2 kcal/(mol K) for the ZBA – cobalt(III)pentamminenitrate complex. The entropy value for the Cobalt (III) pentamminenitrate at higher concentrations is more favorable at lower concentrations. This variation in the trend could be related to the
structural change seen at higher concentrations of cobalt (II) complex, or due to hydration effects occurring for short DNA oligomers. This trend is reversed for the other cobalt (III) complexes revealing that the entropy became less favorable as the concentration of the cobalt (III) complex increases.

Overall the thermodynamic trends indicated that the stability of the Z8A oligomer increases as a function of cobalt (II) concentration, and the increase were enthalpically driven. In fact, across the entire range of data, enthalpy/entropy compensation was observed. As the enthalpy became favorable, the entropy became unfavorable with the total effect of the stability is driven by the enthalpic contributions.

The heat capacity of the DNA complex at various [Co(NH₃)₂Cl]⁺ concentrations were measured by DSC and these measurements produced the following thermodynamic trends (Figure 78). The data represented in table 18 provides the enthalpic, entropic and Gibbs free energy values. The thermodynamic trend indicated that the stability of the Z8A oligomer increases as a function of cobalt (III) concentration. The enthalpy term became less favorable from 60.7 kcal/mol for the Z8A oligomer in SPB to 52.0 kcal/mol in the presence of 200 μM [Co(NH₃)₂Cl]⁺ and more favorable to 66.5 kcal/mol in the presence of 400 μM [Co(NH₃)₂Cl]⁺. This trend is also seen in the optical melting experiments in which the enthalpy value becomes more favorable as the concentration of the[Co(NH₃)₂Cl]⁺ increases.
The heat capacity of the DNA complex at various [Co(NH$_3$)$_2$(H$_2$O)]$^{3+}$ concentrations were measured by DSC and these measurements produced the following thermodynamic trends (Figure 78). The data represented in Table 15 provides the enthalpy, entropy and Gibbs free energy related to the Z8A oligomer and the [Co(NH$_3$)$_2$(H$_2$O)]$^{3+}$ complex. The thermodynamic trend indicated that the stability of the Z8A oligomer increases as a function of cobalt (III) concentration. The enthalpy term became more favorable from 60.7 kcal/mol for the Z8A oligomer in SPB to 64.4 kcal/mol in the presence of 200 µM [Co(NH$_3$)$_2$(H$_2$O)]$^{3+}$ and even more favorable to 71.3 kcal/mol in the presence of 400 µM [Co(NH$_3$)$_2$(H$_2$O)]$^{3+}$. This trend is also seen in the optical melting experiments in which the enthalpy value becomes more favorable as the concentration of the
[Co(NH₃)₆(H₂O)]³⁺ increases. The overall Gibbs free energy trend is seen in both the DSC and optical melting experiments, in which an enthalpy value becomes more favorable as the concentration increases, compensated by the entropy providing a favorable Gibbs free energy value.

Figure 79: Representative DSC thermograms of the Z8A oligomers in 200 and 400 μM [Co(NH₃)₆(H₂O)]³⁺

Representative DSC thermograms for the Z8A - [Co(NH₃)₆(H₂O)]³⁺ series of molecules at (A) 200 and (B) 400 μM. Each sample is reconstituted in the appropriate cobalt (III) solution, heated to 90°C, equilibrated for 48 hours in 4°C. Then each sample is dialyzed versus the cobalt (III) solution, and resuspended in Phosphate Buffer, pH 7.0. A total of 8 scans are performed for each sample from 15 to 100°C with a scan rate of 0.5°C per minute. Nitrogen was purged into the DSC to ensure low humidity at higher temperatures.

The heat capacity of the DNA complexes at various Co(NH₃)₆(H₂O)₂ concentrations were measured by DSC and these measurements produced the following thermodynamic trends (Figure 75). The enthalpy term for the Co(NH₃)₆(H₂O)₂ series for the Z8A oligomer has a value of 62.1 kcal/mol at 200 μM Co(NH₃)₆(H₂O)₂ and 64.5 kcal/mol at 400 μM Co(NH₃)₆(H₂O)₂ concentration. The values obtained for the DSC are in very
good agreement with the van't Hoff analysis in which the van't Hoff values were 63.5 and 67.2 kcal/mol.

Figure 80: Representative DSC thermograms of the Z8A oligomers in SPB
Representative DSC thermograms for the Z8A in SPB. Each sample is reconstituted in the appropriate cobalt (III) solution, heated to 95°C, equilibrated for 48 hours in 4°C. Then each sample is dialyzed versus the cobalt (III) solution, and resuspended in Phosphate Buffer, pH 7.0. A total of 8 scans are performed for each sample from 15 to 100°C with a scan rate of 0.5°C per minute.Nitrogen was purged into the DSC to ensure low humidity at higher temperatures.

The DSC values obtained for the Z8M oligomer have different trends associated with them than the trends previously seen with the Z8A oligomer. The heat capacity of the DNA oligomer Z8M is shown below in figure 76 in SPB solution. The enthalpic value obtained for the Z8M oligomer is 60.7 kcal/mol. Compared to the Van't Hoff value of 64.1 kcal/mol the DSC values are within the ±5% error between DSC and van't Hoff values.
Figure 81: Representative DSC thermograms of the Z8M oligomers in SPB

Representative DSC thermograms for the Z8M in SPB. Each sample is reconstituted in the appropriate cobalt (III) solution, heated to 95°C, equilibrated for 48 hours in 4°C. Then each sample is dialyzed versus the cobalt (III) solution, and resuspended in Phosphate Buffer, pH 7.0. A total of 8 scans are performed for each sample from 15 to 100°C with a scan rate of 0.5°C per minute. Nitrogen was purged into the DSC to ensure low humidity at higher temperatures.

The heat capacity of the cobalt (III) hexammine—DNA complex were measured by DSC and these measurements produced the following thermodynamic trends (Figure 77). The enthalpy term became more favorable from 62.4 kcal/mol for the Z8M oligomer in SPB to 42.1 kcal/mol in the presence of 200 μM cobalt (III) hexammine and 64.3 kcal/mol in the presence of 400 μM. One important note is that according to the CD data, at high concentrations of cobalt (III) hexammine the Z8M oligomer induces the B-to-Z conformational change. Therefore, the DSC experiments are observing the Z-form duplex at high salt going to single strand at high salt. This will later help all for a thermodynamic cycle to be created using model independent methods.
Figure 82: Representative DSC thermograms of the Z8A oligomers in 200 μM [Co(NH₃)₆]²⁺
Representative DSC thermograms for the Z8A - [Co(NH₃)₆]²⁺ series of molecules at 200 μM. Each sample is reconstituted in the appropriate cobalt (III) solution, heated to 95°C, equilibrated for 48 hours at 4°C. Then each sample is dialyzed versus the cobalt (II) solution, and reconstituted in Phosphate Buffer, pH 7.0. A total of 8 scans are performed for each sample from 15 to 100°C with a scan rate of 0.5°C per minute. Nitrogen was purged into the DSC to ensure low humidity at higher temperatures.

The heat capacity of the DNA complex at various NaCl concentrations were measured by DSC and these measurements produced the following thermodynamic trends (Figure 78). Similar to the cobalt (III) hexammine studies with Z8M oligomers, the NaCl studies reveal similar interactions seen by circular dichroism. At high salt concentrations the Z8M oligomer induces a structural change, and therefore the DSC experiments were investigating the duplex at high salt in the Z conformation as it denatures to a single strand at high salt. Previous work done to investigate the B-to-Z transition by Chaires and Sturtevant calculated the enthalpic contributions to the Z-to-SS transition using DSC. The data obtained by Chaires and Sturtevant show three distinct transitions in their DSC thermogram in the temperature range between 15 – 110 °C. The experiments we
performed did not yield three transitions, but only one transition. The cause of this is related to the size of the oligomers studied. Chaires and Staton used a poly (GC) oligomer consisting of hundreds of base pairs, while our Z8M oligomer is only involved in 8 base pair hydrogen bonding.

Figure 83: Representative DSC thermograms of the Z8M oligomers in 4 M NaCl
Representative DSC thermograms for the Z8M in 4 M NaCl. Each sample is reconstituted in the appropriate cobalt (III) solution, heated to 95°C, equilibrated for 48 hours in 4°C. Then each sample is dialyzed versus the cobalt (III) solution, and resuspended in Phosphate Buffer, pH 7.0. A total of 8 scans are performed for each sample from 15 to 100°C with a scan rate of 0.5°C per minute. Nitrogen was purged into the DSC to ensure low humidity at higher temperatures. This particular experiment involves a different conformational change. We assume the DNA oligomer is in its Z-form at 4 M NaCl as shown by CD. Therefore, the transition is from Z-form to single-strand.

The melting temperature of the Z8M oligomer is slightly higher for the cobalt (II) hexamine – DNA complexes than the parent Z8M oligomer. The Z8M oligomer in the presence of the cobalt (III) pentamminechloro complex, at low concentrations, is less favorable than the parent strand, but as the concentration increases, the enthalpy value becomes more favorable. This trend is seen for each of the Z8M - cobalt (III) complexes, at low concentration the enthalpy value is unfavorable but as the concentration increases,
the enthalpy value becomes more favorable. This trend is consistent with the UV-melt data that indicated that the enthalpy became more favorable as a function of cobalt (III) concentration. The heat capacity of the Co(NH$_3$)$_3$Cl – DNA complexes were measured by DSC and these measurements produced the following thermodynamic trends (Figure 79). The enthalpy for the Z8M - Co(NH$_3$)$_3$Cl samples became more favorable, and increased from 62.4 kcal/mol for the parent oligomer, to 73.5 kcal/mol for the 400 μM Co(NH$_3$)$_3$Cl – Z8A complex. The observed enthalpy trend is consistent with the same parameter measured by UV-melt techniques, as the concentration of the Co(NH$_3$)$_3$Cl increases the enthalpy values becomes more favorable.

