Spring 1999

Thermodynamic and Binding Studies of Ligand-Carcinogen DNA Targets

Qianying Liang
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Thermodynamic and Binding Studies of

Ligand-Carcinogen DNA Targets

by

Qianying Liang

May 1999

A Dissertation submitted to the faculty of Seton Hall University,
South Orange, New Jersey,
In partial fulfillment of the requirements for the degree of doctor of philosophy
in the Department of Chemistry.

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APPROVED

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In Charge of Major Work

Member of Dissertation Committee

Member of Dissertation Committee

Approved for the Chemistry Department
Chairperson, Department of Chemistry
To my parents
ACKNOWLEDGEMENT

It is with great pleasure that I record my deep sense of gratitude and sincere thanks to Professor Richard D. Sheardy for his support, guidance and patience throughout course of this research. He played an ideal role of a friend, philosopher and guide in shaping up the research of this dissertation. I am grateful to Dr. Daniel Huchtial, Dr. John Sowa and Dr. Mark Chiu for their willingness to serve on my thesis committee. Thanks are due to my peers and best friend, Dave Calderone, Steve Marrotta for their help when I needed it the most.

I also wish to thank all other members of the research group, Ben Otokiti, Terisita Ortega, Enrique Dilone, Andy Anantha and Mike Hicks, for their willingness to help and cooperate at all times.

Finally, and most importantly, I would like to record the contributions made by my parents, younger brother and Yang Wang, in my education, whatever I have achieved in my life is because of their love, understanding and supporting for me. This thesis is devoted to them.
ABSTRACT

A series of synthetic DNA oligomers were designed that possess potential high affinity carcinogen binding sites. We have examined their thermal stabilities and investigated the relationship between thermal stability and the binding affinity of a simple porphyrin to the DNA. The design addressed how base sequence context influenced the conformational properties of the DNA. All oligomers were variations of a sequence known to covalently bind the carcinogens acetylacetoxy-amino-fluorene (AAAF) and N-nitro-quinoline (NQO). The thermal stability was assessed by (1) determination of the thermodynamic parameters (i.e., enthalpy, entropy and Gibb’s free energy) for the duplex to single strand transition, (2) theoretical calculations based on an established model, and (3) the stability dependence on NaCl concentration. Thermodynamic parameters were determined via application of van’t Hoff analysis of the duplex concentration dependence of melting temperature ($T_m$) obtained from optical melting studies. Theoretical predictions of duplex stability considered both hydrogen bonding and nearest neighbor contributions. While hydrogen-bonding interactions contributed to the observed thermodynamic parameters for duplex formation for these duplexes, the base stacking played the major role in the free energy of duplex formation. The salt dependence of the melting temperature, shown through linear $T_m$ versus log [Na$^+$] plots, can be used to calculate the differential ion binding term, $\Delta n$, which represents the release of counterions from the duplex upon denaturation. The value of $\Delta n$ provides indirect evidence that the sequence context of DNA influences how many Na$^+$ ions bind the DNA. The results indicated that duplexes possessing CTTTC, CTTCC and CTTAC segments exhibited much weaker binding affinities toward Na$^+$. 
The interaction specificities of tetrakis(4-N-methylpyridyl)-21H,23H-porphrin (H₂TMPyP) with selected DNA duplexes was then investigated visible and CD absorption studies. The interactions were followed by measuring the visible spectra at 400-500 nm over a range of temperatures and Na⁺ concentrations. The polycationic H₂TMPyP may bind to DNA through via intercalation into DNA base pairs, outside stacking or both. The data indicated that H₂TMPyP interacted with the carcinogen binding sequences and the induced circular dichroism spectra of the porphyrin at the Soret region suggested that the porphyrin was intercalated into the DNA base pairs at high [DNA]/[porphyrin] ratios. Analysis of the binding isotherms from experimentally determined Scatchard plots indicated a sequence specificity in the binding. The effects observed by changing the base pair adjacent to the proposed high affinity binding site may occur as a result of differential base stacking, differences in the minor groove environment and/or subtle conformational alterations at the interaction site.

For the intercalative binding mode, the binding free energies for porphyrin-DNA interactions are quantitated by van’t Hoff analyses. The favorable binding free energy arises primarily from a large, negative enthalpic contribution for two of the four sequences studied. The binding of the porphyrin, with a +4 charge, was quite sensitive to ionic strength. Analysis of the interaction as a function of ionic strength can be used to dissect the total free energy of binding into electrostatic and non-electrostatic components in light of the polyelectrolyte theory. This analysis demonstrated that the electrostatic component was highly dependent upon ionic strength while the non-electrostatic component was independent of ionic strength. The results were discussed in terms of how the conformational properties of a segment of DNA influence its ligand binding properties.
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A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4,
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\[
\log K_{obs}/\log[NaCl] = -Z\psi, \quad Z \text{ is the charge on the ligand and } \psi \text{ is the fraction of counterions associated with each DNA phosphate.}
\]

A) oligomer C1, B) oligomer C5.

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LIST OF ABBREVIATION

$\varepsilon$ the bulk dielectric constant

$\psi$ the fraction of counterions associated with phosphate in DNA

$\xi$ dimensionless structural parameter

$\Psi$ the overall fraction of monovalent cation associated with each phosphate

$\Delta \Delta$ the difference between experimental result and theoretical calculation, duplex to single strand state functions (the second $\Delta$ denotes $\Delta G^0, \Delta H^0, \Delta S^0$)

$\Delta G^0_{\text{exp}}$ the free energy determined by experiments

$\gamma$ the number of small molecules bound per macromolecule at equilibrium, 

$\gamma = C_b/C_m$

$\Delta G^0_{\text{cal}}$ the predicted free energies, which take into account stacking and hydrogen-bonding contributions, using Doktycz et al. (1992)

$\Delta G(g)$ the deviation from average stacking, in solvent $g$, from Table 3 of Doktycz et al. (1992)

$\Delta G_{\text{stack}}^0(j,g)$ total stacking free energy of oligomer $j$ in solvent $g$

$[A]$ concentration of free A at equilibrium

$[MX]$ monovalent salt concentration

$[Na^+]$ the sodium concentration

$[P]$ concentration of free macromolecule

$[PA]$ concentration of complex

$\varepsilon_{422}$ extinction coefficient at 422 nm

$\varepsilon_{422,b}$ extinction coefficient at 422 nm of fully bound (i.e., in the presence of excess
DNA) porphyrin

$\varepsilon_{422,f}$ extinction coefficient at 422 nm of free porphyrin

A adenine

$\Delta A_{422}$ the difference between absorbances in the absence and presence of porphyrin

A-T adenine:thymine, DNA base pair has two hydrogen bonds

b charge spacing

C cytidine

$C_b$ binding concentration of porphyrin

CD circular dichroism spectroscopies

$C_f$ free concentration of porphyrin in the presence of excess DNA

$\text{CH}_3\text{CN}$ acetonitrile

$C_m$ concentration of macromolecule

$C_T$ concentration of carcinogen target DNA duplexes

CuTMPyP4 metalloporphyrin [copper(II) $meso$-$tetra(N$-$methyl$-$4$-$pyridyl)$porphyrin]

D a DNA binding site in the presence of excess monovalent cation

dG:dC guanine:cytosine, DNA base pair has three hydrogen bonds

DMT - 4',4' dimethoxytrityl

DNA deoxyribonucleic acid

G guanosine

G-C guanine:cytosine, DNA base pair has three hydrogen bonds

$\Delta G^o, \Delta H^o, \Delta S^o$ Gibb's free energy, enthalpy, entropy change

$\Delta G_{obs}$ observed binding free energy
\( \Delta G^0_{\text{hb}}(\text{cal}) \) hydrogen-bonding free energy

\( \Delta G^0_{\text{stack}}(\text{cal}) \) base stacking free energy

\( \Delta G_{\text{pe}} \) the contribution of polyelectrolyte to the free energy

\( \Delta G_i \) the 'non-electrostatic' contribution to the binding free energy, \( \Delta G_i = \ln K_i \).

\( \Delta G_{vH}^0, \Delta H_{vH}^0, \Delta S_{vH}^0 \) The van't Hoff transition Gibb’s free energy, enthalpies and entropies

\( H_2\text{TMPyP} \) meso-tetrakis(4-N-methylpyridyl)-21H,23H-porphine (H2TMPyP)

\( K \) the equilibrium binding constant

\( k \) Boltzmann's constant

\( K_{\text{obs}} \) the apparent ligand binding constant

\( K_e \) the equilibrium binding constant from 'non-electrostatic' contribution

\( L \) a cationic ligand

\( \text{M}^+ \) monovalent cation

\( n \) the number of binding sites on each macromolecule

\( \Delta n \) the differential ion binding which represents the release of counterions upon denaturation

\( N_i(j) \) number of times an \( n-n \) bp doublet appears in the sequence of oligomer \( j \)

\( \text{NMR} \) Nuclear Magnetic Resonance

\( \text{PCR} \) The polymerase chain reaction
\( q \) the protonic charge

\( r \) the number of moles of porpyrin bound per mole of DNA base pair,

\[ r = C_b/[\text{DNA}] \]

\( R \) the gas constant

RNA Ribonucleic acid

\( \Psi_s \) Debye-Huckel screening process

\( T \) the temperature in degrees Kelvin

T thymidine

\( T_m \) the midpoint temperature for a cooperative helix-coil transition is defined as the temperature at one-half of the absorbance change after correction for pre- and post-transitional base lines

TMpyP(4) N-alkyl substituent of meso-tetrakis(4-N-alkylpyridinium-4-yl)porphyrin

UV/Vis Ultraviolet/visible spectroscopy

\( Z \) the charge on the ligand
Chapter I
INTRODUCTION

1.1 Historical Background

The rapid growth of the human genome project as well as novel therapeutics based on either recombinant DNA technology or direct gene therapy has resulted in widespread use of synthetic oligonucleotides and in vivo genetic manipulations. DNA is the genetic material in living cells. DNA is believed to be the molecular target of a number of carcinogens. Certain classes of carcinogens, mutagens and dyes can interact with DNA during biological replication and RNA biosynthesis. These carcinogen and DNA interaction can alter cell metabolism, diminish, and in some cases terminate cell growth or mutate cell growth to cause different kinds of cancers.

1.1.1 DNA Structure

In 1953, Watson and Crick demonstrated that deoxyribonucleic acid (DNA) adopts double helical structure from the X-ray fiber diffraction data. DNA is a linear polymer built up of monomeric units, the nucleotides. A nucleotide consists of three molecular fragments: sugar, nitrogenous aromatic heterocycle; and phosphate. The sugar, deoxyribose, is in a cyclic, furanoside form and is connected by β-glycosyl linkage with one of four heterocyclic bases to produce the four normal nucleosides (Figure 1): adenosine (A), guanosine (G), cytidine (C) and thymidine (T). In double helix (Figure 2), the adenine:thymine (A-T) base pair has two hydrogen bonds while the guanine:cytosine (G-C) base pair has three (Figure 1).

DNA oligomers are stabilized through base-base interactions: hydrogen bonding and base stacking. The base-base interactions include: (a) those horizontal with the base pairs due
to hydrogen bonding and (b) those perpendicular to the base planes from base stacking stabilized mainly by London dispersion forces and hydrophobic effects. Hydrogen bonds are mainly electrostatic in character. They play a key role in the stabilization of nucleic acid secondary structures arising from base-pairing interactions. The stability increases with the proportion of dG:dC base pairs as a result of the three hydrogen bonds formed by this pair. The base stacking is also important for the stabilization of nucleic acid helices. Dipole-dipole interactions, interacting π-electron systems, and induced dipole-dipole interactions appear to be important in vertical base stacking. In addition, evidence has been presented for the contribution of London dispersion forces and for base stacking in aqueous solution, hydrophobic forces also are involved.

Figure 1  Watson-Crick hydrogen bonding.
Figure 2  Schematic drawing of the DNA double helix.
The physicochemical properties are major parameters for the determination of the DNA structural information. The affinity of the binding between an oligonucleotide and its target complementary sequence is characterized by the melting temperature, Tm, of the double-stranded nucleic acid that is formed. The Tm is the temperature at which 50% of the double strand has dissociated into its two single strands. Thermodynamic methods have been used to obtained the enthalpies, entropies and free energies changes for the duplex to single strand transition for DNA, which is sequence-context-dependent.

Thermal denaturation studies of DNA have revealed that the melting temperature, Tm (Martin, 1971) of a DNA double helix depends on strand length (Porschke, 1971; Blake, 1987; Aboul-ela, 1985), strand concentration (Marky, 1987; Gaffney, 1989; Gotoh, 1981), base sequence (Breslauer, 1986; Delcourt, 1991; Marmur, 1970), and ionic strength of added salt (Cantor, 1980). Experimental studies indicate that double helix stability can be predicted in terms of the standard Gibb’s free energy change, \( \Delta G^0 = \Delta H^0 - T \Delta S^0 \), if the standard enthalpy and entropy changes (\( \Delta H^0 \) and \( \Delta S^0 \)) are known for the melting of each nearest-neighbor doublet of base pairs in DNA (Breslauer, 1986). Normal B-form helical DNA, with Watson-Crick base pairs stabilized by nearest neighbor base stacking, has 10 possible kinds of nearest-neighbor doublets. Thermodynamic studies of sequence-dependent conformational states present in naturally occurring DNA polymers from studies on specially designed and synthesized oligonucleotides indicated that purine-purine stacks are most stable (Cantor, 1980; Bloomfield, 1974; Hinz, 1974). Solubility experiments in bi-phasic systems and NMR data show that stacking interactions between purine and pyrimidine bases follow the trend of higher stacking free energy, purine-purine > pyrimidine-purine > pyrimidine-pyrimidine.
1.1.2 DNA Synthesis:

The polymerase chain reaction (PCR) technique allows for an extremely small amount of DNA to be amplified geometrically until necessary quantities are obtained. For biological studies, polydeoxyribonucleotides with defined sequences longer than those accessible by chemical methods are often required. Cloning techniques allow the desired sequences to be inserted into a cell line to obtain a large DNA sequence, gene or group of genes.

The organic synthesis of oligodeoxynucleotides has been the center of interest of many research groups in the past decade due to usage in genetic engineering (Engels, 1989). In order to study nucleic acid structure at the molecular level, the methods for chemically synthesizing DNA have been successfully developed over the past 30 years. The chemical synthesis of oligodeoxyribonucleotides has become a rapid, efficient routine laboratory procedure since the development of solid-phase methods and commercial availability of reagents (Itakura, 1984). The increasing availability of synthetic DNA sequences together with the advent of molecular cloning techniques has had a profound effect on many biological studies. Synthetic DNA has become an indispensable tool in modern biology laboratories. Commercially available automated solid support DNA synthesizers can affordably make DNA molecules of less than 100 bases in useful micromole scale quantities. The synthesis via phosphoramidite method developed (Caruthers, 1985), as the phosphite triester method, is the most efficient method for preparing oligodeoxynucleotides. It entails the 5'-OH group of the growing DNA chain reacting with a nucleoside 3'-β-cyanoethyl N,N-diisopropylphosphoramidite with catalysis by 1H-tetrazole and the resulting phosphite triester being oxidized immediately with I₂ to the phosphotriester. The coupling yield in the method is
>95%, (Efcavitch, 1985) and a synthesis cycle only takes about 8 min. It is possible in this way to construct oligomers having up to 175 nucleotides using an automatic DNA synthesizer (Efcavitch, 1987).

For the solid support, silica gel or control pore glass supports are used with a 10-20 atom linker to the oxygen of 3' end residue. The 3'-hydroxyl is a secondary alcohol that can be chemically modified with a phosphitylating reagent. In order to avoid side reactions and also to increase the solubility of the coupling units, almost all functional groups of the nucleotide are protected. The following protecting groups have been used for each base that has an amino group on a heterocyclic ring: benzoyl for adenine and cytosine, and isobutyryl for guanine during the automation cycle. (Khorana, 1979) (Figure 3A). The automation involves a cycle of four steps:

1) Detritylation of the 5' end

The 4', 4' -dimethoxytrityl (DMT) group is removed from the coupling reaction product under acidic conditions to obtain a new 5' -hydroxyl group for next coupling reaction. Trichloroacetic acid is used in this step.

2) Activation and Coupling

The monomer-coupling unit (nucleoside phosphoramidite) is activated in the presence of 1H-tetrazole to obtain activated phosphoramidite and then reacted with the 5' -hydroxyl component to give the phosphite intermediate on the support. The reaction is complete in a few minutes and average coupling yield is more than 95%.

3) Capping failed sites

Any unreacted 5' -hydroxyl component is reacted with acetic anhydride to give a nonreactive component. It takes two minutes to complete the reaction. This step is essential in order to simplify the purification step.

4) Oxidation of phosphite to phosphate
Figure 3  Organic synthesis of oligodeoxynucleotides.
A) Protection/Deprotection of the nucleoside bases dG, dA, dC, and 5' primary hydroxyl group of the ribose.
Figure 3 Organic synthesis of oligodeoxynucleotides.

B) Phosphoramidite activation (i), coupling (ii), capping of the failure sequences (iii), oxidation of phosphite to phosphate (iv), removal of β-cyanoethyl group (v), and detritylation (vi).
Figure 3 Organic synthesis of oligodeoxynucleotides.