Figure 84: Representative DSC thermograms of the Z8M oligomers in 200 and 400 μM [Co(NH$_3$)$_3$Cl]$^{2+}$

Representative DSC thermograms for the Z8A - [Co(NH$_3$)$_3$Cl]$^{2+}$ series of molecules at (A) 200 and (B) 400 μM. Each sample is reconstituted in the appropriate cobalt (III) solution, heated to 95°C, equilibrated for 48 hours in 4°C. Then each sample is dialyzed versus the cobalt (III) solution, and resuspended in Phosphate Buffer, pH 7.0. A total of 8 scans are performed for each sample from 15 to 100°C with a scan rate of 0.5°C per minute. Nitrogen was purged into the DSC to ensure low humidity at higher temperatures.
The heat capacity of the Co(NH$_3$)$_2$H$_2$O$^-$–DNA complexes were measured by DSC and these measurements produced the following thermodynamic trends (Figure 85). The enthalpy for the Z8M - Co(NH$_3$)$_2$H$_2$O samples became more favorable, and increased from 62.4 kcal/mol for the parent oligomer, to 65.3 kcal/mol for the 400 µM Co(NH$_3$)$_2$H$_2$O–Z8A complex. The observed enthalpy trend is consistent with the same parameter measured by UV-melt techniques, as the concentration of the Co(NH$_3$)$_2$H$_2$O increases the enthalpy values becomes more favorable. The melting temperatures of the cobalt (III) complex and the Z8A oligomer increase as the concentration of the Co(NH$_3$)$_2$H$_2$O increases. This is a similar trend seen in the optical melting experiments.

Figure 85: Representative DSC thermograms of the Z8M oligomers in 200 and 400 µM [Co(NH$_3$)$_2$(H$_2$O)]$^+$

Representative DSC thermograms for the Z8M - [Co(NH$_3$)$_2$(H$_2$O)]$^+$ series of molecules at (A) 200 and (B) 400 µM. Each sample is reconstituted in the appropriate cobalt (III) solution, heated to 95°C, equilibrated for 48 hours in 4°C. Then each sample is dialyzed versus the cobalt (III) solution, and resuspended in Phosphate Buffer, pH 7.0. A total of 8 scans are performed for each sample from 15 to 100°C with a scan rate of 0.5°C per minute. Nitrogen was purged into the DSC to ensure low humidity at higher temperatures.
4.2.3 Thermodynamic Parameters by Isothermal Titration Calorimetry (ITC)

Isothermal Titration Calorimetry provides a model independent method in determining the initial ion binding energy between the cobalt (III) complexes and the Z8A and Z8M oligomers. ITC also provides a method to investigate the binding events between cobalt (III) complexes and DNA molecules. The values reported in Table 18 are values obtained by ITC at 25°C for both Z8A and Z8M oligomer.

Table 2B: Thermodynamic Values Obtained for the Interaction Between Various Cobalt (II): complexes and the Z8A and Z8M oligomers Determined by ITC.

<table>
<thead>
<tr>
<th>Z8A</th>
<th>Cobalt (II) Complex</th>
<th>ΔH (kcal/mol)</th>
<th>K_a (mM)</th>
<th>n</th>
<th>ΔG (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K(Co(NH)3)3[Br2(NH2)2O]</td>
<td>9.23</td>
<td>3.51</td>
<td>1.03</td>
<td>7.67</td>
<td></td>
</tr>
<tr>
<td>K(Co(NH)3)3[Br(NH2)2O]</td>
<td>8.04</td>
<td>3.20</td>
<td>1.52</td>
<td>8.88</td>
<td></td>
</tr>
<tr>
<td>K(Co(NH)3)3[Cl2(NH2)2O]</td>
<td>9.52</td>
<td>2.53</td>
<td>3.43</td>
<td>1.49</td>
<td></td>
</tr>
<tr>
<td>K(Co(NH)3)3[Cl(NH2)2O]</td>
<td>9.47</td>
<td>2.49</td>
<td>4.70</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td>K(Co(NH)3)3[ClO4]</td>
<td>8.04</td>
<td>0.902</td>
<td>3.09</td>
<td>0.53</td>
<td></td>
</tr>
</tbody>
</table>

The ITC binding isotherms shown in Figure 81 represent the integrated raw data for each of the cobalt (III) complexes involving Z8A and Z8M oligomer. The binding equilibrium (K), the Gibbs free energy values and the stoichiometric values (n) were calculated from.
the fit of the data points. The enthalpy value was obtained by taking the tip asymptote and the bottom asymptote and calculating the difference between the two.

Figure 86: Representative ITC Binding Isotherms for the Interaction between each of the cobalt (III) complexes and the Z8A and Z8M Oligomer

The ITC binding isotherms represent the integrate data for each of the (A) Z8A and (B) Z8M – Cobalt (III) Complexes. Each experiment uses 400 µL of the cobalt (III) solution as the solvent at 25°C with a stir rate speed of 300 rpm, and each titration had a volume of 5 - 10 µL with a maximum number of titration set to 45.

The trend in table 20 presents that the cobalt (III) complexes with one labile ligand are more enthalpically favorable than the cobalt (III) complexes with two labile ligands. If comparing the values from cobalt (III) hexammine and the other cobalt (III) complexes, a definite trend is the apparent. The trend related to the enthalpy is that the interaction between the cobalt (III) complex and either Z8A or Z8M goes as follows: Co(NH$_3$)$_6$ < Co(NH$_3$)$_2$Cl$_2$ < Co(NH$_3$)$_2$(H$_2$O)$_2$ < Co(NH$_3$)$_2$H$_2$O < Co(NH$_3$)$_2$Cl$\cdot$H$_2$O. It is apparent that the different ligands effect the interaction with the DNA oligomers. The initial mode of binding is thought to be electrostatic and therefore any bulky type complexes such as the Co(NH$_3$)$_2$Cl$\cdot$H$_2$O interacts with the DNA oligomers producing a favorable enthalpic contribution.
The binding equilibrium \( K \) is an effect method in determining the strength of the bond between the cobalt (III) complex and the DNA oligomer. Typically electrostatic interactions are fairly weak compared to other types, and producing low \( K \) values. The higher the \( K \) value, the more cooperative the ITC isotherm is. The trend seen for the \( K \) value is as follows: \( \text{Co(NH}_3)_6 < \text{Co(NH}_3)_4 \text{Cl} < \text{Co(NH}_3)_2 \text{Cl} < \text{Co(NH}_3)_2 \text{H}_2 \text{O} < \text{Co(NH}_3)_4 \text{H}_2 \text{O} \). The initial ion binding interaction between NaCl or Co(NH₃)₆ and DNA oligomers is caused by the positive charge of the cobalt (III) complex and the negatively charged phosphate backbone. Theoretically the greater the charge of the complex, the better the initial ion binding event. This theory can also be applied to when comparing various ligands. The more labile the ligand, the better chances of the cobalt (III) complex is of binding electrostatically, or preferentially binding covalently to the N7 of the guanine base.

4.3 The B-to-Z Transition

The research observes conformational changes due to "salt" effects brought on by NaCl and cobalt (III) hexamine. The two DNA oligomers, Z8A and Z8M, are both double stranded under low salt concentrations, but only the Z8M oligomer induces the Z conformation at higher concentrations of the "salt". Circular Dichroism, differential scanning Calorimetry, and isothermal titration Calorimetry are employed to determine the thermodynamics related to the B-to-Z transition.

Behe and Felsenfeld demonstrated that methylation of the cytidine residue dramatically
enhances the facility of the B-to-Z transition in the alternating GC polymer as evidenced by much lower concentrations of Z-inducing agent required to attain the transition midpoint. For shorter oligomers, such as Z8A and Z8M, the number and order of GC repeats is also crucial for the ease of the transition. Several groups have reported the enthalpy of the B-to-Z transition obtained by a variety of experimental techniques. The goal of this project is to determine the enthalpy of the transition using Isothermal Titration Calorimetry based on the following premises. We have demonstrated by the CD studies that Z8M will undergo the B-to-Z transition using either Na or [Co(NH$_3$)$_6$]$^{3+}$ but Z8A does not undergo the transition at [Na$^+$] less than 2.0 M or cobalt (III) hexamine concentration less than 200 mM. Hence, any enthalpy differences between Z8A and Z8M in the thermograms obtained in their titrations with these inducers must be due to conformational transitions.

4.3.1 Proposed Thermodynamic Cycle

The data obtained from the SVD analysis provided us insight into the number of transitions involved in the B-to-Z transition. As previously stated from the NSR versus [salt], the transition is a three-state transition. Theoretically, a thermodynamic cycle can be generated to observe the various energetics related to the B-to-Z transition.

For instance, if we are able to determine the energetics related to a DNA oligomer as it goes from a duplex at low salt to a duplex at high salt through a possible titration experiment such as ITC. If we can then perform a melting experiment such as DSC to observe the energetics related to the duplex at low salt to single strand at low salt, and
duplex at high salt to single strand at high salt, a thermodynamic cycle can be generated and by difference a value can be obtain the energetics between the single strand at low salt and the single strand at high salt (Figure 82).

**Figure 87: Schematic Representation of the Proposed Thermodynamic Cycle**

![Diagram showing thermodynamic cycle](image)

This thermodynamic cycle can be applied for the enthalpy, entropy and free energy relationship. In fact the goal of this project is to successfully determine the energetics by a model independent method, such as DSC or ITC.

### 4.3.2 Fit All Application (ΔG)

Non-linear least-squares analysis of transition curves utilizes the software package FitAll (research Edition, vol 5, MTR Software, Toronto, Ontario, Canada). Subroutines were added for specific transition models, and the goodness of fit included both the usual statistical considerations as well as the careful examination of residual plots.

The analysis of the CD spectra of the ZSM oligomer that induces the B-to-Z transition by either NaCl and cobalt (III) hexammine is used to determine the free energy of transition
using the FitAll Software. As previously described, the B-to-Z transition is a three state transition, therefore a three state model must be incorporated to analyze the transitions. If we consider the reaction:

$$\text{B} \rightarrow \text{I} \rightarrow \text{Z}$$

Where B represents the initial right-handed deoxyoligonucleotide conformation, I an intermediate conformation, and Z the final conformation. Each transition is assumed to be non-cooperative, an assumption that is justified since the lengths of the molecules used are well below the known cooperative units for the B-to-Z transition in polynucleotides. $K_1$ and $K_2$ define the NaCl/CcHalt (III)hexammine-dependent equilibrium constants for the first and second transition steps. In their simplest form, these equilibrium constants are defined as:

$$\text{Equation 71:} \quad K_1 = \frac{[I]}{[B]} = \frac{(a_1 a_2)}{n_1}, \quad K_2 = \frac{[Z]}{[I]} = \frac{(a_1 a_2)}{n_2}$$

Where $a$ refers to the salt activity, $n_i$ refers to the salt activity at the transition midpoint ($i = 1$ or 2), and $n_2$ is a phenomenological coefficient that includes contributions from differential ion and water uptake and release. The parameter $n_i$ is not to be taken as or confused with, the parameter $\gamma_2$ utilized in polyelectrolyte theories based on counterion condensation. The conservation relation for the three-state model is:

$$\text{Equation 72:} \quad \gamma_i = [B] + [I] + [Z]$$

216
By substitution of the relations in equation 71 into equation 72, followed by rearrangement, it is easily shown that:

\[ [B] = \frac{C_\text{e}}{(1 + K_1 + K_2)} \]

Equation 58, 59, and 60 were incorporated into an algorithm suitable for the analysis of the experimental transition curves shown in figure 86 by non linear least-square methods. The procedure is as follows. The parameters to be fit are \( a_1, a_1', a_1'', \) and \( n_2. \) \( C_\text{e} \) is known.

At any wavelength and any salt concentration, the apparent molar ellipticity \( \theta_{app} \) is assumed to be additive, such that:

\[ \theta_{app} = [\theta_\text{a}(B)] + [\theta((I)) + \theta(Z)]/C_\text{e} \]

Where \( \theta_{\text{ox}} \) refers to the molar ellipticity for the species \( x = b, I, z. \) The normalized spectral response is then given by equation 61. Previous graphs representing the best fits obtained for the experimental data were shown and the parameters are listed in table 21.

The values obtained for the \( \Delta G \) of the transition are for the Z-inducer NaCl are listed in table 20. The values of \( \Delta G_1 \) are obtained from the \( K_1 \) values and the values for \( \Delta G_2 \) are from the \( K_2 \) values. The sum of \( \Delta G_1 + \Delta G_2 \) is equal to the total free energy (\( \Delta G_{\text{tot}} \)). The \( \Delta G_{\text{tot}} \) is equal to the free energy of the B-to-Z transition.
Table 21: Summary of the Fitting Parameters for the B-to-Z transition.

The symbols $a_1$ and $a_2$ indicate the salt activity at the transition midpoint for the B-to-Z transition, while the $n_1$ and $n_2$ indicate the corresponding exponential factors shown in equation 58.

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>$a_1$</th>
<th>$n_1$</th>
<th>$a_2$</th>
<th>$n_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>0.47 ± 0.02</td>
<td>0.56 ± 0.2</td>
<td>0.75 ± 0.1</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>[Co(NH$_3$)$_6$]$^{3+}$</td>
<td>0.12 ± 0.02</td>
<td>6.3 ± 0.3</td>
<td>0.43 ± 0.01</td>
<td>2.7 ± 0.2</td>
</tr>
</tbody>
</table>

Table 22: Summary of Free Energy Changes of the B-to-Z transition Using NaCl as the Z-inducer.

The free energy of change is related to the concentration of NaCl. These values were obtained by a FitAll program that utilized a non-linear least-square analysis of the transition curves.

<table>
<thead>
<tr>
<th>[Na]$^+$ (M)</th>
<th>$\Delta G_1$ (kcal/mmol)</th>
<th>$\Delta G_2$ (kcal/mmol)</th>
<th>$\Delta G_m$ (kcal/mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>-0.02</td>
<td>+0.86</td>
<td>+0.84</td>
</tr>
<tr>
<td>1.0</td>
<td>-0.3</td>
<td>-0.61</td>
<td>-0.91</td>
</tr>
<tr>
<td>2.0</td>
<td>-0.5</td>
<td>-2.1</td>
<td>-2.5</td>
</tr>
<tr>
<td>3.0</td>
<td>-0.6</td>
<td>-3.0</td>
<td>-3.6</td>
</tr>
<tr>
<td>4.0</td>
<td>-0.7</td>
<td>-3.6</td>
<td>-4.3</td>
</tr>
<tr>
<td>5.0</td>
<td>-0.8</td>
<td>-4.0</td>
<td>-4.8</td>
</tr>
</tbody>
</table>

The values obtained for the $\Delta G$ of the transition for the Z-inducer cobalt(III) hexammine are listed in table 21. The values were determined for a three-state transition as previously described. Each $\Delta G$ value is obtained at the appropriate concentration of the Z-inducers.

218
Table 23: Summary of Free Energy Changes of the B-to-Z transition Using [Co(NH₃)₆]²⁺ as the Z-inducer.

The free energy of change is related to the concentration of [Co(NH₃)₆]²⁺. These values were obtained by a FitAll program that utilized a non-linear least-square analysis of the transition curves.

<table>
<thead>
<tr>
<th>[Co(NH₃)₆]²⁺ (µM)</th>
<th>ΔG₁ (kcal/mbp)</th>
<th>ΔG₂ (kcal/mbp)</th>
<th>ΔG₃ (kcal/mbp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>-2.7</td>
<td>+0.87</td>
<td>-1.83</td>
</tr>
<tr>
<td>50</td>
<td>-5.3</td>
<td>-0.24</td>
<td>-5.5</td>
</tr>
<tr>
<td>100</td>
<td>-7.9</td>
<td>-1.3</td>
<td>-9.2</td>
</tr>
<tr>
<td>125</td>
<td>-8.7</td>
<td>-1.7</td>
<td>-10.4</td>
</tr>
<tr>
<td>150</td>
<td>-9.4</td>
<td>-2.0</td>
<td>-11.4</td>
</tr>
<tr>
<td>200</td>
<td>-10.5</td>
<td>-2.5</td>
<td>-13.0</td>
</tr>
</tbody>
</table>

This method provides a model-dependent technique to determine the free energy of a three-state transition.

4.3.3 Differential Scanning Calorimetry

The DSC results shown in table 22 are for the duplex of the Z-form and the duplex of the B-form going to the single-strand conformation at various concentrations of Na⁺. The values are obtained from the CpCalc software and buffer solution is subtracted from data. The values provide insight into the stability related to the Z-form versus the B-form. It also provides useful information about the energetics regarding the melting of DNA oligomers at high salt versus oligomers at low salt concentration.
Table 24: The DSC-derived thermodynamic parameters for the Z8A and Z8M oligomers.

The thermodynamic parameters of the Z8A and Z8M oligomers, derived from differential scanning calorimetry measurements, are shown below as a function of Na+ concentration. For all thermodynamic parameters listed below the assumed direction is the duplex to single-strand transition.

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>[Na+] (mM)</th>
<th>( T_m ) (°C)</th>
<th>( \Delta H ) (kcal/mol)</th>
<th>( \Delta S ) (cal/mol K)</th>
<th>( \Delta G ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z8A</td>
<td>115</td>
<td>64.7</td>
<td>8.71</td>
<td>26.2</td>
<td>0.90</td>
</tr>
<tr>
<td>Z8M</td>
<td>115</td>
<td>73.3</td>
<td>5.23</td>
<td>14.1</td>
<td>1.03</td>
</tr>
<tr>
<td>Z8A</td>
<td>2</td>
<td>68.3</td>
<td>10.6</td>
<td>31.1</td>
<td>1.33</td>
</tr>
<tr>
<td>Z8M</td>
<td>2</td>
<td>82.5</td>
<td>9.11</td>
<td>23.0</td>
<td>1.24</td>
</tr>
</tbody>
</table>

4.3.4 Isothermal Titration Calorimetry

Examination of the raw calorimetric data in figure 88 reveals that the titrations of both Z8A and Z8M with Na+ are endothermic while those with Co(NH)3Cl3 are exothermic at 25°C as are the respective heats of dilutions.
Figure 88: ITC Titrations of NaCl into Z8A and Z8M Oligomer

The top portion is the heat of dilution associated with the NaCl titrating into SFB solution. The middle graph depicts the energy related to the interaction between the Z8A oligomer and NaCl. The lower graph represents the titration of NaCl with the Z8M oligomer. All experiments were performed at 25°C with a stirrer speed of 300 rpm, and each titration had a volume of 5 - 10 µl with a maximum number of titrations set to 45. Each experiment was performed in sets of three to ensure reproducibility.
Figure 99: ITC Titrations of Cobalt (II) hexammine into Z8A and Z8M Oligomer

The top portion is the heat of dilution associated with the [Co(NH₃)₆]²⁺ titrating into SM solution. The middle graph depicts the energy released in the interaction between the Z8A oligomer and [Co(NH₃)₆]²⁺. The lower graph represents the titration of [Co(NH₃)₆]²⁺ with the Z8M oligomer. All experiments were performed at 25°C with a stirring speed of 500 rpm, and each titration had a volume of 3-10 µl with a maximum number of titration set to 45. Each experiment was performed in sets of three to ensure reproducibility.