B) Phosphoramidite activation (i), coupling (ii), capping of the failure sequences (iii), oxidation of phosphite to phosphate (iv), removal of β-cyanoethyl group (v), and detritylation (vi).
The phosphite intermediates are converted to the phosphotriesters by treatment with I_2 / H_2O for a minute.

The reagent lines and column are washed with acetonitrile between each step. The synthesis scheme is depicted in the Figures 3. This ends the cycle, leaving a resin supported dinucleotide with a dimethoxytrityl group on the 5' end. The cycle is repeated until the programmed sequence is completed. Deblocking of fully protected oligonucleotides include the following steps:

(1) Phosphate group

The phosphate protecting group is the β-cyano-ethyl group which is hydrolyzed using concentrated ammonium hydroxide. This converts the phosphate triester to the diester and acrylonitrile, CH_2=CH-CN.

(2) Amino group

The following protecting groups have been used for each base that has an amino group on a heterocyclic ring: benzoyl for adenine and cytosine, and isobutyryl for guanine. (Khorana, 1979) (Figure 3). These protecting groups are removed by treatment with concentrated ammonium hydroxide at 55 °C for 18 h. (or three days at room temperature) (Figure 3).

(3) 5'-Hydroxyl group

The 4',4'-dimethoxytrityl (DMT) group is used for the protection of the 5'-hydroxyl group and the detritylation is performed by treatment with 80% acetic acid during the purification step.

The final product resulting from solid-phase synthesis is the mixture of desired tritylated polynucleotide and a series of truncated shorter polynucleotides. The still tritylated (full sequence) strand is separated from failure sequences and protecting groups by a trityl
select reverse phase HPLC as described in the methods (Sheardy, 1986). A final reverse HPLC preparative run separates any dimethoxytrityl alcohol from the very pure oligonucleotide. In this manner, very pure DNA can be obtained for studies. The purity can be checked by electrophoresis on a nondenaturing polyacrylamide gel.

1.2 Small Molecule – DNA Interactions

Small molecules interacting with DNA involve different binding processes which include both covalent bonding and various non-covalent binding modes. Non-covalent binding of small organic molecules to duplex DNA is of interest for use in antitumor, antiviral, and antibiotic applications (Myers, 1994; Roche, 1994; Cullinane, 1994; Kusakabe, 1993; Liu, 1993; He, 1993; Silva, 1993; Pindur, 1993).

The study of small molecule-oligonucleotide duplex complexes has been carried out to elucidate basic structure-function relationships with the eventual goal of performing a rational design of sequence specific DNA-binding molecules (He, 1993). Small molecule-oligonucleotide duplex complexes have been studied by several techniques including NMR (Tinoco, 1971), X-ray crystallography (Tinoco, 1973), footprinting by gel electrophoresis (Freier, 1986), FTIR, linear dichroism, flow dichroism (Banville, 1985 and Gabbay 1979) and circular dichroism (Anantha, 1998).

Polyelectrolyte Theory

Monovalent cations bind to DNA through both condensation and Debye-Huckel screening processes. The counterion condensation process was first described by Manning (1972, 1978, 1979). Condensation is described using a dimensionless structural parameter, $\xi$: 
where $q$ is the protonic charge, $\varepsilon$ is the bulk dielectric constant, $k$ is Boltzmann's constant, $T$ is the temperature in degrees Kelvin, and $b$ is charge spacing. Additional cations bind to DNA by a Debye-Huckel screening process ($\Psi$'s) (Record, 1978), so that the overall fraction of monovalent cation associated per phosphate is given by:

$$\Psi = \Psi_c + \Psi_s = [1-(2\xi)^{-\frac{1}{2}}]$$

For B-form DNA, the distance of two negatively charged phosphates is 3.4 Å and the average fraction of monovalent cation associated with each phosphate is $\Psi = 0.88$. The cations bound in this manner are non-specific. These cations surrounding the DNA helix and are free to diffuse within the condensed counterion layer.

Positively charged ligands binding to DNA is thermodynamically linked to the binding of monovalent cations. Record et al (1978) provided a useful formulation of salt effects on ligand binding.

$$\left(\frac{\delta \ln K_{obs}}{\delta \ln [MX]}\right) = -Z\psi$$

where $Z$ is the charge on the ligand. The apparent ligand binding constant, $K_{obs}$, is often observed to be strongly dependent on the monovalent salt concentration ([MX]). Wilson & Lopp (1979) first described the necessary modification of Record's theory to include ion
release from the DNA structural transition. For a cationic ligand (L) binding to a DNA site (D) in the presence of excess monovalent cation (M\(^+\)), C is the complex, the thermodynamic equilibrium is:

\[
D + L = C + Z\psi[M^+] \tag{4}
\]

The equilibrium binding constant for above equation is:

\[
K = ([C][M+]Z\psi)/([D][L]) \tag{5}
\]

For the association of a cationic ligand with the DNA polyanion with intercalation in excess of monovalent cation:

\[
\ln K_{obs} = \ln K_i - Z\psi \ln [MX] \tag{6}
\]

The observed binding free energy at that salt concentration may then be dissected into its non-electrostatic and polyelectrolyte contributions, using equation

\[
\Delta G_{obs} = \Delta G_i + \Delta G_{pc} \tag{7}
\]

where the 'non-electrostatic' contribution to the binding free energy \(\Delta G_i = \ln K_i\) is independent of salt concentration, and refers to the hypothetical standard state of 1 M monovalent cation concentration. \(\Delta G_i\) represents the energy contribution from interactions
other than the polyelectrolyte effect, including, for example, hydrogen bond, hydrophobic and van der Waals stacking interactions. The polyelectrolyte contribution to the free energy is described by:

\[ \Delta G_{pe} = Z'PRT\ln[X] \]  

There are three binding modes for small molecule interacting with DNA through groove binding, intercalation, and exterior electrostatic binding.

**Grove Binding** through hydrogen bonding, van der Waals forces, steric effect and electrostatic interactions have been suggested to account for the sequence selectivity of most small molecules (Pelton, 1990; Pelton, 1989; Freier 1986). The oligonucleotide duplex forms due to hydrogen bond formation, hydrophobic interactions, and electrostatic forces in the presence of stabilizing counterions (Pasternack, 1983). The AT-rich minor groove is narrower than average; their widths depend both on the length and the nature of the AT sequence. Other sequence-dependent localized features of base pair and base pair steps (such as propeller and helical twist), will affect the geometric relationships between hydrogen bond donors/acceptors on successive bases. Minor groove binders are typically crescent-shaped linear molecules that fit into the DNA duplex minor groove of four or five successive A, T base pairs (Figure 4A).

**Intercalation** occurs when planar aromatic molecules insert between adjacent DNA or RNA base-pairs. To accommodate the intercalator, the base pairs must separate, lengthening the helix, and increasing the phosphate spacing at the intercalation site. The increase in the phosphate spacing \( b \) (equation 1) leads to a decrease in the dimensionless parameter \( \xi \).
A) Structures of some groove-binding drugs

**Figure 4:** Structures of small molecules that can interact with DNA
A) Structures of some groove-binding drugs
B) Structures of some DNA-intercalating molecules
B) Structures of some DNA-intercalating molecules

Figure 4: Structures of small molecules that can interact with DNA
A) Structures of some groove-binding drugs
B) Structures of some DNA-intercalating molecules
(equation 1), and a decrease in the fraction of monovalent cations associated per phosphate. For intercalators (Figure 4B), then, there are two contributions to the polyelectrolyte effect that result in cation release, one from the binding of the charged ligand, and a second from the increased phosphate spacing resulting from the structural change in the DNA helix.

Certain classes of drugs, carcinogens, mutagens, and dyes intercalate between adjacent base-pairs of DNA and change the physical properties of a double helix. DNA length is increasing when a small molecule inserts into the base pairs and may unwind DNA helix (Figure 5). Since intercalators interrupt RNA synthesis (transcription) or DNA synthesis (replication), some have been used as anticancer drugs while others are carcinogenic.

**Electrostatic binding** occurs with all nucleic acid and is very sensitive to changes in the ionic environment due to nucleic acids’ high charge. G-C base pairs are more electron-rich than A-T base pairs. Thus, electron-deficient planar molecules that can stack with individual base pairs bind preferentially to G-C base pairs. There is less base stacking in B-DNA between 3′-pyrimidine, 5′-purine dinucleoside steps than 3′-purine, 5′-pyrimidine steps, meaning that the dinucleoside sequences CpG, TpG, CpA, TpA (of the 3′-pyrimidine, 5′-purine type) are more readily unstacked and therefore planar molecules bind intercalatively to them in preference. The methyl group of thymine can also play an important role in sequence readout by being able to make stable van der Waals non-bonded contacts with hydrophobic chromophores or contribute steric effects in destabilizing the resulted DNA-ligand complex.

Small molecules which interact with DNA in a sequence-dependent fashion take advantage of DNA differences in backbone and phosphate conformations and, hence, can act as recognition signals. Perhaps more important are differences in the distance between
Figure 5  Schematic representing the secondary structure of normal DNA, regular sugar-phosphate backbone (left) and DNA containing intercalated planar molecules, (right) (Bloomfiled, 1974).
phosphate groups that necessarily result from backbone conformational differences, which can be recognized by specific hydrogen bonding/electrostatic interactions with small molecules.

The electronic characteristics of an individual base pair produces an electric field and electric potential in its vicinity. These electronic features are distinct for the various classical DNA structural types, as well as for changes in oligonucleotide sequences. Recent studies find that B-DNA has an electrostatic potential minimum occurring in the major groove of oligo (dG),(dC) sequences, the minor groove of oligo (dA),(dT) sequences and the 5’-AATT sequence in the Dickerson-Drew dodecamer. These all have more negative potentials than those around the phosphate groups, which explains why cationic ligands and basic protein side-chains cluster in the grooves rather than around the phosphates. This electrostatic factor is important in directing an incoming ligand to a region of sequence; small local variations in net charge and field around an individual base pair then could assume some importance, especially for electrophilically driven covalent bonding.

**Scatchard Equation and Scatchard Plot**

The Scatchard equation is used to study small molecule binding to a macromolecule (Fiel, 1979). The binding can be measured by any method. The equilibrium constant for binding molecule (A) to the macromolecule (P) can be calculated from the total concentration of macromolecule.

\[ P + A \rightleftharpoons PA \]  \hspace{1cm} (9)

The equilibrium has the equilibrium constant \( K \):
\[ K = \frac{[PA]}{[P][A]} \]  \hspace{1cm} (10)

where \( P \) is a macromolecule with a single binding site for \( A \). If we assume activities can be replaced by concentrations, then

- \([PA]\) = concentration of complex at equilibrium
- \([P]\) = concentration of free macromolecule at equilibrium
- \([A]\) = concentration of free \( A \) at equilibrium

The equation for \( K \) can be simplified and generalized by introducing \( \gamma \), the number of small molecules bound per macromolecule at equilibrium.

\[ \gamma = \frac{C_b}{C_m} \]  \hspace{1cm} (11)

where \( \gamma \) is average number of ligand \( (A) \) bound per macromolecule, \( C_b \) is the concentration bound \( A \) and \( C_m = \) concentration of macromolecule. Writing the expression for \( K \) in terms of \( \gamma \):

\[ K = \frac{C_b}{(C_m - C_b)} C_f \]  \hspace{1cm} (12)

\[ = \frac{\gamma}{(1-\gamma)} C_f \]  \hspace{1cm} (13)

or

\[ \frac{\gamma}{C_f} = K (1 - \gamma) \]  \hspace{1cm} (14)

The equilibrium constant is written with the assumption that i) only one molecule of \( A \) is bound per macromolecule meaning that \( \gamma \) can vary only from 0 (no \( A \) bound) to 1 (each macromolecule has bound an \( A \)). However, many macromolecules have more than one
binding site for a ligand. Here, $\gamma$ can vary from 0 to $n$, the number of binding sites on each macromolecule. If the sites are identical and independent, it is very easy to generalize the equation. If there are $n$ identical and independent binding sites per macromolecule, each site has the same binding equilibrium constant, $K$, and that binding at one site does not change the binding at another site. We can then replace $\gamma$ in the above equation for the equilibrium constant above by $\gamma/n$:

$$\frac{\gamma/n}{C_r} = K (1 - \gamma/n) \quad (15)$$

or

$$\gamma / C_r = K (n - \gamma) \quad (16)$$

The Scatchard plot of $\gamma / C_r$ versus $\gamma$ should give a straight line with slope of minus $K$, $y$ intercept of $nK$, and $x$ intercept of $n$. If a Scatchard plot does not give a straight line, this indicates that the binding sites are not identical or not independent.

1.3 DNA Sequences

The use of chemical probes provides a way of obtaining the molecular level information in accessible structural problems in DNA and DNA-drug recognition. The chemical carcinogens, N-acetoxy-N-acetyl-2-aminofluorene, AAAF, (Winkle, 1981; Bases, 1983; Fuchs, 1983; Combates, 1992; Mallamaci, 1992) and 4-nitro-quinoline oxide, NQO, (Rodolfo, 1994; Winkle, 1989; Winkle, 1990 and Winkle, 1991), have been shown to target specific bases within particular DNA sequences. Winkle et al used restriction enzyme inhibition (Figure 6) studies to map the locations of high affinity binding sites of AAAF on various native DNAs, such as pBR322, phiX174 and SV40. Their studies suggest the consensus sequence C1 binds the
N-acetoxy-N-acetyl-2-aminofluorene (acetoxyAAF)

4-nitro-quinoline oxide (NQO)

Figure 6   Chemical structure of carcinogen
carcinogen AAAF with high affinity to only one G base (indicated with the arrow) (Combates, 1992; Mallarnaci, 1992; Winkle, 1989; Winkle, 1990 and Winkle, 1991)

5'-GTCAGCTCTTGCTGCG-3'
3'-CAGTCGAAACGACGC-5'

In order to ascertain that the above 16 bp DNA represents the carcinogen target sequence, the C1 oligomer was cloned into the plasmids pKSM and pBR322 in locations shown not to contain carcinogen binding sites (Figure 7). Restriction mapping and restriction inhibition studies indicated that the insertion sites became high affinity carcinogen binding sites. Analogues of C1, shown in Table 1, were synthesized in order to address the effect of sequence context on the binding reaction. Since these sequences represent variations of the consensus sequence, C1, whereby the base pair 5' to the high affinity G site has been altered, their conformational properties are also of biological interest.

The focus of this work was to determine whether a correlation between DNA oligomers' thermodynamic stabilities and the binding affinities of both carcinogens and drugs exists. The use of a small molecule to probe the DNA high affinity binding sites can give information on the stability of carcinogen target DNA sequence and to help us to understand better the role of carcinogens and pharmaceutical agent design.

Methods based on molecular biology, such as restriction enzyme kinetics assays and cloning have been used extensively to determine the sequence selectivity of carcinogen target. Such assays are extremely sensitive and may be quantitative; however, they do not yield direct information concerning the structure of the ligand-DNA adducts. NMR spectroscopy
Figure 7  Cloning carcinogen target DNA (C1) into plasmid pBR322
Table 1: Carcinogen Target Oligomers

<table>
<thead>
<tr>
<th></th>
<th>Oligomers</th>
</tr>
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<tbody>
<tr>
<td>C1:</td>
<td>GTCAGCT\textbf{CTTGCTGCG} CAGTCGAG\textbf{AAGACGACGC}</td>
</tr>
<tr>
<td>C2:</td>
<td>GTCAGCT\textbf{CGTGCTGCG} CAGTCGAG\textbf{GCACGACGC}</td>
</tr>
<tr>
<td>C3:</td>
<td>GTCAGCT\textbf{CATGCTGCG} CAGTCGAG\textbf{TACGACGC}</td>
</tr>
<tr>
<td>C4:</td>
<td>GTCAGCT\textbf{CCTGCTGCG} CAGTCGAG\textbf{GACGACGC}</td>
</tr>
<tr>
<td>C5:</td>
<td>GTCAGCT\textbf{CTGGCTGCG} CAGTCGAG\textbf{ACCGACGC}</td>
</tr>
<tr>
<td>C6:</td>
<td>GTCAGCT\textbf{CTAGCTGCG} CAGTCGAG\textbf{ATCGACGC}</td>
</tr>
<tr>
<td>C7:</td>
<td>GTCAGCT\textbf{CTCGCTGCG} CAGTCGAG\textbf{AGCGACGC}</td>
</tr>
<tr>
<td>C8:</td>
<td>GTCAGCT\textbf{CTTTCTGCG} CAGTCGAG\textbf{AAAGACGC}</td>
</tr>
<tr>
<td>C9:</td>
<td>GTCAGCT\textbf{CTTCCTGCG} CAGTCGAG\textbf{AAGGACGC}</td>
</tr>
<tr>
<td>C10:</td>
<td>GTCAGCT\textbf{CCTACTGCG} CAGTCGAG\textbf{CATGACGC}</td>
</tr>
<tr>
<td>C11:</td>
<td>GTCAGCT\textbf{GATGCTGCG} CAGTCGAG\textbf{TACGACGC}</td>
</tr>
<tr>
<td>C12:</td>
<td>GTCAGCT\textbf{ACTGCTGCG} CAGTCGAG\textbf{TACGACGC}</td>
</tr>
</tbody>
</table>
and X-ray crystallography enable detailed structure characterization but are time consuming and require large amounts (millimoles) of material. UV thermal melting studies on these oligomers can provide detailed information about these sequences and can reveal the carcinogen target process.