Subtraction of the heats of dilution from the respective raw data, followed by integration results in the isotherms shown in the figure. Titrations of Z8A with either Na⁺ or [Co(NH₃)₆]³⁺ results in fairly broad isotherms while the titration of Z8M with either inducer appears to have a sharp, cooperative transition.

222
Figure 90: The Binding Isotherms of NaCl titrated into Z8A and Z8M Oligomer

The green data points represent the Z8A oligomer, while the blue data points represent the Z8M oligomer. All experiments were performed at 25°C with a stirrer speed of 300 rpm, and each titration had a volume of 5 - 10 µl with a maximum number of titration set to 45. Each experiment was performed in sets of three to ensure reproducibility.

Figure 91: The Binding Isotherms of [Co(NH₃)₆]³⁺ titrated into Z8A and Z8M Oligomer

The red data points represent the Z8A oligomer, while the blue data points represent the Z8M oligomer. All experiments were performed at 25°C with a stirrer speed of 300 rpm, and each titration had a volume of 5 - 10 µl with a maximum number of titration set to 45. Each experiment was performed in sets of three to ensure reproducibility.

In addition, titration of Z8M with Na⁺ is more endothermic than titration of Z8A while titration of Z8A with [Co(NH₃)₆]³⁺ is more exothermic than titration of Z8M. The enthalpies for these titrations are shown in Table 2.5.

223
Table 25: The ΔH, ΔG, TΔS values obtained by ITC at 25°C for NaCl and [Co(NH₃)₆]³⁺.

The ΔG value is obtained from the Gibbs free equation that relates the K<sub>i</sub> value to ΔG. The enthalpy is a model independent value, and the entropy is determined by the Gibbs free equation, ΔG = ΔH - TΔS.

<table>
<thead>
<tr>
<th>Z Inducer</th>
<th>ΔG (kcal/mmol)</th>
<th>ΔH (kcal/mmol)</th>
<th>-TΔS (kcal/mol K bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺ 2.25 M</td>
<td>-2.8</td>
<td>+0.70</td>
<td>-3.5</td>
</tr>
<tr>
<td>[Co(NH₃)₆]³⁺ 125 μM</td>
<td>-10.4</td>
<td>+0.72</td>
<td>-11.1</td>
</tr>
</tbody>
</table>

We assume that the difference in total enthalpy between Z9A and Z8M upon titration with either Na⁺ or [Co(NH₃)₆]³⁺ is the transition enthalpy. In other words:

**Equation 75:** \[ \Delta H_{\text{tr}} = \Delta H_{\text{obs}} + \Delta H_{\text{in}} \]

Where \( \Delta H_{\text{obs}} \) is the observed calorimetric enthalpy for Z8M, \( \Delta H_{\text{in}} \) is the enthalpy for the transition itself and \( \Delta H_{\text{in}} \) is the enthalpy for all other possible processes such as uptake or loss of Na⁺, uptake or loss of water and the binding of [Co(NH₃)₆]³⁺ when titrating with that inducer. The assumption is that \( \Delta H_{\text{in}} \) is the same for Z8M and Z8A. In other words:

**Equation 76:** \[ \Delta H_{\text{in}} = \Delta H_{\text{in,Z8M}} - \Delta H_{\text{in,Z8A}} \]

As seen in table 25, the enthalpy of the transition is 700 cal/mol base pair when induced with Na⁺ and 730 cal/mol base pair when inducing with [Co(NH₃)₆]³⁺. Adding validity
to our approach is the comparison of our results of previously published work. Our transition enthalpy of ca 0.70 kcal/mole base pair obtained at 25°C using either Na⁺ or [Co(NH₃)₆]³⁺ compares quite favorably with the 0.61 ± 0.02 kcal/mole base pair of Chaires and Sturtevant, although obtained by different techniques.

### Table 26: The Enthalpy of the B-to-Z transition determined by ITC

<table>
<thead>
<tr>
<th>Z Inducer</th>
<th>ΔH_{ZBA} (kcal/mole bp)</th>
<th>ΔH_{ZBM} (kcal/mole bp)</th>
<th>ΔH_{BZ} (kcal/mole bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>0.92</td>
<td>1.62</td>
<td>0.70</td>
</tr>
<tr>
<td>[Co(NH₃)₆]³⁺</td>
<td>-3.85</td>
<td>-3.13</td>
<td>0.72</td>
</tr>
</tbody>
</table>

#### 4.3.5 Heat Capacity

The definition of the heat capacity is the heat required to change the temperature of a substance one degree. Heat capacity is mathematically defined as the ratio of a small amount of heat \(\Delta Q\) added to the body, to the corresponding small increase in its temperature \(dT\):

\[
C = \left(\frac{\Delta Q}{dT}\right)_{\text{cond.}} = T \left(\frac{dS}{dT}\right)_{\text{cond.}}
\]

Equation 77:
Hea capacity originates from changes in surface hydration in the free and the complexed molecules and from changes in molecular vibrations. In order to determine the heat capacity associated with the B-to-Z transition, an ITC experiment must be performed at various temperatures for the Z8A oligomer using either NaCl or Cobalt (III) hexamine as the Z inducer as shown in figure 90 and 91.

### Table 27: The ΔH, ΔS, ΔG, and n values at 25°, 35°, 45°, and 55°C obtained by ITC for the Z8A and Z8M Oligomer in the presence [Co(NH3)6]3+

Each experiment involved titrating in 400 μM [Co(NH3)6]3+ into either the Z8A or Z8M oligomer at various temperatures. This provided a method to determine the heat capacity related to these interactions and also to investigate any change in the enthalpy due to the temperature. The ΔH values were determined as previously explained for each of the four temperatures.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>K (M × 10^-3)</th>
<th>n</th>
<th>ΔH (kcal/mole)</th>
<th>ΔS (cal/mole °C)</th>
<th>ΔG (kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.03 ± 0.01</td>
<td>1509.9 ± 0.05</td>
<td>5.21 ± 0.29</td>
<td>-8.35 ± 0.22</td>
<td>-8.35 ± 0.21</td>
</tr>
<tr>
<td>25</td>
<td>0.03 ± 0.05</td>
<td>1621 ± 0.06</td>
<td>1.01 ± 0.04</td>
<td>-4.68 ± 0.12</td>
<td>-4.68 ± 0.07</td>
</tr>
<tr>
<td>35</td>
<td>1620 ± 0.07</td>
<td>1642 ± 0.01</td>
<td>5.68 ± 0.30</td>
<td>-6.99 ± 0.30</td>
<td>-6.99 ± 0.29</td>
</tr>
<tr>
<td>45</td>
<td>1621 ± 0.05</td>
<td>1653 ± 0.05</td>
<td>5.68 ± 0.28</td>
<td>-6.99 ± 0.30</td>
<td>-6.99 ± 0.29</td>
</tr>
<tr>
<td>55</td>
<td>1620 ± 0.06</td>
<td>1620 ± 0.05</td>
<td>5.90 ± 0.34</td>
<td>-4.29 ± 0.35</td>
<td>-4.29 ± 0.34</td>
</tr>
</tbody>
</table>

226
Table 38: The ΔH, ΔS, ΔG, and n values at 25, 35, 45, and 55 °C obtained by 1TC for the ZKA and ZBM oligomer in the presence NaCl.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>K (mol/L)</th>
<th>n</th>
<th>ΔH (kcal/mol)</th>
<th>ΔS (kcal/mol K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZKA</td>
<td>ZBM</td>
<td>ZKA</td>
<td>ZBM</td>
<td>ZKA</td>
</tr>
<tr>
<td>15</td>
<td>160.08 ± 0.02</td>
<td>260.34 ± 0.02</td>
<td>5.06 ± 0.26</td>
<td>0.92 ± 0.10</td>
</tr>
<tr>
<td>25</td>
<td>703.35 ± 0.04</td>
<td>532.93 ± 0.03</td>
<td>5.06 ± 0.35</td>
<td>0.82 ± 0.09</td>
</tr>
<tr>
<td>35</td>
<td>228.74 ± 0.03</td>
<td>401.12 ± 0.02</td>
<td>5.9 ± 0.25</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td>45</td>
<td>481.84 ± 0.05</td>
<td>501.61 ± 0.05</td>
<td>5.94 ± 0.34</td>
<td>1.19 ± 0.23</td>
</tr>
<tr>
<td>55</td>
<td>2197.02 ± 0.10</td>
<td>708.60 ± 0.08</td>
<td>5.1 ± 0.23</td>
<td>0.62 ± 0.14</td>
</tr>
</tbody>
</table>

Figure 92: The Binding Isotherms of NaCl titrated into ZKA and ZBM Oligomer at Various Temperatures

The experiments were performed at 15, 25, 35, 45, and 55°C. All experiments were performed with a stirring speed of 300 rpm, and each titration had a volume of 5 - 10 μl with a maximum number of titration set to 45. Each experiment was performed in 5 sets of three to ensure reproducibility. From these binding isotherms, the enthalpy, entropy, and the free energy of the interactions is calculated.
Figure 93: The Binding Isotherms of [Co(NH₃)₆]²⁺ titrated into Z8A and Z8M Oligomer at Various Temperatures

The experiments were performed at 15, 25, 35, 45, and 55°C. All experiments were performed with a stirring speed of 300 rpm, and each titration had a volume of 5 - 10 μl with a maximum number of titration set to 4⁸. Each experiment was performed in sets of three to ensure reproducibility. From these binding isotherms, the enthalpy, entropy and the free energy of the interactions is calculated.

![Graph 1: Binding Isotherm of Z8A](image1.png)

![Graph 2: Binding Isotherm of Z8M](image2.png)

The heat capacity is calculated by plotting the ΔH versus the temperature (K) and fitting the data points as shown in figure 93. According to equation 77, the slope is equal to the heat capacity related to cobalt (III) hexamine and NaCl with the Z8A and Z8M oligomer.

228
Table 29: The ΔH at 25°C, 35°C, 45°C, and 55°C obtained by ITC for the Z8A and Z8M Oligomer in the presence of NaCl.

Each experiment involved titrating in 4 M NaCl into either the Z8A or Z8M oligomer at various temperatures. This provided a method to determine the heat capacity related to these interactions and also to investigate any changes in the enthalpy due to the temperature. The ΔH values were determined as previously explained for each of the four temperatures.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Na⁺</th>
<th>Z8A (kcal/mol bp)</th>
<th>Z8M (kcal/mol bp)</th>
<th>ΔH (kcal/mol bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.92 ± 0.09</td>
<td>1.62 ± 0.09</td>
<td>0.70 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>1.73 ± 0.06</td>
<td>1.96 ± 0.14</td>
<td>0.23 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>3.23 ± 0.11</td>
<td>2.36 ± 0.22</td>
<td>-0.87 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>4.66 ± 0.15</td>
<td>3.05 ± 0.14</td>
<td>-1.6 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

Table 30: The ΔH at 25°C, 35°C, 45°C, and 55°C obtained by ITC for the Z8A and Z8M Oligomer in the presence of [Cu(NH₃)₄]²⁺.

Each experiment involved titrating in 40 μM [Cu(NH₃)₄]²⁺ into either the Z8A or Z8M oligomer at various temperatures. This provided a method to determine the heat capacity related to these interactions and also to investigate any changes in the enthalpy due to the temperature. The ΔH values were determined as previously explained for each of the four temperatures.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Co²⁺</th>
<th>Z8A (kcal/mol bp)</th>
<th>Z8M (kcal/mol bp)</th>
<th>ΔH (kcal/mol bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>-3.66 ± 0.34</td>
<td>-3.05 ± 0.31</td>
<td>0.72 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>-4.78 ± 0.26</td>
<td>-3.58 ± 0.30</td>
<td>1.2 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>-5.69 ± 0.36</td>
<td>-4.20 ± 0.29</td>
<td>1.9 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>-6.25 ± 0.36</td>
<td>-4.66 ± 0.38</td>
<td>1.6 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>
The heat capacity values shown in Table 31 were calculated by the equation:

\[ \Delta C_p = \frac{d(\Delta H)}{dT} = \tau \frac{d(\Delta S)}{dT} \]

Equation 78:

The values obtained for the heat capacity related to NaCl and ZnA and ZnM are 0.13 and 0.047 kcal/mol K. The values obtained using cobalt (II) hexamine for the ZnA oligomer is -0.055 and a value of -0.081 kcal/mol K for the ZnM oligomer.
Table 31: Heat Capacity Values for the DNA oligomers Z8A and Z8M for NaCl and [Co(NH₃)₆]³⁺

The values were obtained from the plots of DH versus temperature, and analyzed by Origin to obtained the best fit line of the data. The slope of the best fit line, provided the values for the heat capacity related to NaCl and [Co(NH₃)₆]³⁺

<table>
<thead>
<tr>
<th></th>
<th>Na⁺</th>
<th>[Co(NH₃)₆]³⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z8A</td>
<td>+0.13 kcal/mol K</td>
<td>-0.055 kcal/mol K</td>
</tr>
<tr>
<td>Z8M</td>
<td>+0.047 kcal/mol K</td>
<td>-0.081 kcal/mol K</td>
</tr>
</tbody>
</table>

4.3.6 Enthalpic Thermodynamic Cycle

An enthalpic thermodynamic cycle is essential to understanding the interactions related to the B-to-Z transition. Using the first proposed thermodynamic cycle shown in figure 87, we now can take the values obtained from the DSC, and ITC and complete a thermodynamic cycle for enthalpy of the B-to-Z transition as seen in figure 95 and 96. The difference in enthalpy values for Z8A and Z8M duplex at high and low NaCl concentration versus the single strand oligomer at high and low NaCl is obtained by differential scanning calorimetry (Z8A = 8.7/10.6 kcal/mol bp and Z8M = 5.2/8.1 kcal/mol bp). The ITC values represent the enthalpic difference between the duplex Z8A and Z8M oligomer at low NaCl versus the duplex Z8A and Z8M oligomer at high salt (Z8A = 0.92 kcal/mol bp, Z8M = 1.6 kcal/mol bp).

231
Figure 95: Enthalpic Thermodynamic Cycle for the Z8A Oligomer

The values obtained from DSC and ITC experiments give way to a complete thermodynamic cycle for the various transitions the Z8A oligomer can be involved in under low and high NaCl conditions.

Z8A (duplex, low salt) 0.92 kcal/mol bp
8.7 kcal/mol bp
Z8A (duplex, high salt) 10.6 kcal/mol bp

Z8A (single strand, low salt) 2.5 kcal/mol bp
Z8A (single strand, high salt)

Figure 96: Enthalpic Thermodynamic Cycle for the Z8M Oligomer

The values obtained from DSC and ITC experiments give way to a complete thermodynamic cycle for the various transitions the Z8M oligomer can be involved in under low and high NaCl conditions.

Z8M (duplex, low salt) 1.6 kcal/mol bp
5.2 kcal/mol bp
Z8M (duplex, high salt) 8.1 kcal/mol bp

Z8M (single strand, low salt) 4.5 kcal/mol bp
Z8M (single strand, high salt)

Since the enthalpic contribution is additive, the value obtained for the difference between the Z8A or Z8M single strand oligomer at low NaCl concentration versus the single strand oligomer at high NaCl concentration is 2.8 kcal/mol bp and 4.5 kcal/mol bp. The data was calculated by taking the difference between the DSC results (10.6 kcal/mol bp - 8.7 kcal/mol bp = 2.8 kcal/mol bp) for the Z8M oligomer and the same was done for the Z8M oligomer (8.1 kcal/mol bp - 5.2 kcal/mol bp = 4.5 kcal/mol bp).

232
The same can be done by using the cobalt (III) hexamine salt, filling in the data obtained by the DSC and ITC experiments (Figure 97 and 98).

**Figure 97: Enthalpic Thermodynamic Cycle for the Z8A Oligomer ([Co(NH₃)₆]⁺)**

The values obtained from DSC and ITC experiments give way to a complete thermodynamic cycle for the various transitions the Z8A oligomer can be involved in under low and high [Co(NH₃)₆]⁺ conditions.

Z8A (duplex, low salt) \[ \text{8.7 kcal/mol bp} \] \[ \text{Z8A (duplex, high salt)} \[ \text{11.2 kcal/mol bp} \]

Z8A (single strand, low salt) \[ \text{6.26 kcal/mol bp} \] Z8A (single strand, high salt)

Once more, by taking the mathematical difference between the DSC results for single strand oligomers at low Cobalt (III) hexamine concentration versus high Cobalt (III) hexamine concentration values of 6.39 kcal/mol bp for the Z8A oligomer and 3.33 kcal/mol bp for the Z8M oligomer are obtained. This method provides a model independent mode of calculating the enthalpic contributions related to the Z8A and Z8M oligomer at high and low NaCl and Cobalt (III) hexamine concentrations.
4.3.7 Osmolyte Studies (NaCl)

Hydration is an important component of DNA structure, stability, and dynamics. As corollary to this, if there are differences in the number of water molecules interacting with different DNA structures, then conformational transitions will depend on the availability of water. The B to Z transition is triggered by changes in relative humidity. The left-handed Z form of poly(dG-dC) and poly(dG-dC) was reported as being less hydrated than the right-handed B form. No direct evidence has linked hydration and the B-to-Z equilibrium. We know that larger bulkier hydrophobic cations and anions are effective at inducing the transition, which suggests an indirect role acting by the bulky water properties and the differences in the B-to-Z hydration energies.

A method to observe water activity is using an osmolyte. An osmolyte is a neutral solute that does not directly bind to the polynucleotide. When these solutes are excluded from the water surrounding the polynucleotide, an osmotic stress is created that favors conformations that exclude less solute. The magnitude of the effect will depend on the water activity, osmotic stress of the solution, and the difference in the number of solute-
excluded waters between the two conformations.

The neutral solute betaine, is used to investigate the water activity associated with the B-to-Z transition. This neutral solute stabilizes the Z-conformation, and forces the equilibrium to the Z conformation. Studies done by Chen et al., showed that binding of cobalt (II) hexammine to poly(dG-m5dc) at low ionic strength had linear dependence of the fraction of Z form on the amount of cobalt hexammine added, therefore showing that the free energy difference between the B and Z forms is linearly related to the osmotic concentration, or chemical potential. Also, the differences in electrostatic double layer energies is not the basis for the transition. Instead, it seems that these solutes are probing differences in solute-excluding waters of hydration between the two conformations. This text set of experiments uses a model independent method (ITC) to investigates the thermodynamics related to the hydration of the Z8M oligomer due to the addition of NaCl, and the neutral solute, betaine (N,N,N-trimethylglycine Hydroxide). By using equation 79 taken from the Hofmeister series, the number of water molecules can be determined.