1.4 Porphyrin - DNA interactions

It is very important to provide a clear mechanism of carcinogen DNA binding for clinical and pharmaceutical studies. Such studies may provide valuable information and models to apply to the design of antitumor agents.

The interactions of DNA with drugs are of particular pharmacological importance. Chemical probes can attack specific base sites; these can be useful in defining the precise sites of protection resulting from drug binding to a DNA sequence. Further, it has been demonstrated that intercalative drugs compete with the DNA template primer in the polymerase system. These properties initiated this project to use a porphyrin (H₂TMPyP) (Figure 8) as a probe of the DNA oligomers of this project.

Applications of porphyrins in medicine have been found, and porphyrins have extensively been used in laboratory studies of DNA structure and function. The porphyrin, H₂TMPyP, has been shown to bind with high affinity native DNA. It has been used as a pharmaceutical agent whose mode of bioactivity is through interaction with DNA. Porphyrins have been used as chemotherapeutic agents, such as hematoporphyrin which has been used in conjunction with photodynamic therapy to detect and destroy neoplastic cells (Van den Bergh, 1986). Indium 111-labeled porphyrins have been used for lymph node imaging (Foster, 1985) and administration of boron cluster-porphyrin derivatives and neutron bombardment have the potential
Figure 8  Chemical structure of chemical structure of H$_2$TMPyP (5,10,15,20-Tetrakis(1-methyl-4-pyridyl)-21H-porphine, tetra-p-tosylate salt)
to destroy melanoma cells (Vaurn, 1982).

The H₂TMPyP has been shown that intercalate into double stranded DNA and these interactions can be monitored conveniently by UV/Vis spectroscopy in order to calculate binding constants (Fiel, 1982 and 1994). Porphyrins show significant absorption in the visible spectral range. This property can be used to study quantitative aspects of the interaction of porphyrins with DNA which result in absorbance changes. The advantages of absorption spectroscopic titration are:

(1) Simple experimental procedures with solution.
(2) High accuracy of the measurements.
(3) Information about solution species, as well as temperature and ionic strength dependencies.
(4) Linear relations between the measured absorbance and species concentrations which simplifies theoretical approaches for the binding studies.

After determination of the basic spectra, the equilibrium binding constant, the size of the ligand binding site and the thermodynamic parameter can be obtained (interpreted) by the Scatchard plots using various models.

The most extensively studied DNA binding porphyrin is meso-tetrakis (N-methylpyridinium-4-yl) porphyrin, H₂TMPyP. Pasternack studied the influence of ionic strength on the binding of a water soluble porphyrin to nucleic acids (Pasternack, 1986). Their results indicated that the binding of H₂TMPyP to poly(dG-dC) and calf thymus DNA is dependent on the ionic strength. For calf thymus DNA, the binding profile is not completely compatible with the predictions of condensation theory. Whereas the avidity of binding decreases with increasing [Na⁺], of greater interest is the relocation of the porphyrin from GC-
rich regions to AT-rich regions as the ionic strength increases.

Kuroda studied the effect of the charged sidechains of porphyrin interactions with native DNA. The results indicated that meso-tetrakis[4-N-methylpyridiniumyl- and meso-tetrakis[4-N-(2-hydroxyethyl)pyridiniumyl-porphyrin bound and intercalated similarly into DNA as measured by helix stabilization and DNA unwinding studies in the presence of DNA topoisomerase I (Kuroda, 1990). The presence of charged sidechains on the porphyrin rather than their identity appears to be critical for efficient DNA intercalation.

A theoretical two-mode binding model for porphyrin binding to natural DNA has been presented (Feng, 1990). One of the binding modes is assumed to be base sequence specific with binding sites n base-pairs long. The other binding mode has binding sites which consist of only one base-pair and can involve cooperativity. The results show that the fraction of porphyrin bound in the non-specific mode reaches a maximum at certain input DNA to porphyrin concentration ratios. The value of this maximum decreased, and its position shifted to higher DNA to porphyrin concentration ratios for binding in the high ionic strength buffer. The value of the cooperativity parameter obtained through the fitting process suggests that the non-specific binding is positively cooperative. The results are compared with the data analyzed using other techniques.

Computations of the interactors of tetracationic porphyrin, tetra-(4-N-methylpyridyl)-porphyrin, T₄MPyP, with the hexanucleotides d(CGCGCG)₂ and d(TATATA)₂, demonstrate that T₄MPyP manifests a significant preference for intercalation in its complex with d(CGCGCG)₂ but for non intercalative binding in the minor groove in its complex with d(TATATA)₂ (Hui, 1990). The model suggests that intercalation and groove binding may be viewed as two potential wells on a continuous energy surface. In agreement with
experiment, the computations indicate that in the case considered here, the deepest well is associated with intercalation.

Binding studies on the effect of the N-alkyl substituent of meso-tetrakis(4-N-alkylpyridinium-4-yl)porphyrin cations have been carried out on various native DNA and synthetic polynucleotides by using Resonance Raman, NMR, and visible spectroscopies, as well as viscosity and equilibrium dialysis studies (Gray, 1991). Their results suggested that TPrpyP(4) binds to GC regions of DNA in the same intercalative manner as TMpyP(4) with the N-alkyl substituent extended into the solvent. For AT regions of DNA, the binding of TMpyP(4) and TPrpyP(4) is nonintercalative.

The experimental data for two-mode binding of porphyrin to DNA cannot be fit easily using existing theoretical models. The models that have been used so far are either a straight line (Fiel, 1979; M.J. Carvlin 1983) or the McGhee-von Hippel model (Pasternack, 1983; Dougherty, 1985). When obvious multi-mode binding and/or base sequence specificity is involved, the McGhee-von Hippel model does not fit the data well for single-mode binding to a homogeneous lattice (McGhee, 1974). The discrepancy between the prediction of the model and the experimental data is more conspicuous in the region of high bound porphyrin to DNA concentration ratios. Most binding data are published by using Scatchard plots. But the McGhee-von Hippel model predicts that the plots should intersect the horizontal axis, and that the intercept is equal to the number of base-pairs ‘covered’ by the porphyrin (McGhee, 1974) For binding in low ionic strength buffers, the two-mode nature of the binding becomes so significant that the model cannot fit the data at all (Carvlin 1983; Dougherty, 1985).

Many other theoretical models have developed to tackle multiple-mode binding. They apply to various situations such as binding to alternating binding sites (Hill, 1957), to two
types of completely independent binding sites (Dourlent, 1975; Schwarz, 1977), to sites of identical sizes (Dourlent, 1975; Terrell, 1980; Ghosh, 1986) or non-specific sites with neighbor exclusivity and cooperativity (Schwarz, 1977; Schwarz 1979; Hill, 1978; Pincus 1981). None of the existing models is entirely suitable for the current problem, which involves two-mode binding with one of the binding modes being base sequence specific. These two binding modes are completely independent.

A theoretical two mode binding model have been presented for certain water-soluble cationic porphyrins metal derivative of Cu(II)TMPyP-4 and Cu(II)TMPyP-3 binding to natural DNA (Dougherty, 1985) and Ni^{2+} (Biochemistry 1994, 33, 417-426). One mode is intercalation, which has a CG preference (Pasternack, 1983; Ford, 1987; Fiel, 1980; Kelly, 1985; Marzilli, 1986), while the other is an external, or 'outside' binding mode which is largely electrostatic (Pasternack, 1983, Pastrenack, 1986; Ford,1987; Dougherty, 1985). Recent studies show that intercalation versus outside binding may also be influenced by the charge on the porphyrin core (Marzilli et al., 1992; Kuroda et al., 1990)

X-ray structural studies of the metalloporphyrin (CuTMPyP4) [copper(II) meso-tetra(N-methyl-4-pyridyl)porphyrin]:[d(CGATCG)] complex reports that a base flips out of a DNA helical stack after metalloporphyrin intercalates into the DNA oligomer. The copper atom near the helical axis is located within the helical stack. The porphyrin binds by normal intercalation between the C and G of 5' TCG 3' and by extruding the C of 5' CGA 3'. The DNA forms a distorted right-handed helix with only four normal cross-strand Watson-Crick base pairs. Two pyridyl rings are located in each groove of the DNA. The complex appears to be extensively stabilized by electrostatic interactions between positively charged nitrogen atoms of the pyridyl rings and negatively charged phosphate oxygen atoms of DNA (Lipscomb, 1996).
Intercalation by a porphyrin into adjacent specific sites is minimal. Footprinting experiments showed well-separated regions of DNA protected by H$_2$TMPyP-4 (Ford, 1987). An intercalated porphyrin, therefore, will have little chance of interacting with other intercalated porphyrins. In contrast to intercalation, outside binding, being largely an electrostatic interaction between porphyrin cations and charged groups of DNA phosphate backbone, can occur at any base-pair, including those forming potential intercalating sites. These two binding modes are mutually exclusive: once a site is occupied by a porphyrin in a certain mode, it can not be occupied by another porphyrin in any other binding mode.

Marzilli et al. have concluded that H$_2$TMPyP-4 intercalation requires a unique site, 5'-CG-3', based on NMR studies on H$_2$TMPyP-4 binding to poly[d(G-C)$_2$] and five other oligonucleotides (Marzilli, 1986). A molecular modeling study shows two partly intercalated positions for H$_2$TNPyP-4 in a T-A site (Kelly, 1985). In their early work, Fiel et al. (1980) suggested there might be three binding modes: one due to intercalation and the other two due to outside binding. Later studies suggest that the major outside binding, which produces a positive visible band in the CD spectra, occurs preferentially in AT-rich regions of DNA (Dougherty, 1985; Pastrenack, 1986).

A purely electrostatic interaction should be weakened in a buffer of high ionic strength because the presence of a large amount of Na$^+$ screens the electrostatic interaction in a manner like the Debye-Hückel effect and thus reduces the strength of the interaction. EPR experiments also showed evidence of suppressed outside binding in high ionic strength buffers (Ford, 1987). Other experiments have shown that this major outside binding mode is actually enhanced in a high ionic strength buffer (Dougherty, 1985; Fiel, 1980).
This dissertation contains a two part study involving the chemical syntheses of specific DNA oligomers as model target systems for carcinogen and drug binding studies. The focus of this work was to assess whether a small reversible DNA binding molecule, such as a porphyrin, could also discriminate between the sequences in a manner similar to the carcinogen binding. This project focused on establishing a correlation between the DNAs' thermodynamic stabilities and the binding affinities of both carcinogens and drugs. The basic premise here is that the conformation and dynamics of the DNA duplex in the vicinity of the high affinity site are responsible for the reactivity observed. The use of a reporter molecule to probe the carcinogen target DNA high affinity binding sites may help us to understand better the behavior of these carcinogens.

This study involves the chemical syntheses of specific DNA oligomers as model target systems for carcinogen and drug binding studies. Ultraviolet/visible (UV/vis) and circular dichroism (CD) spectroscopies were utilized to examine the structure and thermal stability of carcinogen target DNA-porphyrin complexes which form. The dependence of the binding on ionic strength and temperature was also investigated in order to evaluate and dissect the binding free energy.
Chapter II

EXPERIMENT AND MATERIALS

2.1 Oligonucleotide Synthesis and Purification

DNA Synthesis and Preparation: The DNA oligomers were synthesized on a 1.0 μM scale via the phosphoramidite method on an Applied Biosystems 380B DNA Synthesizer with purification by trityl select Radial Pak C18 column reverse phase HPLC (Figure 9A-F). The final product resulting from solid-phase synthesis is a mixture of polynucleotides (desired oligomer) and a series of failure, shorter polynucleotides. The desired oligomer has the intact 4',4'-dimethoxytrityl (DMT) group in the resulting mixture. The resulting mixture is analyzed on a reverse-phase Radial Pak C18 column using a gradient of increasing acetonitrile with decreasing TEA buffer. The first eluted peak contains polynucleotides corresponding to the failure sequences and the second peak is the desired product (Figure 9A).

After the first preparative HPLC separation by using triethylammonium acetate buffer (0.1 M, pH 7.0) in which the amount of acetonitrile, CH$_3$CN, was varied between 15% and 35%, the tritylated sequence was separated from failures (Figure 9B). Detritylation of the sequence with 0.1M acetic acid was then carried out followed by extraction the free dimethoxytrityl alcohol with diethylether to obtain the desired sequence. The result was conformed by reverse phase HPLC (Figure 9C).

After removal of the DMT group from the second peak, the desired product is further purified by repeating the reversed-phase chromatography using a gradient of acetonitrile. The second HPLC purification preparative run was to remove any DMTr-OH from the pure oligonucleotide which uses the same triethylammonium acetate buffer (0.1 M, pH 7.0) and an
Figure 9  Purification of oligonucleotides by HPLC on a reversed-phase column. A) Isolation of DMT-oligonucleotide. B) A DMT-oligonucleotide was isolated by using gradient of CH$_3$CN (5%-15%). C) The detritylation of the sequence was confirmed by HPLC. D) The final product after removal of the DMT group was confirmed. E) The final product after removal of the DMT group was purified by using gradient of CH$_3$CN (3%-10%). F) Final purification of desired oligonucleotides is characterized by HPLC.
Figure 9 Purification of oligonucleotides by HPLC on a reversed-phase column. A) Isolation of DMT-oligonucleotide. B) A DMT-oligonucleotide was isolated by using gradient of CH$_3$CN (5%-15%). C) The detritylation of the sequence was confirmed by HPLC. D) The final product after removal of the DMT group was confirmed. E) The final product after removal of the DMT group was purified by using gradient of CH$_3$CN (3%-10%). F) Final purification of desired oligonucleotides is characterized by HPLC.
Figure 9  Purification of oligonucleotides by HPLC on a reversed-phase column. A) Isolation of DMT-oligonucleotide. B) A DMT-oligonucleotide was isolated by using gradient of CH$_3$CN (5%-15%). C) The detritylation of the sequence was confirmed by HPLC. D) The final product after removal of the DMT group was confirmed. E) The final product after removal of the DMT group was purified by using gradient of CH$_3$CN (3%-10%). F) Final purification of desired oligonucleotides is characterized by HPLC.
CH₃CN range are from 8% to 20% (Figure 9D, 9E).

Oligomers were characterized by analytical HPLC (Figure 9F). The pure oligomers were exhaustively dialyzed vs water and then lyophilized to dryness. The lyophilized DNA samples were reconstituted in milli-Q-water. Individual duplexes were generated by mixing equal amounts of the complementary strands and heating at 80 °C for two minutes followed by slow cooling. Total concentration of all oligomer species in the samples, C_T, is given per base pair. Samples were prepared by reconstituting lyophilized samples of the duplexes in standard phosphate buffer.

The 16 base pair oligomers synthesized and purified are listed in Table 1.

2.2 Thermodynamic Stability Studies

2.2.1 Optical Melting Studies

Ultraviolet absorbance methods were used to characterize the thermodynamics of melting of the series of 16 bp DNA sequences. The thermally induced denaturation of each DNA duplex was investigated by using two different protocols. The thermal denaturations were determined by monitoring the absorbance at 268 nm as the temperature was ramped from 25 °C to 95 °C using the Temperature Programming software of the Gilford Response II UV/VIS spectrophotometer (Ciba-Corning, Oberlin, OH) equipped with a thermoset cuvette holder at a rate of 0.3 °C/min. The melting temperature Tm is defined as the temperature at which the fracture of single strands, α, is equal to 0.5. The melt profiles were transferred to an external PC as an ASCII file via Procomm Software version 2.4.1 (Datastorm Technologies Inc., Columbia, MO),
sorted and reheaded in order to be analyzed by GODIFF software (Turbo-Basic, Borland International) for transition temperatures and thermodynamic parameters (Marky & Breslauer, 1987). The program GODIFF can be used to calculate the first derivative, or Tmax, of the melt profile. The melting temperatures, Tm, were equal to Tmax.

The first set of experiments were to determine how the base pair changes in these sequences affect the thermal stabilities of the DNA sequences at a fixed NaCl concentration (115mM). For these experiments, the lyophilized DNA samples were reconstituted in in 115 mM Na⁺ buffer (15 mM phosphate, 100mM Na⁺, pH 7.0). Each DNA duplex was melted at various concentrations to determine van't Hoff enthalpies and entropies. The DNA concentration ranged from 1x10⁻³ M to 2x10⁻⁵ M in single strand bases.

For the second set of experiments, the influence of [NaCl] on the thermal stabilities of these oligomers were considered by determining the Tm of each oligomer in 10 mM phosphate buffer at a constant DNA concentration as a function of NaCl concentrations of 15, 115, 150 and 215 mM.

2.2.2 Thermal Analysis

Objectives to be considered in the study on the thermodynamic stability of the DNA sequences are as follows:

(1) Theoretical Calculation (Doktycz's theoretical analysis)

Since hydrogen bonding and base stacking in a specific DNA sequence influences the drug binding activity and helix stability, the predicted thermodynamic free energies of the DNA sequences were calculated according to Doktycz et al (1992) which takes into account both stacking and hydrogen bonding free energies (Doktycz, 1992).
Calculation of $\Delta G_{\text{cal}}$

The dinucleotide permutations in the sequence of the oligomers alter both the hydrogen-bonding scheme and base stacking in the region adjacent to the specific binding site. These alterations will influence the magnitudes of the thermal melting free energies.