Equation 79: \[
\frac{dK_{\text{eq}}}{dM} = -\frac{RT}{55.6 (\Delta N_{\text{H2O}})}
\]

When you plot \( \ln [K] \) versus [Betaine], the slope is equal to \( \Delta N_{\text{H2O}} \). The \( K \) value is obtained by isothermal titration calorimetry. Figure 99 are the binding isotherms for the Z8A and Z8M oligomer at 25°C at 0, 0.1, 0.5, and 1.0 M betaine. Table 32 has the numerical values for the titration of NaCl into Z8A and Z8M. By taking the difference

235
Figure 99: ITC Binding Isotherms at Various Osmolyte Concentrations

The binding isotherms provide enthalpy values for the interaction of NaCl and the Z8A and Z8M oligomer under 0, 0.1, 0.5, and 1.0 M of the osmolyte, betaine. This experiment investigates how water activity may play a role in the B-to-Z transition, and will provide insight into how the thermodynamics is effected due to water activity changes. All experiments were performed at 25°C, with a stirrer speed of 300 rpm, and each titration had a volume of 5 - 10 μl with a maximum number of titration set to 45. Each experiment was performed in sets of three to ensure reproducibility. From these binding isotherms, the enthalpy, entropy and the free energy of the interactions is calculated.

between the enthalpy values for Z8A and Z8M, the enthalpy of the transition at each of the [osmolyte] can be determined as shown in table 32.
Table 32: Calorimetric Enthalpy Values Obtained for the B-to-Z transition at Various Osmolyte Concentrations using the Z-inducer NaCl.

The ΔH values obtained from the isothermal titration calorimetry experiments were calculated following the same assumptions used to calculate the B-to-Z transition, without the osmolyte. The concentration of the osmolyte varied from 0.1 M to 1.0 M. Each sample was reconstituted in SPB that included the appropriate concentration of the osmolyte. 4 M NaCl was then titrated into the sample cell and the heat evolved provided information pertaining to the water accessibility of the B-
to-Z transition.

<table>
<thead>
<tr>
<th>[Betaine]</th>
<th>ΔH_{ZSA} (kcal/mol bp)</th>
<th>ΔH_{ZIM} (kcal/mol bp)</th>
<th>ΔH_{HZ} (kcal/mol bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 M</td>
<td>0.92</td>
<td>1.62</td>
<td>0.70</td>
</tr>
<tr>
<td>0.1 M</td>
<td>0.88</td>
<td>1.51</td>
<td>0.63</td>
</tr>
<tr>
<td>0.5 M</td>
<td>0.58</td>
<td>1.03</td>
<td>0.45</td>
</tr>
<tr>
<td>1.0 M</td>
<td>0.23</td>
<td>0.52</td>
<td>0.59</td>
</tr>
</tbody>
</table>

The K value is determined by the fit of the binding isotherm. The K value is then plotted versus the concentration of the osmolyte and a graph is generated revealing the number of water molecules released for a certain experiment. The number of water molecules is determined by the slope of the best fit line of the data as shown in figure 100.
Figure 100: Plot of log K versus [osmolyte]
The change in enthalpy for the Z8A oligomer is represented in graph A, in which each data point represents the equilibrium constant for that particular osmolyte concentration. The change in enthalpy for the Z8M oligomer is represented in graph B, in which each data point represents the equilibrium constant for that particular osmolyte concentration. The slope of the line provides insight into the water activity as NaCl interacts with the DNA oligomers.

The calculated values for the number of water molecules released is 0.93 for the Z8A oligomer and 5.37 for the Z8M oligomer as shown in Table 33.

Table 33: Values for the number of water molecules released.
The values obtained from the plots in Figure 90, provide a method to determine the number of water molecules released due to a conformational change. These values were determined by Isothermal Titration Calorimetry according to Parsegian, plotting log K versus the concentration of the osmolyte.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>ΔωH (Betaine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z8A</td>
<td>0.93 ± 0.05</td>
</tr>
<tr>
<td>Z8M</td>
<td>5.37 ± 0.12</td>
</tr>
</tbody>
</table>

238
5 Discussion/Conclusions

5.1 Structural Studies

5.1.1 Purpose

There has been much discussion about the thermodynamic effects related to metal complexes that bind to DNA oligomers. Many researchers have observed cobalt (III) complexes specifically that preferentially bind to DNA molecules via a covalent linkage to the N7 of the guanine or by electrostatic interaction. When these cobalt (III) complexes bind to DNA molecules a conformational transition is very common. For instance, the B-to-Z transition is a conformational change due to a hydration effect caused by cobalt (III) complexes displacing water molecules located on the phosphate backbone. (Figure 101)

Figure 101: Interestrand and Intrastrand Binding Associated with Cobalt (III) Complexes

Schematic of the Z8M oligomer in which a cobalt (III) complex is binding to the N7 location on the guanine either through an interstrand or intrastrand manner. Either of the two possibilities could occur depending on the lability of the ligand attached to the cobalt core, the environmental effects, and the sequence of the DNA strand.

Pohl and Javin first determined that this conformational change is entropically driven, giving values of ± 1.0 kcal/mol for the enthalpic contributions related to the structural
change. Recent studies done by Chaires and Sturtevant have used model independent methods to obtain the enthalpic values of the Z-DNA to B-DNA transition. This is the first attempt to use a model independent method such as ITC to determine the enthalpic values for the B-to-Z transition in situ.

5.1.2 Differences Between the Z8A and Z8M Oligomer

The two DNA oligomers studied differ by one small aspect, and that is the Z8M oligomer is methylated. This methylation leads to different results when compared to the analogous strand, Z8A. The CD data for the cobalt (III) - Z8A complex show slight variation in the CD spectra as a function of concentration and temperature. This slight variation unfortunately does not provide substantial evidence that the DNA molecules have drastically altered structures. On the other hand, the Z8M oligomer is involved in various structural changes at higher concentrations depending on the cobalt (II) complex used. Cobalt (II) hexammine is a cobalt (III) complex that is involved in inducing the structural change in the DNA oligomer Z8M but not in the oligomer Z8A. The cause of this difference is related to the methylation of the cytosine located on the Z8M oligomer. This large bulky group promotes the hydration effect, forcing the DNA molecule to switch to a conformation that is more favorable under the certain environmental conditions. In the Z8A oligomer, hydration is not seen because more of the cobalt (III) hexammine is necessary to hydrate the abundant water molecules located.

The cobalt (III) hexammine and NaCl studies provide information pertaining to the B-to-Z transition for short DNA oligomers. Behe and Felsenfeld first determined that cobalt
(III) hexammine would induce the conformational change in poly (GC) strands, but little was known about the thermodynamics related to the conformational change brought about from cobalt (III) hexammine. NaCl was also a Z-inducer that had been studied quite a bit, and values for the enthalpic contributions to the B-to-Z transition vary depending on strand length and technique.

**Figure 10:** Schematic Representation of the B-to-Z transition

The schematic is a representation of the Z8M oligomer that includes water molecules in the red, and cobalt (III) hexammine in the blue. The figure on the left depicts the DNA oligomer before the cobalt (III) hexammine electrostatically binds to the negatively charged phosphate backbone. The figure on the right depicts the DNA molecule after the cobalt (III) hexammine binds to the outer sphere of the DNA molecule causing the water molecules to be displaced and forcing the equilibrium to shift towards the Z-form.

The native gel studies of the Z8A and Z8M oligomers and the Cobalt (III) - DNA

241
complexes show Z8A and Z8M having similar mobilities on the gel. The size, and shape of each of the DNA oligomers are the same, therefore the size to charge ratio should also be the same. This relationship seen in the native gels, provides information pertaining to the charge to charge ratio between the Z8A and Z8M oligomer. Structural changes caused by the introduction of a cobalt (III) complex are due to specific interactions, other than electrostatic interactions. If one DNA oligomer contained a higher size to charge ratio, then the relationship between structural change and structural charge would be prevalent.

5.1.3 Temperature Dependent CD

The temperature dependent CD results reveal that for all the cobalt (III) complexes except cobalt (II) hexammine, temperature played a little role. In fact, up to a temperature of 55°C, little or no change in the CD spectra was apparent. This is caused by the cobalt (III) complex stabilizing the Z8A and Z8M oligomer by some type of interaction. A temperature above 70°C was required to notice any substantial structural changes in the CD spectra. The stability of the cobalt (III) – DNA complexes were tested by optical melting studies, and differential scanning calorimetry experiments.

5.1.4 Singular Value Decomposition

Singular value decomposition provided insight into the number of transition out particular model was involved in. This method of determining the number of transitions mathematically proved to be the ideal method. The SVD analysis also backed up the FitAll data which was unable to be fitted with a typical two-state model. Instead the plot of the NSR versus concentration of the Z-inducer is fitted with more of a three-state
model which was determined previously by SVD analysis. The residual plots provided the extra support to confirm that the there are three major species in the B-to-Z transition.

5.2 Thermodynamic

5.2.1 Purpose
The interactions between cobalt (III) complexes and DNA sequences have been studied by many laboratories. While some laboratories have studied the thermodynamics stability through model independent methods, none have attempted to understand the underlying forces that stabilize DNA oligomers by model independent methods. The goal was similar to the structural studies, to define the thermodynamic properties of each cobalt (III)–DNA complex and determine if a correlation exists between the stability and the cobalt (III) complex or between the two DNA oligomers, Z8A and Z8M.

5.2.2 UV Melts vs Calorimetry
Van't Hoff analysis is a typical means of determining thermodynamic parameters for DNA molecules that have a two-state transition. Though it has been used for other hairpin and quadruplex species, ideally the van't Hoff analysis is used for duplex to single-strand transitions. Calorimetry on the other hand provides a relatively fast and effective method to determine the thermodynamics related to any type of transition that is caused by DNA oligomers.