Doktycz et al. (1992) recently published a method to predict thermal melting free energies ($\Delta G_{\text{cal}}$), which takes into account stacking and hydrogen-bonding contributions.

(i) By using this method, the contribution of base stacking free energies for these oligomers may be calculated

$$\Delta G_{\text{stack}}^{\alpha}(j, g) = \sum N_i(j) \delta G_i(g)$$  \hspace{1cm} (17)

where $\Delta G_{\text{stack}}^{\alpha}(j, g)$ -- total stacking free energy of oligomer $j$ in solvent $g$, $N_i(j)$ -- number of times an $n$-$n$ bp doublet appears in the sequence of oligomer $j$, $\delta G_i(g)$ -- the deviation from average stacking, in solvent $g$; values used were obtained from Table 3 of Doktycz et al. (1992).

**example:**

C9: CTTCC

GAAGG

$\Delta G_{\text{stack}}^{\alpha}(\text{C9, 115 mM Na}^+) = \Delta G_{\text{CT}} + \Delta G_{\text{TT}} + \Delta G_{\text{TC}} + \Delta G_{\text{CC}}$

$$= 248.8 + (-196.0) + (-273.4) + 150.2$$

$$= -70.4 \text{ cal}$$

(ii) The contribution of hydrogen bonding to the total calculated $\Delta G_{\text{cal}}$ can be evaluated using

$$\Delta G_{\text{hb}}^{\alpha}(j, g) = \Delta S [N_{\text{AT}}(j)(T_{\text{AT}}(g) - T) + N_{\text{GC}}(j)(T_{\text{GC}}(g) - T)]$$  \hspace{1cm} (19)
where

$$T_{AT} = 355.55 + 7.95\ln[Na^+]$$  \hspace{1cm} (20)

$$T_{GC} = 391.55 + 4.89\ln[Na^+]$$  \hspace{1cm} (21)

$T = 310$ K, $\Delta S = -24.8$ cal/kmol, which is independent of sequence and sodium ion concentration and

$N_{AT}(j), N_{GC}(j) --$ number of AT and GC base pairs in sequence $j$

**example:**

C9: CTTCC

GAAGG

$$T_{AT} (115mM Na^+) = 355.55 + 7.95\ln[115] = 393.27$$  \hspace{1cm} (22)

$$T_{GC} (115mM Na^+) = 391.55 + 4.89\ln[115] = 414.75$$  \hspace{1cm} (23)

$$\Delta G^0_{hb}(C9, 115mM [Na^+]) = (-24.8)[2(393.27-310)+3(414.75-310)]$$

$$= 6685.9 \text{ cal}$$  \hspace{1cm} (24)

The mathematical analysis of the theoretical calculation free energy of carcinogen target DNA oligomers can be obtained by the following equation.

$$\Delta G^0_{cal}(\text{cal}) = \Delta G^0_{\text{stack}}(\text{cal}) + \Delta G^0_{hb}(\text{cal})$$  \hspace{1cm} (25)

### 2.3 Drug Binding Studies

The porphyrin free base $H_2TMPyP$ (5,10,15,20-Tetrakis(1-methyl-4-pyridyl)-21$H$-porphine, tetra-$p$-tosylate salt) was purchased from Aldrich and was used without further
purification. Concentrations for the porphyrin were calculated spectrophotometrically using \( \varepsilon_{424} = 2.26 \times 10^5 \text{ M}^{-1}\text{cm}^{-1} \). Porphyrin solutions were prepared in the 115 mM Na\(^+\) buffer (15 mM phosphate, 100mM Na\(^+\), pH 7.0) buffer as the DNA oligomers for all experiments.

Oligomer C1, C4, C5 and C10 are used to study the reversible binding to H\(_2\)TMPyP.

C1: 
- GTCAGCT \textit{CTTGCTGCG}
- CAGTCA\textit{GAGCGACGC}

C4: 
- GTCAGCT \textit{CCTGCTGCG}
- CAGTCA\textit{GGAGCGACGC}

C5: 
- GTCAGCT \textit{CTGGCTGCG}
- CAGTCA\textit{GACCGACGC}

C10: 
- GTCAGCT \textit{CGTACTGCG}
- CAGTCA\textit{GACTGACGC}

2.3.1 UV/Vis spectrophotometer Binding Analysis

The binding of H\(_2\)TMPyP with the different DNAs was monitored by UV/VIS spectroscopy with a Gilford Response II spectrometer (Ciba-Corning, Oberlin, OH) with a thermostated cell holder by titrating small volume of concentrated oligonucleotide duplex into dilute porphyrin. All spectra were acquired at room temperature. Both the drug and DNA were dissolved in 0.01 M sodium phosphate
buffer, pH 7.0, and 115 mM sodium chloride. Absorbance readings and spectra were obtained at 25 °C, except when otherwise noted. For each oligomer, a solution of DNA ([DNA] = 0.5 - 0.8 mM in bases) and H₂TMPyP ([H₂TMPyP] = 5.13 μM) was titrated into a solution of H₂TMPyP at the same porphyrin concentration and the spectrum was recorded from 400- 500 nm at 0.5 nm intervals after exhaustively mixing. The spectrum of the free porphyrin before addition of the DNA/porphyrin solution was also recorded.

The binding of the porphyrin to each oligomer duplex in phosphate buffer at a variety of Na⁺ concentrations at 25 °C was also monitored via the UV/VIS titration method. These experiments provided quantitative data to describe the factors contributing to the stability of the complex formed between the porphyrin based drug and the target DNA.

2.3.2 Circular Dichroism Spectroscopy.

CD spectra of the porphyrin/DNA complex were obtained in a manner similar to the visible titration described above but at only four different [DNA]/[porphyrin] ratios. Solution of DNA/porphyrin and free porphyrin were prepared in buffer with 100 mM Na⁺ and 15 mM phosphate to give a final concentration of porphyrin = 5.13 μM. The circular dichroism spectrum for each porphyrin/DNA complex at 25 °C was recorded on an AVIV 62A DS Circular Dichroism Spectropolarimeter (Aviv Associates Inc., Lakewood, NJ). Aviv Software (version 4.1t) was used for base line correction, scaling, and smoothing the curve by least squares polynomial fit (up to 10th
order). The wavelength region used was 400-550 nm to monitor the porphyrin Soret band.

2.3.3 Optical Melting Studies

The influence of the presence of the porphyrin on the thermal stabilities of these oligomers were carried by determining the $T_m$ of each oligomer in 115 mM NaCl, 10 mM phosphate, pH = 7.0 buffer at [DNA] = 5.0 μM and at concentrations of porphyrin of 9.63, 12.5, 15.4 and 30.8μM.
Chapter III

RESULTS AND DISCUSSION

3.1 Optical melting studies

3.1.1 *UV Thermal Profiles.*

The primary goal of these studies was to assess the effect of base permutations adjacent to the carcinogen binding site on the thermal stability of the DNA duplexes. Thermal stabilities and characteristics of helix-coil transitions for the various oligomers can be determined from the temperature dependence of their UV spectra. Thermal denaturation studies of all oligomers at 115 mM Na\(^+\) and different duplex concentrations were carried out by monitoring the absorbance at 268 nm as a function of temperature. This wavelength contains the maximum hyperchromicity for these DNA duplexes.

Figure 10 shows typical melting curves for the denaturation of a DNA duplex at constant DNA concentration and Na\(^+\) concentrations ranging from 15 to 215 mM. The midpoint temperature \((T_m)\) for a cooperative helix-coil transition is defined as the temperature at one-half of the absorbance change after correction for pre- and post-transitional base lines. All profiles were thermally reversible, giving \(T_m\) values within one degree for both heating and cooling cycles. Although thermodynamic parameters can be directly obtained via melting curve analysis, many of the melting curves did not have a sufficient upper baseline to accurately calculate \(T_m\). Examination of the first derivative plots of the melting curves shown in Figure 10 indicates that all melting transitions could be analyzed as two state transitions. \(T_m\) values were obtained from the inflection points of the first derivative plots of the melting curves. Table 2 lists \(T_m\) values for these duplexes.
Figure 10  Melting curve (absorbance vs temperature) for the carcinogen target DNA oligomer. The experiment conditions are pH 7.0, 115 mM sodium phosphate buffer with an oligomer concentration of 6 μM.
Table 2  The Influence of [Na+] on the Tm of the Carcinogen Binding Sequences

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Tm (°C) at [Na+] indicated&lt;sup&gt;a&lt;/sup&gt;</th>
<th>δTm/δlog[Na+]&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15mM</td>
<td>55mM</td>
</tr>
<tr>
<td>C1</td>
<td>CTTGC</td>
<td>46.4</td>
</tr>
<tr>
<td>C2</td>
<td>CGTGC</td>
<td>51.0</td>
</tr>
<tr>
<td>C3</td>
<td>CATGC</td>
<td>48.7</td>
</tr>
<tr>
<td>C4</td>
<td>CCTGC</td>
<td>48.3</td>
</tr>
<tr>
<td>C5</td>
<td>CTGGC</td>
<td>52.1</td>
</tr>
<tr>
<td>C6</td>
<td>CTAGC</td>
<td>46.4</td>
</tr>
<tr>
<td>C7</td>
<td>CTCGC</td>
<td>50.0</td>
</tr>
<tr>
<td>C8</td>
<td>CTTTC</td>
<td>43.6</td>
</tr>
<tr>
<td>C9</td>
<td>CTTCC</td>
<td>45.7</td>
</tr>
<tr>
<td>C10</td>
<td>CTTAC</td>
<td>43.8</td>
</tr>
<tr>
<td>C11</td>
<td>GTTGC</td>
<td>48.6</td>
</tr>
<tr>
<td>C12</td>
<td>ATTGC</td>
<td>52.5</td>
</tr>
<tr>
<td>C13</td>
<td>TTTGC</td>
<td>57.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Experimental melting temperature (Tm) values reported are ± 0.3 °C.  
<sup>b</sup> Obtained over the range of 15-215 mM Na⁺. Data are obtained melting experiments at [DNA] = 5.0 x 10⁻⁵ M, 10 mM phosphate, pH = 7.0 buffer.
In all cases, the $T_m$ rises with the increase concentration of Na$^+$ with ranging from 15 mM to 215 mM.

3.1.2 Experimental Data Analysis (Van't Hoff Analysis)

Determination of thermal parameters for the oligomer via analyses of the $T_m$ versus $\ln C_T$ plots are more reliable than curve analysis (Marky and Breslauer, 1987). The helix stabilities of the carcinogen target DNA molecules were determined by extracting thermodynamic parameters from melting curves via optical methods described above. A two-state method is assumed and the melting curve involves a transition from a duplex to a single strand DNA in an equilibrium thermodynamic condition. Thus the highest point on the first-derivative curve ($T_m$) was used for construction of the van't Hoff plots. Figure 11 (A to L) shows plots of $1/T_m$ versus $\ln C_T$ for carcinogen target DNA duplexes in 115 mM Na$^+$.

The solid lines represent the results of the linear regression of the plotted data. The least-squares linear regression analysis of these data resulted in the $r^2 > 0.99$. The van't Hoff transition enthalpies, entropies and Gibb's free energies can be determined via equation (26) and (27):

$$1/T_m = (R/\Delta H_{vH}^0) \ln C_T/4 + \Delta S_{vH}^0/\Delta H_{vH}^0$$ (26)

$$\Delta G_{vH}^0 = \Delta H_{vH}^0 - T \Delta S_{vH}^0$$ (27)

Slight deviations from two-state behavior should have minimal effects on the enthalpies and entropies determined via $1/T_m$ versus $\ln C_T$ plots (Gaffney and Jones, 1989). The
Figure 11  The van't Hoff plots ($1/T_m$ versus $\ln C_T/4$) for the carcinogen target DNA oligomer in 10 mM phosphate buffer (pH=7.0) at 115 mM Na$^+$ concentration.

A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 11  The van't Hoff plots ($1/T_m$ versus $\ln C_T/4$) for the carcinogen target DNA oligomer in 10 mM phosphate buffer (pH=7.0) at 115 mM Na$^+$ concentration.
A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 11 The van’t Hoff plots ($1/T_m$ versus $\ln C_{7/4}$) for the carcinogen target DNA oligomer in 10 mM phosphate buffer (pH=7.0) at 115 mM Na$^+$ concentration.

A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 11  The van't Hoff plots ($1/T_m$ versus $\ln C_{\gamma/4}$) for the carcinogen target DNA oligomer in 10 mM phosphate buffer (pH=7.0) at 115 mM Na$^+$ concentration.
A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
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A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 11  The van't Hoff plots ($1/T_m$ versus $\ln C_{7/4}$) for the carcinogen target DNA oligomer in 10 mM phosphate buffer (pH=7.0) at 115 mM Na\(^+\) concentration. A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
master equilibrium thermodynamic equations provide a means to evaluate the van't Hoff enthalpies and the entropies of the transitions.

Table 3 lists the thermodynamic parameters calculated from the slopes and $y$-intercepts of these plots. The errors in determining the enthalpy and entropy values of the carcinogen binding sequences' thermal denaturations from the van't Hoff plots of are typically $\pm 5\%$.

The experimental data indicate that the sequence context influences the $\Delta G_{\text{H}}$ values through compensations both $\Delta H_{\text{H}}$ and $\Delta S_{\text{H}}$. Analysis of the data presented in Figure 11 resulted in thermodynamic parameters (Table 3) which indicate that the duplexes possessing the -CCTGC- and -CGTGC- segments are the most stable at 115 mM Na$^+$. The relative stability order of the central sequences for these carcinogen target DNA duplexes are CCTGC > CGTGC > CTTCC > CTGGC > CATGC > CTTGC > GTTGC > CTTAC > CTTTC > CTGGC > ATGGC > CTAGC.

The thermodynamic profiles shown in Table 3 show that alteration of a single dinucleotide step within a 16 bp sequence can significantly alter the energetics of duplex melting. These changes in free energy of helix denaturation with sequence context arise from alteration of both the enthalpic and entropic contributions to the total free energy.

### 3.1.3 Theoretical Calculation

Through application of Doktycz's theoretical model, the predicted thermodynamic free energies of the carcinogen target duplexes were calculated by taking into account both stacking and hydrogen bonding free energies (Docktycz et al., 1992). In these calculations, only the base pairs involved in the permuted region were considered. Here, the contributions of
Table 3  Thermodynamic Parameters for the Carcinogen Binding Sequences from Experimental Thermal Denaturations

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$\Delta S^\circ$ (eu)$^a$</th>
<th>$\Delta G^\circ$ (kcal/mol)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1: CTTGC</td>
<td>116.9</td>
<td>328.6</td>
<td>15.0</td>
</tr>
<tr>
<td>C2: CGTGC</td>
<td>130.7</td>
<td>368.4</td>
<td>16.5</td>
</tr>
<tr>
<td>C3: CATGC</td>
<td>131.6</td>
<td>374.5</td>
<td>15.5</td>
</tr>
<tr>
<td>C4: CCTGC</td>
<td>127.4</td>
<td>357.3</td>
<td>16.6</td>
</tr>
<tr>
<td>C5: CTGGC</td>
<td>112.9</td>
<td>314.0</td>
<td>15.6</td>
</tr>
<tr>
<td>C6: CTAGC</td>
<td>80.8</td>
<td>222.2</td>
<td>11.9</td>
</tr>
<tr>
<td>C7: CTCGC</td>
<td>95.1</td>
<td>262.4</td>
<td>13.7</td>
</tr>
<tr>
<td>C8: CTCCC</td>
<td>116.9</td>
<td>332.3</td>
<td>13.9</td>
</tr>
<tr>
<td>C9: CTCCC</td>
<td>125.0</td>
<td>352.5</td>
<td>15.7</td>
</tr>
<tr>
<td>C10: CTTAC</td>
<td>119.7</td>
<td>339.7</td>
<td>14.4</td>
</tr>
<tr>
<td>C11: GTTGC</td>
<td>103.0</td>
<td>285.5</td>
<td>14.5</td>
</tr>
</tbody>
</table>

$^a$ 1 kcal = 0.239 kJ, $^b$ $\Delta G^\circ$ values are calculated at 37°C. The data are obtained at 115 mM Na+, 10 mM phosphate, pH = 7.0 buffer.
hydrogen bonding ($\Delta G^0_{\text{hb}}$) and nearest neighbor or stacking ($\Delta G^0_{\text{stack}}$) were determined via the protocols described elsewhere. The theoretical values thus obtained for the hydrogen bonding and stacking contributions and the total free energy in 115 mM Na$^+$ are shown in Table 4. The calculated data indicated that the stacking free energy varies according to this order: $\text{-GTTGC-} > \text{-TTTGC-} > \text{-ATTGC-} > \text{-CATGC-} > \text{-CGTGC-} > \text{-CTTGC-} > \text{-CTCGC-} > \text{-CTTTCC-} > \text{-CCTGC-} > \text{-CTGGC-} > \text{-CTTAC-} > \text{-CTAGC-}$. The hydrogen bonding free energy are more than 10 times of the base stacking free energy in the magnitude. Calculation of the hydrogen-bonding free energies indicates the following order $C_2, C_4, C_5$ and $C_7 > C_1, C_3, C_6, C_9$ and $C_11 > C_8, C_10$ and $C_12$ for total hydrogen bond stability. Thus, even though $\text{-CATGC-}$ is more stably stacked than $\text{-CTTGC-}$, hydrogen bonding is the predominant factor in the favored overall stability of $\text{-CATGC-}$. Furthermore, since the hydrogen bonding is similar for these three set of duplexes: $\text{-CTTGC-} = \text{-CATGC-} = \text{-CTAGC-} = \text{-CTTCC-} = \text{-GTTGC-}$, $\text{-CGTGC-} = \text{-CCTGC-} = \text{-CTGGC-}$ and $\text{-CTTTCC-} = \text{-CTTAC-} = \text{-ATTGC-}$; the difference in the experimentally determined $\Delta G^0$ between those oligomers are entirely due to differences in stacking. The total calculated $\Delta G^0$ total for each oligomer can be calculated by using the $\Delta G^0_{\text{stack}}$ and $\Delta G^0_{\text{hb}}$ values from Table 4.