The calorimetric and van't Hoff derived data presented in tables 34 and table 35 are shown slight variations in their values. This is typical when comparing van't Hoff values to

243
calorimetric values. The percent error associated with van't Hoff values are $+5\%$. Though the calorimetric values are different than the van't Hoff, the values are within $+5\%$ of each other as shown below.

| Table 34: Comparison between van't Hoff and calorimetric enthalpies for the ZnM Oligomer |
|---------------------------------------------|-----------------|-----------------|-----------------|
| ZnM                                        | $\Delta H_{\text{cal}}$ (kcal/mol) | $\Delta H_{\text{vH}}$ (kcal/mol) | %               |
| NaCl                                        |                               |                              |                 |
| 2 M NaCl                                    | -67.38 ± 0.4               | 70.2 ± 0.4                  | 4.18            |
| 4 M NaCl                                    | -76.87 ± 0.2               | 76.1 ± 0.2                  | 1.00            |
| [Co(NH$_3$)$_6$]Cl$_2$                       |                               |                              |                 |
| $\Delta H_{\text{cal}}$ (kcal/mol)          | $\Delta H_{\text{vH}}$ (kcal/mol) |
| 200 μM                                      | -40.1 ± 0.2                | 51.2 ± 0.7                  | 22.8            |
| 400 μM                                      | 66.5 ± 0.6                 | 55.4 ± 0.7                  | 5.22            |
| [Co(NH$_3$)$_2$Cl]Cl$_2$                     |                               |                              |                 |
| $\Delta H_{\text{cal}}$ (kcal/mol)          | $\Delta H_{\text{vH}}$ (kcal/mol) |
| 200 μM                                      | -67.4 ± 0.3                | 52.0 ± 0.6                  | 22.8            |
| 400 μM                                      | -70.2 ± 0.3                | 66.5 ± 0.7                  | 5.32            |
| [Co(NH$_3$)$_2$(H$_2$O)$_2$NO$_2$]           |                               |                              |                 |
| $\Delta H_{\text{cal}}$ (kcal/mol)          | $\Delta H_{\text{vH}}$ (kcal/mol) |
| 200 μM                                      | -56.1 ± 0.5                | 64.4 ± 0.3                  | 14.7            |
| 400 μM                                      | -62.1 ± 0.1                | 71.3 ± 0.3                  | 14.8            |
| cis-[Co(NH$_3$)$_2$(Cl$_2$O)$_2$]Cl$_2$       |                               |                              |                 |
| $\Delta H_{\text{cal}}$ (kcal/mol)          | $\Delta H_{\text{vH}}$ (kcal/mol) |
| 200 μM                                      | -41.4 ± 0.9                | 62.1 ± 0.8                  | 50.1            |
| 400 μM                                      | -43.5 ± 0.3                | 64.5 ± 0.9                  | 48.3            |
| cis-[Co(NH$_3$)$_2$(Cl)$_2$]Cl$_2$            |                               |                              |                 |
| $\Delta H_{\text{cal}}$ (kcal/mol)          | $\Delta H_{\text{vH}}$ (kcal/mol) |
| 200 μM                                      | -64.3 ± 0.6                | 63.6 ± 0.5                  | 1.08            |
| 400 μM                                      | -66.4 ± 0.4                | 63.2 ± 0.6                  | 4.81            |

244
<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Z8A</th>
<th>ΔH_{in} (kcal/mol)</th>
<th>ΔH_{out} (kcal/mol)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td></td>
<td>-65.43 ± 0.2</td>
<td>60.7 ± 0.6</td>
<td>12.32</td>
</tr>
<tr>
<td>2 M NaCl</td>
<td></td>
<td>-48.17 ± 0.5</td>
<td>51.2 ± 0.7</td>
<td>5.9</td>
</tr>
<tr>
<td>4 M NaCl</td>
<td></td>
<td>-58.38 ± 0.5</td>
<td>55.4 ± 0.7</td>
<td>5.3</td>
</tr>
<tr>
<td>[Co(NH3)6]Cl2</td>
<td></td>
<td>ΔH_{in} (kcal/mol)</td>
<td>ΔH_{out} (kcal/mol)</td>
<td>%</td>
</tr>
<tr>
<td>200 µM</td>
<td></td>
<td>-75.7 ± 0.1</td>
<td>52.0 ± 0.6</td>
<td>7.40</td>
</tr>
<tr>
<td>400 µM</td>
<td></td>
<td>-83.9 ± 0.2</td>
<td>66.5 ± 0.7</td>
<td>4.61</td>
</tr>
<tr>
<td>[Co(NH3)6]Cl2</td>
<td></td>
<td>ΔH_{in} (kcal/mol)</td>
<td>ΔH_{out} (kcal/mol)</td>
<td>%</td>
</tr>
<tr>
<td>200 µM</td>
<td></td>
<td>-71.5 ± 0.7</td>
<td>64.4 ± 0.3</td>
<td>31.2</td>
</tr>
<tr>
<td>400 µM</td>
<td></td>
<td>-72.6 ± 0.2</td>
<td>71.3 ± 0.3</td>
<td>20.54</td>
</tr>
<tr>
<td>[Co(NH3)6(H2 O)2]NO3</td>
<td></td>
<td>ΔH_{in} (kcal/mol)</td>
<td>ΔH_{out} (kcal/mol)</td>
<td>%</td>
</tr>
<tr>
<td>200 µM</td>
<td></td>
<td>-63.5 ± 0.3</td>
<td>62.1 ± 0.8</td>
<td>9.03</td>
</tr>
<tr>
<td>400 µM</td>
<td></td>
<td>-67.2 ± 0.7</td>
<td>64.5 ± 0.1</td>
<td>1.51</td>
</tr>
<tr>
<td>cis-[Co(NH3)6(H2 O)2]Cl2</td>
<td></td>
<td>ΔH_{in} (kcal/mol)</td>
<td>ΔH_{out} (kcal/mol)</td>
<td>%</td>
</tr>
<tr>
<td>200 µM</td>
<td></td>
<td>-68.3 ± 0.5</td>
<td>63.6 ± 0.9</td>
<td>1.74</td>
</tr>
<tr>
<td>400 µM</td>
<td></td>
<td>-68.5 ± 0.5</td>
<td>63.2 ± 0.6</td>
<td>3.80</td>
</tr>
<tr>
<td>cis-[Co(NH3)6(Cl)2]Cl2</td>
<td></td>
<td>ΔH_{in} (kcal/mol)</td>
<td>ΔH_{out} (kcal/mol)</td>
<td>%</td>
</tr>
<tr>
<td>200 µM</td>
<td></td>
<td>-69.43 ± 0.2</td>
<td>60.7 ± 0.6</td>
<td>3.34</td>
</tr>
<tr>
<td>400 µM</td>
<td></td>
<td>-68.5 ± 0.5</td>
<td>63.2 ± 0.6</td>
<td>7.05</td>
</tr>
</tbody>
</table>

245
A number of variations are noticed between the van't Hoff values and calorimetric values. First, as a function cobalt (III) complex the van't Hoff enthalpies are inconsistent with the magnitudes of the calorimetrically derived enthalpies. Second, the data trends of the van’t Hoff data are consistent within an oligomer series as a function of increasing concentration of the cobalt (III) complex. As the concentration of the cobalt (III) complex increases whether it is the van’t Hoff enthalpy or the calorimetric enthalpy, in both methods a trend is found.

Examination of the van’t Hoff revealed systemic problems throughout the data sets that could have accounted for variation in the enthalpy data. These problems are related to the manner through which the alpha slope of the melting transition was determined. Several of the samples had incomplete melting transitions or transitions where the post-transition baseline was difficult to establish with the given software analysis package. Several other data sets had unusual transitions that could have been related to a multiple populations of states through out the melting measurement or multiple transitions of a single sample. Most problematic was determination of the linear range of the alpha plot that was the basis for calculation of the van’t Hoff transition enthalpy plot. Small scale deviations in the slope of this line could produce different enthalpy values.
5.2.3 Isothermal Titration Calorimetry

The enthalpy values obtained by ITC are model independent and provide insight into the initial ion binding energetics. The cobalt (III) complexes interacted with the DNA oligomers in an enthalpically favorable fashion.

Figure 103: Graphic Thermodynamic Comparison Between each of the Cobalt (III) Complexes and the Z8M Oligomer

The values obtained are from the ITC results at 25°C. The red bars depict the enthalpy, the green is for the entropy, and the blue is the free energy.

As the concentration of the cobalt (III) complex increases, the enthalpy values become more favorable. The first set of experiments involves investigating the interactions between the Z8A and Z8M oligomer and cobalt (III) complexes. Tables 27 and 28
provide the values for the enthalpy, entropy, and free energy related to this binding event.

The figure below is a detailed graph of the thermodynamic values obtained by ITC for each of the cobalt (III) complexes and the DNA oligomers.

Figure 104: Graphic Thermodynamic Comparison Between each of the Cobalt (III) Complexes and the ZBA Oligomer

The values obtained are from the ITC results at 75°C. The red bars depict the enthalpy, the green is for the entropy, and the blue is the free energy.

5.2.4 The B-to-Z Transition

5.2.4.1 Fit All Model Dependent

The free energy of the B-to-Z transition was determined by a Fit All program that took into account the number of transitions, the K value, and allowed for the input of variable
to calculate the free energy of a transition at each of the concentrations of the inducer.

The free energy values were plotted versus ln of the concentration of the Z-inducer.