### 3.1.4 Sequence Effects

The most appropriate way to compare oligomers is to calculate the difference in free energies ($\Delta \Delta G^0$) within a series, thereby negating end effects (Doktycz et al., 1992). The thermodynamic studies were carried out to assess the influence of the base permutations on the stabilities of the duplexes. In order to compare the difference of the
Table 4  Theoretically Determined Thermodynamic Parameters for Carcinogen Target Oligomers

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>$\Delta G^o_{\text{stack}}$ (cal/mol)</th>
<th>$\Delta G^o_{\text{hb}}$ (cal/mol)</th>
<th>$\Delta G^o_{\text{cal}}$ (cal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1: CTTGC</td>
<td>543.0</td>
<td>6685.9</td>
<td>7228.9</td>
</tr>
<tr>
<td>C2: CGTGC</td>
<td>659.4</td>
<td>7744.5</td>
<td>8403.9</td>
</tr>
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<td>C3: CATGC</td>
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<td>6685.9</td>
<td>7354.3</td>
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<td>C4: CCTGC</td>
<td>196.8</td>
<td>7744.5</td>
<td>7941.3</td>
</tr>
<tr>
<td>C5: CTGGC</td>
<td>196.8</td>
<td>7744.5</td>
<td>7941.3</td>
</tr>
<tr>
<td>C6: CTAGC</td>
<td>84.9</td>
<td>6685.9</td>
<td>6770.8</td>
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<tr>
<td>C7: CTCGC</td>
<td>469.0</td>
<td>7744.5</td>
<td>8213.5</td>
</tr>
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<td>C8: CTTTC</td>
<td>416.6</td>
<td>5627.3</td>
<td>6043.9</td>
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<td>C9: CTGCC</td>
<td>70.4</td>
<td>6685.9</td>
<td>6756.3</td>
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<tr>
<td>C10: CTTAC</td>
<td>148.9</td>
<td>5627.3</td>
<td>5776.2</td>
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<tr>
<td>C11: GTTGC</td>
<td>1027.0</td>
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<td>7712.9</td>
</tr>
<tr>
<td>C12: ATTAGC</td>
<td>884.6</td>
<td>5627.3</td>
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<td>C13: TTTGC</td>
<td>987.8</td>
<td>5627.3</td>
<td>6615.1</td>
</tr>
</tbody>
</table>

$\Delta G^o_{\text{cal}}$ is calculated binding free energy by using the following equation,

$$\Delta G^o_{\text{cal}}(\text{cal}) = \Delta G^o_{\text{stack}}(\text{cal}) + \Delta G^o_{\text{hb}}(\text{cal})$$

$\Delta G^o_{\text{hb}}(\text{cal})$ is hydrogen-bonding free energy,

$\Delta G^o_{\text{stack}}(\text{cal})$ is base stacking free energy.
The AΔG° values are calculated by subtracting the ΔG° for a particular oligomer from that of the parent oligomer, -CGTGC-. A positive AΔG° indicates that that oligomer is more stable than the parent oligomer. The data indicate that there is excellent agreement for -CATGC- between the experimental and theoretical determinations of AΔG° at 115 mM Na+. There are also a good agreement for -CTTGC-, -CCTGC-, -CTGGC-, -CTTTC-, -CTTCC- and -CTTAC- since the differences between experimental and theoretical calculations of AΔG° are less than 1.0 kcal/mol. The sequences -CTAGC-, -CTCGC- and -ATTGC- are experimentally more stable than the theoretical prediction. The free energy difference between experiment and theoretical calculations range from 2.6-3.0 kcal/mol. These data (Table 5) provide an independent quantitative test of the algorithm presented by Doktycz et al. (1992) for the prediction of DNA helix stability.
## Table 5  Comparison of the Experimental and Theoretical Calculated Carcinogen Binding Sequences' Free Energies

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>( \Delta \Delta G^o_{\text{exp}} ) (kcal/mol)(^c)</th>
<th>( \Delta \Delta G^o_{\text{cal}} ) (kcal/mol)(^c)</th>
<th>( \delta \Delta \Delta G^o ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1: CTTGC</td>
<td>+1.5</td>
<td>+1.2</td>
<td>+0.3</td>
</tr>
<tr>
<td>C2: CGTGC</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>C3: CATGC</td>
<td>+1.0</td>
<td>+1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>C4: CCTGC</td>
<td>-0.1</td>
<td>+0.5</td>
<td>-0.6</td>
</tr>
<tr>
<td>C5: CTGGC</td>
<td>+0.9</td>
<td>+0.5</td>
<td>+0.4</td>
</tr>
<tr>
<td>C6: CTAGC</td>
<td>+4.6</td>
<td>+1.6</td>
<td>+3.0</td>
</tr>
<tr>
<td>C7: CTCGC</td>
<td>+2.8</td>
<td>+0.2</td>
<td>+2.6</td>
</tr>
<tr>
<td>C8: CTTTC</td>
<td>+2.6</td>
<td>+2.4</td>
<td>+0.2</td>
</tr>
<tr>
<td>C9: CTTCC</td>
<td>+0.8</td>
<td>+1.6</td>
<td>-0.8</td>
</tr>
<tr>
<td>C10: CTTAC</td>
<td>+2.1</td>
<td>+2.6</td>
<td>-0.5</td>
</tr>
<tr>
<td>C11: GTTGC</td>
<td>+2.0</td>
<td>+0.7</td>
<td>+1.3</td>
</tr>
<tr>
<td>C12: ATGTC</td>
<td>+4.5</td>
<td>+1.9</td>
<td>+2.6</td>
</tr>
</tbody>
</table>

\(^c\)The \( \Delta \Delta G^o_{\text{exp}} \) (kcal) and \( \Delta \Delta G^o_{\text{cal}} \) (kcal) were determined by subtracting a particular sequence's free energy from that of C2. The experimental values (\( \Delta \Delta G^o_{\text{exp}} \)) are obtained from Table 2 at 115 mM Na\(^+\), 10 mM Phosphate, pH = 7.0 buffer. The theoretical calculated free energy difference (\( \Delta \Delta G^o_{\text{cal}} \)) are obtained from Table 3, taking account of both stacking and hydrogen bonding free energy contributions. \( \delta \Delta \Delta G^o = \Delta \Delta G^o_{\text{exp}} - \Delta \Delta G^o_{\text{cal}} \). A positive sign for the \( \Delta \Delta G^o_{\text{exp}} \), \( \Delta \Delta G^o_{\text{cal}} \) and \( \delta \Delta \Delta G^o \) values indicates that the sequence is more stable than C2.
3.1.5 Salt Effects

One of the goals of this study was to determine the effects of both oligomer and Na\(^+\) concentrations on the conformational states of these oligomers. Thermal denaturation studies of all oligomers at different Na\(^+\) concentrations were carried out in order to investigate the effects of Na\(^+\) on duplex stability. T\(_m\) values were obtained from the inflection points of the first derivative plots of the melting curves. These first derivative plots also indicated that all melting transitions were monophasic.

Figure 12 (A – J) shown the plot of T\(_m\) vs [Na\(^+\)] for all oligomers (at constant DNA concentration). The nonlinear dependence of T\(_m\) on Na\(^+\) has been observed. Similar behavior has been described for native DNA samples (Gruenwedel et al., 1971). T\(_m\) increases quickly at very low salt concentrations (15 to 100 mM) and slowly at higher low salt concentration (100 to 215 mM) of Na\(^+\). The combined effects of dehydration and anion binding at higher salt concentrations for the nonlinear dependence of T\(_m\) on Na\(^+\) has been proposed. (Gruenwedel et al., 1971; Record et al., 1990). For the range of 15-215 mM Na\(^+\), the increase in T\(_m\) may be interpreted in terms of the polyelectrolyte theories of Manning (1978) and Record and co-workers (1978, 1981).

Quantitative evaluation of the effects of Na\(^+\) on the denaturation transitions of DNA duplexes were carried out in the context of counterion condensation polyelectrolyte theory (Manning, 1978; Record et al., 1978, 1981). Using corresponding T\(_m\) values and salt concentrations from melting experiments, the differential ion binding term \(\Delta n\) was determined for each oligomer:
Figure 12  The variation in $T_m$ as a function of $[\text{Na}^+]$ for the carcinogen target DNA oligomer, with $\text{Na}^+$ concentration ranging from 15 mM to 215 mM and $[\text{DNA}] = 5.0 \times 10^{-5} \text{ M}$ in base pairs in 10 mM phosphate buffer (pH=7.0)

A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 12  The variation in $T_m$ as a function of [Na⁺] for the carcinogen target DNA oligomer, with Na⁺ concentration ranging from 15 mM to 215 mM and [DNA] = 5.0 x 10⁻⁵ M in base pairs in 10 mM phosphate buffer (pH=7.0) 
A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 12  The variation in $T_m$ as a function of [Na$^+$] for the carcinogen target DNA oligomer, with Na$^+$ concentration ranging from 15 mM to 215 mM and [DNA] = 5.0 x 10^{-5} M in base pairs in 10 mM phosphate buffer (pH=7.0) A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 12 The variation in $T_m$ as a function of $[\text{Na}^+]$ for the carcinogen target DNA oligomer, with Na$^+$ concentration ranging from 15 mM to 215 mM and $[\text{DNA}] = 5.0 \times 10^{-5}$ M in base pairs in 10 mM phosphate buffer (pH=7.0) A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 12 The variation in $T_m$ as a function of $[\text{Na}^+]$ for the carcinogen target DNA oligomer, with $\text{Na}^+$ concentration ranging from 15 mM to 215 mM and $[\text{DNA}] = 5.0 \times 10^{-5}$ M in base pairs in 10 mM phosphate buffer (pH=7.0) A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6. G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10. K) oligomer C11, L) oligomer C12.
Figure 12 The variation in $T_m$ as a function of $[\text{Na}^+]$ for the carcinogen target DNA oligomer, with Na$^+$ concentration ranging from 15 mM to 215 mM and [DNA] = 5.0 x $10^{-5}$ M in base pairs in 10 mM phosphate buffer (pH=7.0) A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
\[ T_m / \log [Na] = \{2.303RT^2_m / \Delta H_{m}^0 \} \Delta n \] (28)

where \( T_m \) is the temperature at the mid-point of the melting transition, \([Na^+]\) is the molar concentration of monovalent counterion, \( \Delta H_{m}^0 \) is the enthalpy of the melting transition, \( R \) is the gas constant, and \( \Delta n \) is the differential ion binding which represents the release of counterions upon denaturation.

Plots of \( T_m \) vs log \([Na^+]\), over the range of Na\(^+\) concentrations from 15 mM to 215 mM, for all oligomers are shown in Figure 13 (A to L). All DNA samples were prepared in 15 mM sodium phosphate buffer, pH 7.0, with NaCl added to give final total \([Na^+]\) ranging from 15 to 215 mM ([DNA] = 5.0 x 10\(^{-3}\) M in base pairs). The salt dependence of melting temperature, shown through linear \( T_m \) versus log \([Na^+]\) plots, can be used to calculate (from the slope \( \delta T_m / \delta \log [Na^+] \), equation 3) the differential ion binding term. The solid line represent the results of the linear regression of the plotted data. Least squares linear regression analyses of the data resulted in high correlation factors \( (r^2 > 0.99) \) over the range 15-215 mM Na\(^+\) for all oligomers. The slopes obtained for the salt dependent thermal data are shown in Table 6. The slopes calculated reflect the linkage between Na\(^+\) binding and the duplex to single strand transition. The slopes of the lines for the salt dependent thermal data are variable, suggesting that Na\(^+\) to both the duplex and single stranded DNA is sequence context dependent.

As shown in Table 6, the slopes of the regression lines over the range 55-215 mM Na\(^+\) for all the oligomers range from 13.6 to 17.3 except -ATTGC- (7.5) and -TTTGC- (9.7). The sequences -CTTGC- and -CCTGC- are the most sensitive to the Na\(^+\) with the largest slope of 17.3. The value of \( (\delta T_m / \delta \log [Na^+]) \) observed here is in agreement with
Figure 13  The variation in $T_m$ as a function of log [Na$^+$] for the carcinogen target DNA oligomer, with Na$^+$ concentration ranging from 15 mM to 215 mM and [DNA] = 5.0 x 10$^{-5}$ M in base pairs in 10 mM phosphate buffer (pH=7.0)

A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 13 The variation in $T_m$ as a function of log $[\text{Na}^+]$ for the carcinogen target DNA oligomer, with $\text{Na}^+$ concentration ranging from 15 mM to 215 mM and $[\text{DNA}] = 5.0 \times 10^{-5}$ M in base pairs in 10 mM phosphate buffer (pH=7.0)

A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 13  The variation in $T_m$ as a function of log $[\text{Na}^+]$ for the carcinogen target DNA oligomer, with $\text{Na}^+$ concentration ranging from 15 mM to 215 mM and $[\text{DNA}] = 5.0 \times 10^{-5}$ M in base pairs in 10 mM phosphate buffer (pH=7.0)
A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 13  The variation in $T_m$ as a function of log [Na$^+$] for the carcinogen target DNA oligomer, with Na$^+$ concentration ranging from 15 mM to 215 mM and [DNA] = 5.0 x 10$^{-5}$ M in base pairs in 10 mM phosphate buffer (pH=7.0)

A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 13  The variation in $T_m$ as a function of log [Na$^+$] for the carcinogen target DNA oligomer, with Na$^+$ concentration ranging from 15 mM to 215 mM and [DNA] = 5.0 x 10^{-5} M in base pairs in 10 mM phosphate buffer (pH=7.0)  
A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
Figure 13  The variation in $T_m$ as a function of log $[\text{Na}^+]$ for the carcinogen target DNA oligomer, with Na\textsuperscript{+} concentration ranging from 15 mM to 215 mM and [DNA] = 5.0 x 10\textsuperscript{-5} M in base pairs in 10 mM phosphate buffer (pH=7.0)
A) oligomer C1, B) oligomer C2, C) oligomer C3, D) oligomer C4, E) oligomer C5, F) oligomer C6, G) oligomer C7, H) oligomer C8, I) oligomer C9, J) oligomer C10, K) oligomer C11, L) oligomer C12.
## Table 6  The Na\textsuperscript{+} ion Effect on Carcinogen Binding

**Sequences' Thermal Denaturation**

<table>
<thead>
<tr>
<th></th>
<th>$\delta T_m/\delta \log[\text{Na}^+]$</th>
<th>$\Delta H_m^{115\text{mM}}$</th>
<th>$T_m^{115\text{mM}}$</th>
<th>$\Delta n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1:</td>
<td>17.3</td>
<td>116.9</td>
<td>62.4</td>
<td>3.9</td>
</tr>
<tr>
<td>C2:</td>
<td>16.7</td>
<td>130.7</td>
<td>66.3</td>
<td>4.1</td>
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<td>131.6</td>
<td>60.7</td>
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<td>127.4</td>
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</tr>
<tr>
<td>C5:</td>
<td>14.4</td>
<td>112.9</td>
<td>65.4</td>
<td>3.1</td>
</tr>
<tr>
<td>C6:</td>
<td>15.9</td>
<td>80.8</td>
<td>60.6</td>
<td>2.5</td>
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<td>C7:</td>
<td>15.65</td>
<td>95.1</td>
<td>64.1</td>
<td>2.9</td>
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<tr>
<td>C8:</td>
<td>16.8</td>
<td>116.9</td>
<td>59.3</td>
<td>3.9</td>
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<tr>
<td>C9:</td>
<td>16.6</td>
<td>125.0</td>
<td>61.3</td>
<td>4.1</td>
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<tr>
<td>C10:</td>
<td>16.2</td>
<td>119.7</td>
<td>59</td>
<td>3.8</td>
</tr>
</tbody>
</table>

The enthalpy values are obtained from experimental data listed in Table 2. The differential Na\textsuperscript{+} ion binding term ($\Delta n$) are calculated according to the following equation:

$$\delta T_m/\delta \log [\text{NaCl}] = \{2.303RT_m^{2}/\Delta H_m\} \Delta n$$ (Record et al 1978).
that reported for polymeric native DNA (Gruenwedel et al., 1971). This similarity in polyelectrolyte behavior of oligonucleotides and polymers with the expected deviation, is consistent with the behavior reported by Braunlin and Bloomfield (1991) for the melting of an octanucleotide.

As can also be seen from the data in Table 6, values of \( \Delta n \) range from 2.5 to 4.2. The highest value of \( \Delta n \) (4.2 for -CGTGC-) indicates that 4.2 Na\(^+\) ions are released per 16 base pairs of duplex upon melting. That means that 0.13 Na\(^+\) ions are released per phosphate, a value near that observed for the melting of polymeric DNA (Record et al., 1978). Olmsted (Olmsted et al., 1991; 1989) predicted that deviations from polyelectrolyte theory developed for polymers when applying to oligonucleotides arise from non negligible end effects.

3.1.6 SUMMARY

The designed series of synthetic DNA duplexes which possess potential high affinity carcinogen binding sites and the parent carcinogen target sequence C1 has been examined. The thermal stability of these duplexes was considered in light of (1) sequence context effect on thermal stability, (2) theoretical predictions and (3) duplex stability dependence on Na concentration.

Base-base interactions through hydrogen bonding and base stacking have been investigated by thermodynamic methods in order to derive enthalpies, entropies and free energies changes for the series of DNA duplexes. The hydrogen-bonding interactions are the primary contribution to the observed thermodynamic stabilities for these carcinogen binding sequences while the base stacking also plays a significant role.
The salt dependence of melting temperature, shown through linear Tm versus log [Na+] plots, can be used to calculate (from the slope) the differential ion binding term, Δn, which represents the release of centurions per duplex upon denaturation. This is indirect evidence that the different sequence context of the DNA duplexes results in Na⁺ bound differently to the various duplexes. The results indicate that -CTAGC- and CTCGC- exhibits a much weaker binding affinity toward Na⁺. The effects observed by changing the base pair adjacent to the high affinity binding site may result in differential stacking, differences in the minor groove environment and/or subtle conformational alterations at the interaction site.

An additional result from these studies is a thermodynamic description of the profound effect of slight sequence changes on DNA helix stability. The thermodynamic parameters listed in Table 3 indicate that alteration of a single base pair within a 16 bp carcinogen target duplex can alter the energetic of duplex melting. The dependence of the free energy of helix denaturation on sequence context arises from alteration of both the enthalpic and entropic contributions. These data provide an independent quantitative test of the algorithm presented by Doktycz et al. (1992) for the prediction of DNA helix stability. The agreement between the predicted ΔΔG⁰ values and the experimentally determined ΔΔG⁰ values is, in general, excellent (Table 5).
3.2 BINDING ANALYSIS

A number of studies have investigated the interaction specificities of porphyrin based molecules with DNA (Carvlin, 1983; Pasternack, 1983; Banville, 1986; Mukundan, 1994; Lipscomb, 1996; Pethos, 1993; Pasternak, 1997; Sheardy, 1998). Most of these studies used polymeric DNA to demonstrate that porphyrins bound to DNA by either intercalation, outside stacking or groove binding, depending upon DNA base content, ionic strength and pH of the media and whether the porphyrin is metallated or not. (Mukundan, 1994; Pasternak, 1997). Intercalation is the preferred mode of binding of H<sub>2</sub>TMPyP to DNA with GC sites while outside binding without stacking is favored at AT sites at moderate concentrations of porphyrin and ionic strength (Pastemack, 1983; Carvlin, 1983; Pasternak, 1997). It has been shown recently that H<sub>2</sub>TMPyP bound to a quadruplex of T<sub>4</sub>G<sub>4</sub> with a higher affinity than to a duplex [d(CGCGATATCGCG)]<sub>2</sub>. Furthermore, results of CD and fluorescent energy transfer experiments indicate that the porphyrin is stacked with the base tetrads of the quadruplex or base pairs of the duplex (Anantha, 1998). Finally, a recent X-ray structure of a porphyrin-DNA complex revealed that the porphyrin intercalated into the -CG- site of a 5'-TCG-3' trinucleotide step while extruding the C of the complementary 5'-CGA-3' trinucleotide step (Lipscomb, 1996).

3.2.1 UV/Vis Titration studies.

The interactions of porphyrins with DNA could be determined by monitoring the absorption of the porphyrin by progressive titration with carcinogen target DNA
oligomers. Figure 14 showed the typical visible absorption spectra of porphyrin in the absence and presence of carcinogen target DNA oligomer C1. The free porphyrin exhibits the maximal absorption spectrum at 422 nm, the Soret band. As duplex was added, the spectrum was observed to undergo a 17-19 nm shift from 422 to 450 nm, accompanied by a decrease in absorbance of the Soret band of the porphyrin chromophore. An isosbestic point was observed at 432 nm at high concentrations of DNA.

For these studies, the concentration of porphyrin was held constant at 5.0 µM for all titration data and the ratio of [DNA] to [porphyrin] ranges from 0.0 to 15. Titration of the porphyrin with any of the DNA oligomers resulted in a similar decrease in extinction coefficient at the Soret band (422 nm) and a shift in the absorption maximum. An isosbestic point was observed in all cases. The visible spectral data were summarized in Table 7.

The concentration of total porphyrin in the absence of DNA was calculated from

\[ C_T = \frac{A_{422}}{\varepsilon_{422}} \]  \hspace{1cm} (29)

where \( \varepsilon_{422} = 14400 \text{ M}^{-1}\text{cm}^{-1} \) \cite{Pasternack, 1972}. The amount of bound drug could be calculated from the difference in absorbance at 422 nm in absence and presence of different amounts of DNA:

\[ C_b = \frac{\Delta A_{422}}{\Delta \varepsilon_{422}} \]  \hspace{1cm} (30)

Where \( \Delta A_{422} \) was the change in absorbance of the porphyrin in the absence and presence of DNA and \( \Delta \varepsilon_{422} = \varepsilon_{422,f} - \varepsilon_{422,b} \) where the subscripts f and b referred to free
Figure 14 Representative absorption spectra for the interaction of porphyrin with C1 at 20 °C. The free porphyrin exhibits the maximal absorption spectrum. As oligonucleotide (duplex) was added, the spectrum was observed to undergo a 18-nm shift from 422 to 440 nm, accompanied by a decrease in absorbance of the porphyrin chromophore. An isosbestic point was observed at 434 nm. Spectra were obtained by titrating small volumes of concentrated oligonucleotide duplex into dilute porphyrin solution over the range [DNA]/[porphyrin] from 0.3 to 15 at 20 °C. The concentration of porphyrin (5.13 µM) was kept constant during the titration process. Both the DNA and porphyrin were dissolved in 115 mM NaCl, 10 mM phosphate buffer, pH 7.0.
### Table 7. Effect of DNA on the Soret Band of H₂TMPyP

<table>
<thead>
<tr>
<th>Duplex</th>
<th>Δλₘₘ, (nm) hypoehromicity</th>
<th>%</th>
<th>Induced CD&lt;sup&gt;b&lt;/sup&gt; Δε₄₄₉ (M⁻¹cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>18</td>
<td>65.5</td>
<td>-8.42</td>
</tr>
<tr>
<td>C4</td>
<td>19</td>
<td>72.5</td>
<td>-6.06</td>
</tr>
<tr>
<td>C4</td>
<td>17</td>
<td>63.8</td>
<td>-8.59</td>
</tr>
<tr>
<td>C10</td>
<td>18</td>
<td>70.3</td>
<td>-8.59</td>
</tr>
</tbody>
</table>

<sup>a</sup> Δλₘₘ is calculated as λₘₘ - λₘₙ where λₘₙ is the wavelength of maximum absorbance of the Soret band for the free porphyrin and λₘₘ is the wavelength of maximum absorbance of the Soret band for the porphyrin in the presence of excess DNA (i.e., under conditions where the porphyrin is fully bound). % Hypochromicity was calculated using the expression % H = [(εₙ - εₘₙ) / εₙ] X 100% where εₙ and εₘₙ are, respectively, the extinction coefficients of free and fully bound porphyrin at Δλₘₘ, the Soret band at 422 nm.

<sup>b</sup> Δε₄₄₉ is the magnitude of the induced molar ellipticity at 449 nm for [porphyrin]/[DNA duplex] = 11.3.
and fully bound (i.e., in the presence of excess DNA) porphyrin. (Pasternack, 1972). The concentration of free drug was then given by:

\[ C_f = C_T - C_b \]  

(31)

Figure 15 showed the typical binding concentration (i.e., \( C_b \)) for C1 interaction with the porphyrin. The binding concentration for C1 increased rapidly at low molar ratio of DNA to porphyrin. However, at higher ratios, the binding concentration for C1 levels off, displaying saturation. A comparison of the binding concentration of the porphyrin to the various oligomers was shown in Figure 16. Inspection of the data indicated that the binding order is C1>C5>C4>C10 at 115 mM NaCl.

### 3.2.2 CD Spectroscopy

The conformations of the porphyrin-DNA complexes were analyzed by comparison of the circular dichroism spectra of the porphyrin in the absence and presence of the carcinogen target DNA oligomers. The formation of a complex between the porphyrin and DNA was confirmed from the observation of a band emerging in the 400-500 nm region. In general, titration of the porphyrin with DNA resulted in an induced CD band at 449 nm.

CD spectra (millidegrees vs wavelength) of the porphyrin in the presence of C1, C4, C5 and C10 were given in Figure 17, 18, 19 and 20, respectively. The temperature was 25 °C. The buffer contained 115 mM sodium chloride and 10mM phosphate with pH 7.0. Titration of porphyrin with DNA resulted in an induced CD band at 447 nm. The ellipticity in this band increased with increasing [DNA].
Figure 15  Binding concentration for Cl interaction with porphyrin. All binding data were obtained from titration experiment in 115 mM Na\(^+\) and 10 mM phosphate buffer, pH = 7.0 at 25 °C.
Figure 16: Comparison of Binding Concentration for Oligomers Interaction with Porphyrin. All binding data were obtained from titration experiment in 115 mM Na$^+$ and 10 mM phosphate buffer, pH = 7.0 at 25 °C. C1 (▲), C5 (■), C4 (●), C10 (▼)
Figure 17  The CD spectra of porphyrin at 25 °C in the absence and presence of Cl. The data were obtained in 115 mM NaCl, 10 mM phosphate, pH = 7.0 buffer with DNA titration in to 5.13 µM porphyrin solution. The double dot line represents without DNA and the ratio [DNA]/[porphyrin] = 4.62, 6.57, and 11.29.
The CD spectra of porphyrin at 25 °C in the absence and presence of C4. The data were obtained in 115 mM NaCl, 10 mM phosphate, pH = 7.0 buffer with DNA titration into 5.13 μM porphyrin solution. The black line represents without DNA and the ratio [DNA]/[porphyrin] = 4.62, 6.57 and 11.29.
The CD spectra of porphyrin at 25 °C in the absence and presence of C5. The data were obtained in 115 mM NaCl, 10 mM phosphate, pH = 7.0 buffer with DNA titration into 5.13 μM porphyrin solution. The ratio [DNA]/[porphyrin] = 4.62, 6.57, 10.08 and 11.29.
Figure 20  The CD spectra of porphyrin at 25 °C in the absence and presence of C10. The data were obtained in 115 mM NaCl, 10 mM phosphate, pH = 7.0 buffer with DNA titration into 5.3 μM porphyrin solution. The black line represents without DNA and the ratio [DNA]/[porphyrin] = 4.62 and 11.29.
Figure 21 showed the comparison of the induced CD spectra of H₂TMyP in the presence of C1, C4, C5 and C10 with [DNA]/[porphyrin] = 11.29. All oligomers induced similar CD spectra of the porphyrin at 449 nm. The magnitude of the ellipticity at 449 nm was about the same for C1, C5 and C10 and somewhat less for C4 as indicated in Table 7. The induced CD for the porphyrin in the presence of C10 also had a positive peak at 424 nm.

Both visible and CD spectra could be utilized to assess the mode of binding of a porphyrin to a DNA duplex. As seen in Table 7, binding of H₂TMPyP to any of the duplexes resulted in a large bathochromic shift (17 to 19 nm) and a substantial hypochromism (64 to 74 %) in the Soret band. This suggested intercalation of the porphyrin between the base pairs of the duplexes (Pasternack, 1983; Carvlin, 1983; Lipscomb, 1996; Anantha, 1998). Furthermore, the induced negative CD at 449 nm was consistent with intercalation as well (Lipscomb et al, 1996). The presence of the positive induced band in the CD spectrum of C10 at 429 nm might be due to differential stacking and porphyrin groove interactions, possibly the consequence of changes on the minor groove environment and/or subtle sequence-dependent conformational alterations at the intercalation site.

3.2.3 Scatchard plots

In this study, we investigated the interaction specificities of H₂TMPyP with a DNA sequence previously shown to possess a high affinity binding site for the carcinogen NQO and three related DNA oligomers. The rationale was to determine
Figure 21  The CD spectra at 25 °C of porphyrin with carcinogen binding sequence. All data were obtained in 115 mM NaCl, 10 mM phosphate, pH = 7.0 buffer with DNA titration in to 5.13 μM porphyrin solution and the ratio [DNA]/[porphyrin = 11.29, C1 (-), C4 (--), C5 (-.-) and C10 (- -.-).
whether a similar specificity would be observed with a reversible binder such as a porphyrin. In order to avoid porphyrin aggregation in all experiments, the free porphyrin concentration was kept under 15 μM. As shown above, titration of the porphyrin with any of the DNA oligomers results in a decrease in extinction coefficient at the Soret band (422 nm) and a shift in the absorption maximum. An isosbestic point indicated there were only two states in the equilibrium binding process of the porphyrin to DNA. These changes could be utilized as a means to monitor the equilibrium binding of the porphyrin to DNA.

Binding data from absorbance titration experiments were cast into the form of a Scatchard plot of \( r/C_f \) vs \( r \), where \( r \) was the number of moles of porphyrin bound per mole of DNA base pair. Figure 22 showed the typical Scatchard plot for the interaction of the porphyrin with C1. The shapes of resultant Scatchard plot indicated two types of binding; one at low \( r \) values and one high higher \( r \) values. Since a simple 16 bp DNA was used in this titration experiment, both the low \( r \) value data and the high \( r \) value data were fit via a simple, non-cooperative mode, as described as follow equation:

\[
r/C_f = K(1-n \ r) \tag{32}
\]

where \( r = C_p/[DNA] \), was the ratio of number of moles of the bound porphyrin to the moles of base pairs of DNA, \( n \) was the exclusion parameter (number of binding sites/base pair) and \( K \) is the binding constant. \( K \) and \( n \) were determined from using
Figure 22  Scatchard plot for equilibrium titrations of porphyrin with C1. The same \( \varepsilon_b \) was used for all Scatchard plots. These data were obtained from C1 at 25 °C titration with porphyrin over the range of [DNA]/[porphyrin] = 0.3 - 15, 115 mM [Na+] buffer. Two binding modes are observed for porphyrin binding with these short DNA sequences.
linear least-squares fitting of the Scatchard plot; the y-intercept was \( K \) and slope/y-intercept was \( n \). The high degree of linearity of fitting indicated good agreement with the identical-and-independent sites model (Figure 21).

Figure 23 showed a comparison of the Scatchard plots for all four DNA oligomers at 25 °C. Close examination of these plots revealed the beginning of positive slope in the data at higher \( r \) values, which suggested neighbor exclusion (McGhee, 1974). At higher \( r \) values, even more complex behavior was observed (Figure 23) which indicated mixed modes of binding. Hence for the results reported here, only data with \( r \) values less than 0.1 were used.

### 3.2.4 Studies of temperature dependence of binding constant

Titrations of the porphyrin with the various DNA duplexes were also carried out at different temperatures to estimate the contributions of entropy and enthalpy to the total free energy of the porphyrin-DNA interaction. Figure 24 showed the titration of porphyrin with carcinogen target DNA sequence C1 (\( C_b \) vs [oligonucleotide]/[porphyrin]) at 20 °C in the same DNA concentration. A comparison with data obtained at 15 and 40 °C was shown in Figure 25. As shown in Figure 25, the binding concentrations of porphyrin decreased with increasing temperature.

Figure 26 (A, B, C and D) showed the binding concentration for C4 interaction with porphyrin at 15, 20, 30 and 40 °C, respectively, while Figures 27 and 28 were shown for C5 and C10 interacting with porphyrin at these temperature, respectively. In all cases, even at the highest studied temperature (40 °C), over 90% of the DNA
Figure 23  Comparison of the satchard plot for the porphyrin titration with C1, C4, C5 and C10. All data were obtained from titration experiment in 115 mM Na$^+$ and 10 mM sodium phosphate buffer, pH=7.0 at 25 °C. C1 (●), C4 (■), C5 (▼) and C10 (▲).
Figure 24  Binding concentration for the carcinogen target DNA oligomer C1 interaction with porphyrin. All binding data were obtained from titration experiment in 115 mM Na\(^+\) and 10 mM phosphate buffer, pH = 7.0 at 20°C.
Figure 25  Comparison of binding concentration for C1 interaction with porphyrin. All binding data were obtained from titration experiment in 115 mM Na\(^+\) and 10 mM sodium phosphate buffer, pH = 7.0 at 15 (■), 20 (●) and 40 °C (▲).
Figure 26 Binding concentration for the carcinogen target DNA oligomer C4 interaction with porphyrin. All binding data were obtained from titration experiment in 115 mM Na⁺ and 10 mM phosphate buffer, pH = 7.0. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 26 Binding concentration for the carcinogen target DNA oligomer C4 interaction with porphyrin. All binding data were obtained from titration experiment in 115 mM Na⁺ and 10 mM phosphate buffer, pH = 7.0. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 27  Binding concentration for the carcinogen target DNA oligomer C5 interaction with porphyrin. All binding data were obtained from titration experiment in 115 mM Na⁺ and 10 mM phosphate buffer, pH = 7.0. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 27  Binding concentration for the carcinogen target DNA oligomer C5 interaction with porphyrin. All binding data were obtained from titration experiment in 115 mM Na\(^+\) and 10 mM phosphate buffer, pH = 7.0. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 28  Binding concentration for the carcinogen target DNA oligomer C10 interaction with porphyrin. All binding data were obtained from titration experiment in 115 mM Na\(^+\) and 10 mM phosphate buffer, pH = 7.0. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 28  Binding concentration for the carcinogen target DNA oligomer C10 interaction with porphyrin. All binding data were obtained from titration experiment in 115 mM Na\(^+\) and 10 mM phosphate buffer, pH = 7.0. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
were still in the duplex form. The binding drop-off at higher temperatures might be due to thermally induced fluctuations in the double helix.