Figure 105: Plot of Free Energy versus Concentration of Z-inducer
The graph shows a linear correlation between the Gibbs free energy and the concentration of the Z-inducer used.

\[ \Delta G \text{ (obs)} = \Delta G \text{ (calc)} + \Delta G_{\text{corr}} \]

The graph reveals a correlation between the \( \Delta G \) and the concentration of \( \text{Na}^+ \) and \( \text{Co}^{3+} \) due to the linear free energy relationship with the log of the concentration of the Z-inducer. The slope for both \( \text{Na}^+ \) and \( \text{Co}^{3+} \) are \( < 8 \), following a similar trend as the concentration increases. The similarity between the free energy shown for the NaCl and cobalt (III) hexamine inducer, is related to the similar type of binding associated with positively charged species interacting with DNA molecules. If \( \Delta G_{\text{obs, Na}} = \Delta G_{\text{calc, Na}} + \Delta G_{\text{corr}} \), when \( \Delta G_{\text{obs, Na}} = \Delta G_{\text{obs, Co}} \), then \( \Delta G_{\text{calc, Na}} = \Delta G_{\text{calc, Co}} = 0 \), and \( \Delta G_{\text{obs, Na}} = \Delta G_{\text{calc, Co}} \). If the data points were to be extrapolated to determine the point of intercept, or the point at which \( \Delta G_{\text{calc, Na}} \) is obtained, a value of 37.33 kcal/mol and a concentration of \( 1.6 \times 10^{-7} \text{ M} \) would be needed.

249
5.2.4.2 Isothermal Titration Calorimetry

The values obtained by ITC for the Z8M and Z8A oligomers provided the foundation for determining the enthalpic, entropic, and ultimately the Gibbs free energy of the B-to-Z transition. The value obtained for the B-to-Z transition is 700 cal/mol base pair when induced with Na+ and 120 cal/mol base pair when induced by Cobalt (III) hexamine. We determined these values by making certain assumptions, that the difference in total enthalpy between Z8A and Z8M upon titration with either Na+ or Cobalt (III) hexamine is the transition enthalpy. The other assumption is that the DPePe is the same for Z8A and Z8M. Adding validity to our approach is the comparison of our results with previously published work. Our transition enthalphy of ca 0.70 kcal/mol base pair obtained at 25°C using either NaCl or Cobalt (III) hexamine compares more favorably with the 0.61 kcal/mol base pair of Chaires and Sturtevant, although obtained by different techniques. The enthalpy of the B-to-Z conformational transition using ITC is determined and demonstrated that it can be a very useful tool in monitoring conformational
transitions that may not be observed by spectroscopic techniques. ITC also provides the most direct method of determining conformation transition enthalpies.

One uncharacteristic feature that ITC demonstrated is its ability to monitor reactions that are in the calorie to milli calorie range. The sensitivity of the instrument helped in observing small thermodynamic values, and relating the structure to the energetics. One apparent difference in the ITC binding isotherm is seen between the titrations of NaCl and Cobalt (III) hexamine. For the NaCl titrations both oligomers give exothermic peaks as the reactions continues, giving sharp cooperative transitions for the ZnM oligomer and broad uncooperative transitions for the ZnA oligomer.

The reason for the exothermic peaks is related to the titrations of the NaCl into the DNA oligomers. The titrant contained a sodium ion that is surrounded by water molecules. As the titrant is titrated into the solution containing the DNA oligomer, the H-bonds that are holding together the Na-water molecule are being broken. Therefore, the release of heat (exothermic) is due to the breaking of more bonds than the interaction is making.

The titrations of cobalt (III) hexamine into the DNA oligomers produce endothermic peaks due to the making of bonds or associating with the DNA oligomers. This difference in heat evolved is again why water plays an important role in thermodynamics and in biological systems in general.
Figure 197: Schematic Representation of a Sodium Molecule Surrounded by Water

The schematic represents a Na\(^+\) molecule that is bonded by water molecules in solution. When the Na-water molecule is introduced into another solution of lesser Na concentration, the Na-water bond breaks. This occurrence causes the energy value in the TEC experiments to differ between the NaCl titrations and the Cobalt (III) hexammine titrations. If more bonds are being broken then being made, the reaction will have an exothermic isotherm, but if more bonds are being made than broken like in the case of cobalt (III) hexamine, the reaction will be endothermic and produce an endothermic isotherm.

5.2.5 Enthalpic, Entropic and Free Energy Correlations

The trends present in both NaCl and the cobalt (III) hexammine complex are shown in figure 108. The enthalpic contributions are compensated by the entropic contributions as evident by the linear correlation seen when plotting enthalpy versus entropy and fitting by least-square analysis obtaining excellent correlation values (> 95 %) for both the Z8A and Z8M oligomer. Interestingly, both DNA oligomers, Z8A and Z8M follow a similar trend in the presence of cobalt (III) hexammine (Figure 05)

252
Figure 108: Plot of Enthalpy versus Entropy
A thermodynamic compensation is seen between the enthalpy and the entropy of the system for both the Z8A and Z8M oligomer.

Figure 109: Plot of Free Energy versus Temperature
A linear correlation between the free energy and the temperature is shown below for both the Z8A and Z8M oligomer. The red data points represent the data for the Z8M oligomer, that is untreated with NaCl, while the black data points represent the data for the Z8A oligomer.

The calculated ΔG values at each temperature obtained by the ITC data for the Z8A and Z8M oligomer, are plotted to determine if any correlations exist between the ΔG and the temperature. A linear correlation between the ΔG and temperature is found for the interaction between Cobalt (III) hexammine and the DNA oligomers, Z8A and Z8M. This trend is also found with NaCl as shown in table 24 and 25.

253
Figure 110: Plot of Entropy versus Temperature

The graph below reveals a correlation between the entropy and the temperature depending on the DNA oligomer. The entropy associated with the Z8M oligomer represented as the red, has less dependence on the temperature than the Z8A oligomer due to the difference in slope between the two.

The last correlation for the cobalt (III) hexammines and the DNA oligomers, is the correlation between the entropy and the temperature. There is a direct linear correlation between the entropy and the temperature for the DNA oligomers Z8A and Z8M. This correlation is due to changes in hydration brought about by electrostatic interactions between the cobalt (III) complex and the DNA.

5.2.6 Heat Capacity

As mentioned in the previous section, the heat capacity is related to a number of factors such as solvent accessible surface area and vibrational factors. For the B-to-Z transition, the factor most prominent is the change in the solvent accessible surface area brought about from the difference in size between the NaCl and Cobalt (III) hexammine molecule. As shown in table 27, the heat capacity values were obtained for both the Z8A and Z8M oligomer. Using the previous equation to calculate the enthalpy of the B-to-Z
transition, the heat capacity is calculated for the B-to-Z transition. The values of 0.083 for the NaCl interaction and a value of -0.136 for the cobalt (III) hexamine interaction. The difference in heat capacity is associated with the relative size difference between NaCl and Cobalt (III) hexamine. Since heat capacity is related to the accessible surface area, the cobalt (III) hexamine occupies more of the surface than the NaCl molecule would, therefore having a different heat capacity value. Also cobalt (III) hexamine is involved in some form of binding to the oligomers, changing the solvent accessible surface area. The difference in sign is due to the binding event seen in cobalt (III) complexes and not in NaCl.

5.2.7 Osmolyte Studies

Water is an important function in all biological systems. The B-to-Z conformational transition is no exception. In this study Betaine was incorporated to investigate how water played a part in the thermodynamic values of the B-to-Z transition. Previously published work by SKDHSKH provided structural evidence by CD, that as you increase the concentration of Betaine, or other osmolytes, the equilibrium of the conformation transition shifts toward the Z-form. This is a result of further hydrating the system, displacing water molecules with the osmolyte, inducing the conformational change prior to adding a Z-inducer such as NaCl or Cobalt (III) hexamine. The results obtained used an equation by Parsegian for the calculation of the number of water molecules associated with the B-to-Z conformational transition by calculating the K values and plotting it versus the concentration of your osmolyte. As table 33 shows, the number of water molecules released due to the conformational change induced by NaCl is 5.37 for the
Z8M oligomer and 0.93 for the Z8A oligomer. The Z8M oligomer contains a large bulky methyl substituent on each of the cytosine bases.

The difference in the enthalpic value obtained prior to adding the osmolyte and after adding the osmolyte should also provide information pertaining to the energetics of the water molecules. For instance, the enthalpic values for the B-to-Z conformational transition prior to adding the betaine is 0.70 kcal/mol base pair, with the addition of 0.5 M betaine the enthalpic value for the B-to-Z transition decreases to 0.45 kcal/mol bp.

### Table 36: The Enthalpy Difference With the Osmolyte and Without

The table provides the enthalpy of the B-to-Z transition determined by ITC. It also accounts for the energy associated with the release of water at each osmolyte concentration.

<table>
<thead>
<tr>
<th>[Osmolyte]</th>
<th>ΔHΔZ (kcal/mol bp)</th>
<th>ΔΔHΔZ (kcal/mol bp)</th>
<th>ΔΔHΔZ/Δαn (Z8M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 M</td>
<td>0.70</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>0.1 M</td>
<td>0.63</td>
<td>0.07</td>
<td>0.013</td>
</tr>
<tr>
<td>0.5 M</td>
<td>0.45</td>
<td>0.25</td>
<td>0.046</td>
</tr>
<tr>
<td>1.0 M</td>
<td>0.59</td>
<td>0.11</td>
<td>0.020</td>
</tr>
</tbody>
</table>

If the difference between the enthalpic value prior to adding the betaine and after adding the betaine, a value for the enthalpic contributions relative to the water activity can be determined. This value is calculated to be 0.25 kcal/mol base pair. Therefore, if we take the number of water molecules released due to the conformational transition (Z8M) by the calculated value for the enthalpy of water activity we can then obtain the energy.
required to release one molecule of water at 0.5 M betaine using NaCl as the Z-ducer. This value is calculated to be 46.5 cal/mol base pair.

This relationship between sequence and water activity is definitely apparent between the two DNA oligomers studied. Future studies should investigate how cobalt (III) hexamine would compare to the results obtained for the NaCl series. Also, the use of other osmolytes such as sucrose could also help understand the role of water in biological systems and investigate the thermodynamics related to water activity.
6 REFERENCE


258

260


261

262


254


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265