The data from Figures 24-28 were cast in the form of Scatchard plots. The low r values were fit using the simple relationship expressed in equation 26. The plots for C1, C4, C5 and C10 at 15, 20, 30 and 40 °C were shown in Figure 29 (A, B, C and D), Figure 30 (A, B, C and D), Figure 31 (A, B, C and D) and Figure 32 (A, B, C and D), respectively. Analysis of the least squares fit resulted in values of K and n. They were reported in Table 8. The estimated error range of K is ±10%. The binding constants obtained at 15 °C indicate that H2TMPyP binding to C1 was favored over binding to the other oligomers.

Using a van't Hoff approach, plots of ln K vs 1/T were constructed and analyzed to determine the thermodynamic parameters for porphyrin-DNA interactions. The contribution to the observed thermodynamic parameters could be determined from the following equation (Lohman, 1992; Record, 1978; Manning, 1978; Wilson, 1979; Friedman, 1984), assuming the heat capacity effects were zero. The enthalpy was evaluated from the equation:

\[ \frac{\delta \ln K}{\delta (1/T)} = - \frac{\Delta H^o}{R} \]  

The Gibb’s free energy and the entropy were then calculated using following equations:

\[ \Delta G^o = -RT \ln K \]  
\[ \Delta S^o = -(\Delta G^o - \Delta H^o) / T \]
Table 8  Thermodynamic Parameters for the Interaction of H$_2$TMPyP with DNA Duplexes$^a$

<table>
<thead>
<tr>
<th>Duplex</th>
<th>T (°C)</th>
<th>$K_{obs}/10^7$ (M$^{-1}$)</th>
<th>n</th>
<th>$\Delta G_{obs}^o$ (kcal/mol)</th>
<th>$\Delta H_{obs}^o$ (kcal/mol)</th>
<th>T$\Delta S_{obs}^o$ (kcal/mol)</th>
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<tr>
<td></td>
<td>15</td>
<td>10.0</td>
<td>0.09</td>
<td>-10.9</td>
<td>-18.3</td>
<td></td>
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<tr>
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<td>-29.2</td>
<td>-19.0</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.89</td>
<td>0.12</td>
<td>-9.4</td>
<td>-19.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.14</td>
<td>0.12</td>
<td>-8.4</td>
<td>-20.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.1</td>
<td>0.12</td>
<td>-9.6</td>
<td>5.34</td>
<td></td>
</tr>
<tr>
<td>C4</td>
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<td>0.63</td>
<td>0.16</td>
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<td>-4.26</td>
<td>5.04</td>
</tr>
<tr>
<td></td>
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<td>0.14</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.10</td>
<td>0.15</td>
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<tr>
<td></td>
<td>15</td>
<td>4.4</td>
<td>0.14</td>
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<td></td>
</tr>
<tr>
<td>C5</td>
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<td>1.0</td>
<td>0.13</td>
<td>-9.5</td>
<td>-27.2</td>
<td>-17.7</td>
</tr>
<tr>
<td></td>
<td>30</td>
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<td>0.17</td>
<td>-8.6</td>
<td>-19.6</td>
<td>-19.1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.090</td>
<td>0.20</td>
<td>-8.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>C10</td>
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</tr>
<tr>
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<td>0.15</td>
<td>-9.7</td>
<td>8.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.80</td>
<td>0.18</td>
<td>-9.4</td>
<td>7.75</td>
<td></td>
</tr>
</tbody>
</table>

$^a$All data are calculated from titration experiments in standard phosphate buffer with [Na$^+$] = 115 mM at the temperature indicated. The binding constant, $K_{obs}$, and exclusion parameter, n (in binding sites/base pair), were determined by application of the identical-and-independent sites model to Scatchard plots (eq. [32]). The $\Delta G_{obs}^o$ values were calculated using eq. [34], determined at $T = 298$ K, and the $\Delta H_{obs}^o$ and $T\Delta S_{obs}^o$ values were calculated using eq. [33] and [35]. The estimated error in $\Delta G_{obs}^o$ is +0.1 kcal/mol.
Figure 29 Scatchard plot for equilibrium titrations of porphyrin with the carcinogen target DNA oligomer Cl. The same $e_b$ was used for all scatchard plots. These data were obtained in 115 mM NaCl, 10 mM phosphate buffer, pH=7.0, over the range of [DNA]/[porphyrin] = 0.3 - 15. Two binding modes are observed for porphyrin binding with these short DNA sequences. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 29  Scatchard plot for equilibrium titrations of porphyrin with the carcinogen target DNA oligomer C1. The same $s_o$ was used for all Scatchard plots. These data were obtained in 115 mM NaCl, 10 mM phosphate buffer, pH=7.0, over the range of [DNA]/[porphyrin] = 0.3 - 15. Two binding modes are observed for porphyrin binding with these short DNA sequences. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 30 Scatchard plot for equilibrium titrations of porphyrin with the carcinogen target DNA oligomer C4. The same $e_0$ was used for all scatchard plots. These data were obtained in 115 mM NaCl, 10 mM phosphate buffer, pH=7.0, over the range of [DNA]/[porphyrin] = 0.3 - 15. Two binding modes are observed for porphyrin binding with these short DNA sequences. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 30 Scatchard plot for equilibrium titrations of porphyrin with the carcinogen target DNA oligomer C4. The same \( e_b \) was used for all scatchard plots. These data were obtained in 115 mM NaCl, 10 mM phosphate buffer, pH=7.0, over the range of \([\text{DNA}] / [\text{porphyrin}] = 0.3 - 15\). Two binding modes are observed for porphyrin binding with these short DNA sequences. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 31 Scatchard plot for equilibrium titrations of porphyrin with the carcinogen target DNA oligomer C5. The same $\varepsilon_0$ was used for all scatchard plots. These data were obtained in 115 mM NaCl, 10 mM phosphate buffer, pH=7.0, over the range of [DNA]/[porphyrin] = 0.3 - 15. Two binding modes are observed for porphyrin binding with these short DNA sequences. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 31 Scatchard plot for equilibrium titrations of porphyrin with the carcinogen target DNA oligomer C5. The same ε₀ was used for all scatchard plots. These data were obtained in 115 mM NaCl, 10 mM phosphate buffer, pH=7.0, over the range of [DNA]/[porphyrin] = 0.3 - 15. Two binding modes are observed for porphyrin binding with these short DNA sequences. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 32  Scatchard plot for equilibrium titrations of porphyrin with the carcinogen target DNA oligomer C10. The same $e_0$ was used for all scatchard plots. These data were obtained in 115 mM NaCl, 10 mM phosphate buffer, pH=7.0, over the range of [DNA]/[porphyrin] = 0.3 - 15. Two binding modes are observed for porphyrin binding with these short DNA sequences. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 32 Scatchard plot for equilibrium titrations of porphyrin with the carcinogen target DNA oligomer C10. The same $b_0$ was used for all scatchard plots. These data were obtained in 115 mM NaCl, 10 mM phosphate buffer, pH=7.0, over the range of [DNA]/[porphyrin] = 0.3 - 15. Two binding modes are observed for porphyrin binding with these short DNA sequences. A) 15 °C, B) 20 °C, C) 30 °C, D) 40 °C.
Figure 33 (A, B, C and D) showed the van’t Hoff plots of ln $K_{obs}$ vs $1/T$ for the binding of the porphyrin to carcinogen targets C1, C4, C5 and C10 at the range of 15, 20, 30 and 40 °C, respectively. The thermodynamic values contribution for the porphyrin-DNA interactions were summarized in Table 8. For all duplexes, $K$ and the free energy of binding decreased with increasing temperature with C1 having the highest binding constant at 15 °C.

The binding stoichiometry indicated that one porphyrin is bound to every 10 or 11 base pairs (i.e., $1/n$) for intercalation to either C1 while smaller site sizes were observed for C4, C5 and C10 at temperatures < 20 °C from Table 8. The free energies obtained from the determined binding equilibrium constants all fall in the range of 8 to 11 kcal/mol. However, for both C1 and C5, there was a substantial enthalpic contribution to the total free energy, with a significant unfavorable entropic contribution. On the other hand, C4 and C10 both have much smaller, yet favorable, enthalpic contributions than for C1 and C5, while the contributing entropic contributions were moderate but quite favorable.

The average size of binding site for these carcinogen target sequences was 8. Due to the flexibility of oligomer at higher temperatures, the binding site was 5 to 6 for C5 at 30 °C and 40 °C and C10 at 40 °C. That result might be due to the possibility of the porphyrin orientating the pyridyl rings to intercalated into the DNA base pairs, with its preferred affinity site inside the minor groove when temperature was increased.

Examination of the thermodynamic data presented in Table 8 revealed that the binding free energies of the porphyrin to the various DNA are favorable and range
Figure 33  The van't Hoff plots for the porphyrin-DNA the carcinogen target DNA oligomer interaction. All data were obtained from the titration experiments at given temperature (15, 20, 30 and 40 °C) The solid lines are the linear least-squares fits to the experimental data.
A) oligomer C1, B) oligomer C4, C) oligomer C5, D) oligomer C10.
Figure 33 The van't Hoff plots for the porphyrin-DNA the carcinogen target DNA oligomer interaction. All data were obtained from the titration experiments at given temperature (15, 20, 30 and 40 °C) The solid lines are the linear least-squares fits to the experimental data. A) oligomer C1, B) oligomer C4, C) oligomer C5, D) oligomer C10.
The van't Hoff plots for the porphyrin-DNA the carcinogen target DNA oligomer interaction. All data were obtained from the titration experiments at given temperature (15, 20, 30 and 40 °C). The solid lines are the linear least-squares fits to the experimental data.

A) oligomer C1, B) oligomer C4, C) oligomer C5, D) oligomer C10.
Figure 33  The van't Hoff plots for the porphyrin-DNA the carcinogen target DNA oligomer interaction. All data were obtained from the titration experiments at given temperature (15, 20, 30 and 40 °C) The solid lines are the linear least-squares fits to the experimental data.
A) oligomer C1, B) oligomer C4, C) oligomer C5, D) oligomer C10.
from -8.1 to -10.9 kcal/mol over the temperature range studied. However, the DNAs could be classified into two major groups based upon the enthalpic and entropic contributions to the total free energy. The favorable binding free energies for C1 and C5 arose primarily from large, negative enthalpies counterbalancing substantial unfavorable entropic contributions. On the other hand, the favorable free energies observed for C4 and C10 arose from small, though favorable, enthalpic contributions coupled to moderately favorable entropies.

Classic intercalators such as daunomycin and actinomycin demonstrated a preference for GC rich DNA (Chaires, 1990; Roche, 1994; Chen, 1996; Kamitori, 1992; Krugh, 1980). For example, daunomycin selects either 5'-GC-3' or 5'-CG-3' sites flanked on the 5' side by either a T or A base (Chaires, 1990; Roche, 1994) while actinomycin prefers to intercalate into a 5'-GC-3' site (Chen, 1996; Kamitori, 1992), although other sites, such as 5'-GG-3' and 5'-CG-3' may also bind actinomycin (Chen, 1996; Krugh, 1980). As noted above, porphyrins bind via intercalation to GC rich polymeric DNAs (Carvlin, 1983; Pasternack, 1983; Banville, 1986; Mukundan, 1994; Lipscomb, 1996; Pethos, 1993; Pasternak, 1997; Anantha, 1998). In particular, NMR studies have shown that porphyrins prefer to intercalate into 5'-CG-3' sites but this sequence specificity can be modulated by flanking sequences (Banville, 1986; Pasternak, 1997; Marzilfi, 1986; Marzilfi, 1990; Ford, 1987). Examination of the sequences studied here revealed that all oligomers had a 5'-GC-3' sequence at positions 5 and 6 (from the 5'-terminus), with a flanking 5'-A and A-T, and a 5'-GCG-3' sequence at the 3'-terminus, flanked by a 5'-T. Hence, these sites were
virtually identical from oligomer to oligomer. However, each oligomer also had a
unique site: C1 had a 5'-GC-3' which was flanked by a 5'-CTT and 3'-TGC; C4 had a
5'-GC-3' site flanked by a 5'-CCT and a 3'-TGC; C5 has a 5'-GC-3' site flanked by a
5'-CTG and a 3'-CTG; and C10 had a 5'-CG-3' site flanked by a 5'-GCT and a 3'-TAC.

It has been suggested that the stability of the sequences flanking a reactive site
modulate its reactivity (Benight, 1995). Since C1, C4 and C5 all have unique 5'-GC-3'
sites, the differential reactivities might be rationalized in terms of the stability of the
sequence flanking these unique sites as well. For this analysis, we considered only the
5' flanking sequences, since the 3' flanking sequences were identical for these three
oligoners. Thus, a comparison was made for the unique tetrameric sequences
5'-CTTG-3' for C1, 5'-CCTG-3' for C4 and 5'-CTGG-3' for C5 (Benight, 1995). Applying the Doktycz algorithm (Dotkycz, et al 1992), we compared the total free
energy of duplex formation for those three segments. Table 9 showed the results
obtained for this analysis. Keeping in mind that the analysis focused only on the
differences in sequence between the three oligomers, the results indicated that the
sequence flanking the potential binding site in C1 was about 700 cal less stable than
those flanking the potential binding sites in either C4 or C5. Optical melting studies
were also carried out to determine the concentration dependent Tm values and
subsequent thermodynamic parameters for this same oligomer. As shown in Table 9,
experimentally, C1 was less stable than either C4 or C5 by 600 cal/mol, in excellent
agreement with the theoretical comparison. It was interesting to note, however, that
Table 9  Comparison of Theoretical and Experimental Free Energies of Duplex Formation for the Oligomeric Duplexes$^a$

<table>
<thead>
<tr>
<th>Duplex</th>
<th>$\Delta G^\circ_{\text{calc}}$</th>
<th>$\Delta \Delta G^\circ_{\text{calc}}$</th>
<th>$\Delta G^\circ_{\text{exp}}$</th>
<th>$\Delta \Delta G^\circ_{\text{exp}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(kcal/mol)</td>
<td>(kcal/mol)</td>
<td>(kcal/mol)$^b$</td>
<td>(kcal/mol)</td>
</tr>
<tr>
<td>C1</td>
<td>-6.0</td>
<td>-0.0</td>
<td>-15.0 $\pm$ 0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>C4</td>
<td>-6.7</td>
<td>-0.7</td>
<td>-15.5 $\pm$ 0.1</td>
<td>-0.5</td>
</tr>
<tr>
<td>C5</td>
<td>-6.7</td>
<td>-0.7</td>
<td>-15.6 $\pm$ 0.1</td>
<td>-0.6</td>
</tr>
<tr>
<td>C10</td>
<td>na</td>
<td>na</td>
<td>-14.4 $\pm$ 0.1</td>
<td>+0.6</td>
</tr>
</tbody>
</table>

$^a$The theoretical values ($\Delta G^\circ_{\text{calc}}$) were calculated according to the algorithm developed by Dotzyk et al (1992) and the experimental values ($\Delta G^\circ_{\text{exp}}$) were calculated via a van't Hoff approach from concentration dependent thermal denaturation studies. For comparison, the $\Delta \Delta G$ values were calculated by subtracting a particular value from that of C1: a negative sign indicates a more stable duplex.

$^b$Calculated at $T= 37$ °C.
C4 and C5 were nearly identical by both theoretical and experimental free energy calculations.

Higher affinity of porphyrin bound to C5 than to C4 might be due to C5's higher GC content. Porphyrin could bind to the -GG- site as well as to the -GC- site. Although a direct comparison to C10 couldn't be made from a theoretical point of view, data in Table 9 indicated that, experimentally, it was less stable than the other three sequences. Hence, the porphyrin might bind to a less favorable site but the binding free energy was still governed by the overall thermodynamics of the duplex.

3.2.5 Studies of the salt dependency of drug-DNA binding constants

In order to evaluate the salt dependence of porphyrin-DNA binding, the titration experiments were carried out at different concentrations of Na⁺ for the DNA duplexes with highest binding enthalpies for the porphyrin, i.e., C1 and C5. Figure 34 (A, B and C) showed the titration of porphyrin with carcinogen target DNA sequence of C1 (C₀ vs [oligonucleotide]/[porphyrin] over the range of 55-215 mM NaCl and Figure 35 (A, B, C and D) showed the binding concentration of C5 interacting with porphyrin over the same range of NaCl concentrations. For both oligomers, the porphyrin-DNA binding concentration decreased with increasing ionic strength. Since the porphyrin had 4 positive charges, it was not surprising that the porphyrin-DNA interaction was quite sensitive to the ionic strength.

Plots of r vs log Cᵣ for C1 and C5 interacting with the porphyrin as a function of NaCl concentrations were shown in Figure 36 (A and B). The range of
Figure 34 Binding concentration for the carcinogen target DNA oligomer C1 interaction with porphyrin. All binding data were obtained from titration experiment in 55 mM Na\(^+\) and 10 mM phosphate buffer, pH = 7.0 at 25 \(^\circ\)C. A) 15 mM Na\(^+\), B) 115 mM Na\(^+\), C) 150 mM Na\(^+\), D) 215 mM Na\(^+\).
Figure 34 Binding concentration for the carcinogen target DNA oligomer C1 interaction with porphyrin. All binding data were obtained from titration experiment in 55 mM Na\(^+\) and 10 mM phosphate buffer, pH = 7.0 at 25 °C. A) 15 mM Na\(^+\), B) 115 mM Na\(^+\), C) 150 mM Na\(^+\), D) 215 mM Na\(^+\).
Figure 35  Binding concentration for the carcinogen target DNA oligomer C5 interaction with porphyrin. All binding data were obtained from titration experiment in 55 mM Na\(^+\) and 10 mM phosphate buffer, pH = 7.0 at 25 °C.  A) 15 mM Na\(^+\), B) 115 mM Na\(^+\), C) 150 mM Na\(^+\), D) 215 mM Na\(^+\).
Figure 35 Binding concentration for the carcinogen target DNA oligomer C5 interaction with porphyrin. All binding data were obtained from titration experiment in 55 mM Na\(^+\) and 10 mM phosphate buffer, pH = 7.0 at 25 °C. A) 15 mM Na\(^+\), B) 115 mM Na\(^+\), C) 150 mM Na\(^+\), D) 215 mM Na\(^+\).
Figure 36  Plot r vs log \(C_f\) for the porphyrin - DNA interaction as a function of NaCl concentration over the range 55, 115, 150 and 215 mM. All the binding data were obtained from titration experiment in 10 mM phosphate buffer, pH = 7.0 at 25 °C. \(r\) is the number of moles of porphyrin bound per mole of DNA base pair, \(r = C_b/[DNA]\). \(C_b, C_f\) is the bound and free concentration of porphyrin in the presence of excess DNA, respectively. A) oligomer C1, B) oligomer C5.
Figure 36  Plot $r$ vs $\log C_f$ for the porphyrin - DNA interaction as a function of NaCl concentration over the range 55, 115, 150 and 215 mM. All the binding data were obtained from titration experiment in 10 mM phosphate buffer, pH = 7.0 at 25 °C. $r$ is the number of moles of porphyrin bound per mole of DNA base pair, $r = C_b/[\text{DNA}]$. $C_b$, $C_f$ is the bound and free concentration of porphyrin in the presence of excess DNA, respectively.  A) oligomer C1, B) oligomer C5.
concentration was 55, 115, 150 and 215 mM. The temperature was 5 °C. The ratio of number of moles of the bound porphyrin to the moles of base pairs of DNA (\( r = C_o/[DNA] \)) decreased with increasing salt concentration at the same temperature due to the +4 charges of porphyrin. The binding constant as a function of [NaCl] could be obtained from the Scatchard plots shown in Figures 37 and 39. Figure 38 gave a comparison of the data for C1 at different [NaCl].

The stability of complexes with +4 charged ligands (such as the porphyrin) in aqueous solution were determined by the present electrolytes and by competitive ion exchange processes involving cations on carcinogen target DNA. Hence, the binding process had a salt dependence (Lohman, 1992; Record, 1978; Manning, 1978; Wilson, 1979; Friedman, 1984) given by:

\[
\log K_{obs}/\log[NaCl] = -Z\psi \tag{36}
\]

where, \( Z \) was the charge on the ligand and \( \psi \) was the fraction of counterions associated with each DNA phosphate. The binding free energy (\( \Delta G_{obs}^o \)) could now be separated (Lohman, 1992; Chaires, 1993; Chaires, 1996) into electrostatic (\( \Delta G_{pe}^o \)) and non-electrostatic contributions (\( \Delta G_t^o \)). The free energy can be calculated using the following equations.

\[
\Delta G_{obs}^o = \Delta G_t^o + \Delta G_{pe}^o \tag{37}
\]

\[
\Delta G_{pe}^o = Z\psi RT\ln[Na^+] \tag{38}
\]

The salt dependence of free energy \( \Delta G_{obs}^o \) arises from this variation of \( \Delta G_{pe}^o \) and \( \Delta G_t^o \) with salt concentration increased. Figure 40 (A and B) showed the plots of ln \( K \) vs ln [Na⁺] for C1 and C5, respectively. As shown, there was a linear dependence
Figure 37  Scatchard plots for the porphyrin - DNA (C1) interaction. All binding data were obtained from titration experiment in 10 mM phosphate buffer, pH = 7.0 at 25°C. A) 15 mM Na⁺, B) 115 mM Na⁺, C) 150 mM Na⁺, D) 215 mM Na⁺.
Figure 37 Scatchard plots for the porphyrin - DNA (C1) interaction. All binding data were obtained from titration experiment in 10 mM phosphate buffer, pH = 7.0 at 25°C. A) 15 mM Na+, B) 115 mM Na+, C) 150 mM Na+, D) 215 mM Na+.
Figure 38  Scatchard plots for the porphyrin - DNA (C1) interaction as a function of NaCl concentration over the range 55, 115, 150 and 215 mM. All the binding data were obtained from titration experiment in 10 mM phosphate buffer, pH = 7.0 at 25°C. 
55 mM(●), 115 mM(▲), 150 mM(♦) and 215 mM(▼).
Figure 39  Scatchard plots for the porphyrin - DNA (C5) interaction. All binding data were obtained from titration experiment in 10 mM phosphate buffer, pH = 7.0 at 25 °C.  A) 15 mM Na⁺, B) 115 mM Na⁺, C) 150 mM Na⁺, D) 215 mM Na⁺.
Figure 39  Scatchard plots for the porphyrin - DNA (C5) interaction. All binding data were obtained from titration experiment in 10 mM phosphate buffer, pH = 7.0 at 25 °C.  A) 15 mM Na⁺, B) 115 mM Na⁺, C) 150 mM Na⁺, D) 215 mM Na⁺.
Figure 40: Studies of the salt dependency of porphyrin-DNA binding constants. Experimental determination of $\frac{\delta \ln K}{\delta \ln [Na^+]}$ for the DNA oligomer. All data were obtained in 10 mM phosphate buffer, pH=7.0 at 25°C. The dependence of ln $K$ is plotted as a function of ln [Na$^+$]. $K$ is the equilibrium binding constant. [Na$^+$] is the concentration of sodium in the buffer. log $K_{obs}/\log[NaCl] = -Z\psi$, $Z$ is the charge on the ligand and $\psi$ is the fraction of counterions associated with each DNA phosphate. A) oligomer C1, B) oligomer C5.
of the ln $K$ on ln [Na$^+$]. Using eq. (36), where $Z = +4$, and the slope for C1 was 3.03, and 2.12 for C5, we determined $\psi = 0.76$ for C1 and 0.53 for C5. As summarized in Table 10, both the observed free energy of binding and the electrostatic component, dramatically decreased as the concentration of Na$^+$ increased. This was not surprising because of the +4 charges of the porphyrin. Further, the non-electrostatic component was independent of ionic strength and more favorable for C5 than C1. Finally, the binding of the porphyrin to C1 was more sensitive to ionic strength than that to C5.

The salt dependence of the observed binding free energy, $\Delta G^o_{obs}$ for C1 and C5 arose from the variation of $\Delta G^o_{pe}$, as [Na$^+$] was increased. The values obtained for non-electrostatic contribution, $\Delta G^o_i$ to the overall free energy (Table 10) for C1 and C5 were not salt dependent, as expected. The line parameters for the Figure 33 and Figure 34 were listed in Table 11 and Table 12, respectively. Figure 41 (A and B) and the data in Table 10 revealed that at [Na$^+$] less than 200 mM, $\Delta G^o_{pe}$, was the major contributor to the total binding free energy for the binding of H$_2$TMPyP to C1. Since the contribution of $\Delta G^o_{pe}$, was dominant, the complex appeared to be stabilized by electrostatic interactions between positively charged nitrogen atoms of the pyridyl rings and negatively charged phosphate oxygen atoms of DNA. However, the magnitude of $\Delta G^o_i$, indicated that the complex was also stabilized by stacking, hydrophobic and possibly H-bonding interactions. For the binding of H$_2$TMPyP to C5 at all concentrations of sodium, the magnitude of $\Delta G^o_i$ was larger than $\Delta G^o_{pe}$, suggesting that electrostatic binding was less important. The sequence of 5'-CTGGC-3' in C5 had one more GC base pair than 5'-CTTGC-3' in C1. The greater
Table 10  Dissection of the Binding Free Energy for the Interaction of H$_2$TMPyP with DNA Duplex C1 and C5

<table>
<thead>
<tr>
<th>Duplex</th>
<th>[Na$^+$]</th>
<th>$K_{obs}$ / $10^6$</th>
<th>$\Delta G_{obs}^0$</th>
<th>$\Delta G_{pe}^0$</th>
<th>$\Delta G_{t}^0$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>(M$^{-1}$)</td>
<td>(kcal/mol)</td>
<td>(kcal/mol)</td>
<td>(kcal/mol)</td>
</tr>
<tr>
<td>C1</td>
<td>55</td>
<td>37.6</td>
<td>-10.3</td>
<td>-6.7</td>
<td>-3.6</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>3.4</td>
<td>-8.9</td>
<td>-5.0</td>
<td>-3.9</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.89</td>
<td>3.90</td>
<td>-8.1</td>
<td>-4.4</td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>0.18</td>
<td>-7.2</td>
<td>-3.5</td>
<td>-3.7</td>
</tr>
<tr>
<td>C5</td>
<td>55</td>
<td>4.1</td>
<td>-9.0</td>
<td>-3.5</td>
<td>-5.5</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>0.91</td>
<td>-8.1</td>
<td>-2.6</td>
<td>-5.5</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.45</td>
<td>2.05</td>
<td>-7.7</td>
<td>-2.3</td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>0.27</td>
<td>-7.4</td>
<td>-1.9</td>
<td>-5.5</td>
</tr>
</tbody>
</table>

All data are calculated from titration experiments in standard phosphate buffer at 25 °C at the [Na$^+$] indicated. The binding constant, $K_{obs}$ was determined by application of the identical-and-independent sites model to Scatchard plots (eq. [32]). The values of $Z\psi$ were determined according to eq. [36] from the plots shown in Figure 37, the $\Delta G_{obs}^0$ values were calculated according to eq. [33], determined at $T = 298$ K, $\Delta G_{pe}^0$, values were determined using eq. [38] and $\Delta G_{t}^0$, values were determined using eq. [35]. The estimated average error in $\Delta G_{obs}^0$ is ±0.1 kcal/mol and the estimated average error in $\Delta G_{pe}^0$ is +0.2 kcal/mol giving a propagated error in $\Delta G_{t}^0$ of +0.3 kcal/mol.
Figure 41  The binding free energy of the porphyrin-DNA interaction for the DNA oligomer. The total free energy ($\Delta G_{\text{obs}}$), the polyelectrostatic contribution ($\Delta G_{\text{pe}}$) and the non-electrostatic contribution ($\Delta G_t$) is shown as a function of Na$^+$ concentration. $\Delta G_{\text{obs}} = \Delta G_t + \Delta G_{\text{pe}}$.  A) oligomer C1, B) oligomer C5.
Table 11  Line Parameters for the Plots of ln K vs 1/T for Figure 33

<table>
<thead>
<tr>
<th>Duplex</th>
<th>Slope</th>
<th>y-intercept</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>$1.48 \times 10^4$</td>
<td>-33.06</td>
<td>0.9868</td>
</tr>
<tr>
<td>C4</td>
<td>$8.47 \times 10^3$</td>
<td>-13.19</td>
<td>0.9981</td>
</tr>
<tr>
<td>C5</td>
<td>$1.37 \times 10^4$</td>
<td>-30.21</td>
<td>0.9573</td>
</tr>
<tr>
<td>C10</td>
<td>$3.67 \times 10^3$</td>
<td>1.89</td>
<td>0.9457</td>
</tr>
</tbody>
</table>
Table 12  Line Parameters for the Plots of

$\ln K$ vs $\ln [\text{Na}+]$ for Figure 40

<table>
<thead>
<tr>
<th>Duplex</th>
<th>Slope</th>
<th>y-intercept</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>-3.90</td>
<td>6.28</td>
<td>0.9885</td>
</tr>
<tr>
<td>C5</td>
<td>-2.05</td>
<td>9.26</td>
<td>0.9937</td>
</tr>
</tbody>
</table>
magnitude of $\Delta G^\circ$, of C5 compared to C1 at the same salt condition might be partially due to the higher selectivity of porphyrins for G-C base pairs. In fact, the calculated free energy of the nearest neighbor interactions of the 5'-CTTG-3' sequence abutting the unique potential binding site in C1 indicated it was more stable than the sequence in C5, 5'-CTGG-3', by nearly 350 cal/mol. (Dotkycz, 1992). The neighboring base pairs played a role in the binding process.
3.2.6 Summary

The interaction specificities of tetrakis(4-N-methylpyridyl)-21H,23H-porphine (H₂TMPyP) with selected synthetic DNA sequences via visible and CD absorption have been studied. The interactions were followed by measuring the visible spectra at 400-500 nm over a range of temperatures and NaCl concentrations. The data indicated that H₂TMPyP interacted with the duplexes under study and the induced circular dichroism spectra of the porphyrin in the Soret region suggested that the porphyrin intercalated into the DNA base pairs. Analysis of the binding isotherms from experimentally determined Scatchard plots indicated a sequence context effect on the binding. The binding free energies for porphyrin-DNA interactions were derived from van't Hoff analysis. The favorable binding free energy arose primarily from a large, negative enthalpic contribution for two of the four sequences studied. Analysis of the interaction as a function of ionic strength was used to dissect the total free energy of binding into electrostatic and non-electrostatic components in light of the polyelectrolyte theory. This analysis demonstrated that the electrostatic component was highly dependent upon ionic strength while the non-electrostatic component was independent of ionic strength.
CHAPTER IV

CONCLUSION

We designed a series of synthetic DNA oligomers containing potential high affinity carcinogen binding sites and a known carcinogen target sequence CI. The relationship between porphyrin binding affinity and thermal stability has been investigated.

Base-base interactions, which are resulted from hydrogen bonding and base stacking, have been investigated by thermodynamic methods to derive enthalpies, entropies and free energies changes for the duplex to single strand transition. The hydrogen-bonding interactions were the primary contributions to the observed thermodynamic parameters for these carcinogen binding sequences while base stacking provided secondary contributions. The stacking was more important for stabilization of the oligomer for a one-base-pair mutation. The differential ion binding term, Δn (the release of centurions per duplex upon denaturation) was indirect evidence that the different sequence context of the DNA oligomers resulted in differential binding of Na⁺ to the DNA.

An additional result from these studies was that a thermodynamic description of the profound effect of slight sequence change on DNA helix stability. The alteration of a single base pair next to the high-affinity binding site G within a 16 bp carcinogen target sequence can alter the energetic of duplex melting. These changes in free energy of helix melting with sequence arose from alteration of both the enthalpy
and entropy. The agreement between the predicted $\Delta \Delta G^0$ values and the experimentally determined $\Delta \Delta G^0$ values were excellent.

We have also investigated the interaction of tetrakis(4-N-methylpyridyl)porphine ($H_2$TMPyP) with selected synthetic carcinogen DNA sequences via UV/VIS titration studies over a range of temperatures and Na$^+$ concentrations. The UV/Vis spectrum indicated that $H_2$TMPyP interacted with carcinogen binding sequences and the induced porphyrin spectrum of circular dichroism (CD) at Soret region confirmed that the porphyrin was intercalated into DNA base pairs. Analysis of the binding isotherms from experimentally determined Scatchard plots indicated that the two binding models were involved in porphyrin-DNA interactions, intercalation at low binding ratios and outside stacking at high ratios. The results of these studies along with the observed negative induced circular dichroism (CD) at Soret region of $H_2$TMPyP in the presence of DNA were interpreted in terms of porphyrin intercalation into the DNA base pairs.

For the intercalative binding model, the binding energies for porphyrin-DNA intercalation were quantitated by van't Hoff analysis. The entropy increased as the temperature arose for C1, C4 and C5. The favorable binding free energy arose primarily from the large, negative enthalpy. For carcinogen binding sequence, C1, the binding constant at intercalative mode was strong function of ionic strength. The possible molecular contributions to the enthalpy and entropy were discussed. The results suggested that sequence context not only influenced the total free energy of binding but also influenced the electrostatic and non electrostatic contributions.
Further studies with additional sequences were required to determine if the sequence context effect was a general phenomenon.
REFERENCES


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Qianying Liang was born in Tianjin, P. R. China. She completed her B. Eng. Degree in Chemical Engineering from Tianjin University, in July 1988. She received a M. Sc. in biochemistry in December, 1994, from Department of Chemistry, Seton Hall University.