The Dissertation Committee for Suzanne Lai Tobey Certifies that this is the approved version of the following dissertation:

Energetics: The Fundamental Thermodynamic Parameters of Molecular Complexation *via* Electrostatic Interactions in Water

Committee:

Eric V. Anslyn, Supervisor

Philip D. Magnus

Michael J. Krische

Jason B. Shear

Kevin N. Dalby

Energetics: The Fundamental Thermodynamic Parameters of Molecular Complexation *via* Electrostatic Interactions in Water

by

Suzanne Lai Tobey, B.S.

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Dedication

Dedicated to Grandpa, Gram, Grandma, Mom, Dad and my little Bro for their sense of humor, love, support and inspiration for all I have accomplished in my personal and professional pursuits.

Acknowledgements

I would not be composing the acknowledgments section for my dissertation work were it not for my mentor Eric Anslyn. Thank you Eric, for welcoming me into your group and giving me the opportunity to hone my skills as a chemist. I have learned a great deal while under your tutelage, but I have also learned that there is a great deal out there that I have yet to learn, and for that you have truly prepared me. I have always been encouraged by your enthusiasm for chemistry; I walked into your office numerous times feeling a bit discouraged about a particular result, but I always left with a renewed confidence. Thank you for being a wonderful mentor and a friend. See you in Jamaica.

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Paola, you are a great asset to the group and through you I have a greater appreciation and respect for the inorganic/analytical fields. You have been a great help with the pH titrations. In the short time you have been here we have become good friends; if only you had arrived sooner! Thank you for being there.

Finally, I'd like to extend a 'thank you' to the members of the Anslyn Group past and present for numerous discussions. You've made this a great

learning environment, and also given me an opportunity to share discussions pertinent to my research as well as yours. Thank you.

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Suzanne Lai Tobey, PhD

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Supervisor: Eric. V. Anslyn

The thermodynamic parameters that govern the molecular recognition phenomena continue to be a significant area of interest. As a molecular complex forms through non-bonded interactions, quantification of the fundamental enthalpy and entropy changes that occur, offer a more comprehensive understanding of the process. The research presented here specifically explores the energetics of host-guest complexes that form through electrostatic interactions in water at neutral pH. Chapter 1 provides an introduction to molecular recognition and the binding forces that promote binding. Chapter 2 details extensive studies involving phosphate binding to two metalloreceptors; entropy changes are the dominant driving force. In Chapter 3 we report studies focused on determining the thermodynamic origin of cooperative binding; we report the

presence of negative cooperativity having entropy as its origin. Chapter 4 is comprised of extensive investigations into the formation of 1:1 host/guest complexes as well as higher ordered complexes. The predominance of either complex results in different thermodynamic profiles.

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Chapter 1: Introduction and Background

1.0 INTRODUCTION

A molecular recognition event involves the association of organic molecules through non-bonded interactions. In natural systems such associations have both high selectivities and high affinities; this is exemplified by enzyme-substrate and antigen-antibody interactions. The desire to better understand the physical nature of and the driving forces for such interactions is the focus of many researchers in the field of biochemistry and physical biochemistry. The organic chemists' approach involves the design and the study of the associations of smaller, simpler molecules. The use of synthetic systems for molecular recognition studies is often referred to as host-guest chemistry.

One of the research goals within the arena of host-guest chemistry involves the characterization and quantification of the physical forces that drive the molecular associations. Each type of binding interaction has an associated thermodynamic component (ΔG°), which is comprised of both an enthalpy (ΔH°) and an entropy (ΔS°) term. Extensive work by Diederich explored the entropic and enthalpic changes that underlie the hydrophobic binding of aromatic guests to a cyclophane host. Physical studies by both Schmidtchen and Hamilton offered insights into the entropic driving forces of binding *via* electrostatic interactions between carboxylate and guanidinium groups in organic media. Combined, these results have awakened an interest in pursuing more comprehensive thermodynamic studies on host-guest complexation with the goal of deciphering the roles of the host, the guest, the solvent and the counterions, because they contribute to the overall thermodynamic profile of a binding event. Few studies

of this kind involving ion-pairing host-guest binding in water can be found in the literature.

Another aspect of molecular recognition involves the notion of cooperativity. The association of molecules in solution is often a result of multiple types of binding forces acting cooperatively. While this is widely accepted, the thermodynamic origins of this phenomenon are poorly understood. Physical studies on the dimerization of vancomyacin, by Williams, represent one of the first attempts to address the energetics of cooperativity experimentally. Designed synthetic receptors are inherently simple, and have the potential to be used to probe the fundamental thermodynamic parameters of cooperative binding.

The two aspects of molecular recognition chemistry introduced above establish the tone of the research presented in this thesis. The research goals were to: 1) investigate the thermodynamic parameters of host-guest binding promoted by ion-pairing interactions in water, and 2) to identify the thermodynamic origins of cooperativity in host-guest binding that proceeds through ion-pairing interactions in water. The remainder of this chapter is intended to provide a general introduction to host-guest chemistry while providing a background for the specific research presented in subsequent chapters. A substantial amount of detail is presented on the binding forces and their manifestation in terms of thermodynamic parameters. There is also significant detail included on the thermal technique by which binding can be monitored. This is necessary to inspire an appreciation for the principles that govern the binding phenomena and the results stemming from the research reported in this thesis.

1.1 BINDING FORCES

The association of molecules in solution through non-covalent interactions defines the field of molecular recognition. Nature provides the most elegant

examples of molecular recognition in the form of enzyme-substrate complexes, antigen-antibody associations, protein-protein complexes and double stranded composites of nucleic acids to form DNA molecules. The specificity and selectivity demonstrated by such molecular recognition events in nature derive from seemingly simple non-bonded interactions between functional groups on each of the partners. This provides the inspiration for using rationally designed synthetic host molecules to bind target guests in solution with high selectivities and affinities. To achieve this end it is necessary to understand the non-bonded interactions that promote enzyme-substrate associations and incorporate them into the host-guest system design.

The non-bonded interactions that enable molecules in solution to associate with a specific orientation and strength are representative of binding forces. Such forces are often described as being electrostatic interactions or hydrophobic interactions. Electrostatic binding forces include hydrogen bonding, ion-pairing, dipole-dipole, cation-π, charge-dipole, π-π interactions, H-π interactions and van der Waals interactions. Each of these differ in the modes of interaction, strengths, geometries, and driving forces.

1.1.1 Hydrogen Bonding

Hydrogen bonding proceeds through the sharing of a hydrogen atom between a hydrogen bond donor and a hydrogen bond acceptor (Figure 1.0). The most noted examples from nature are the hydrogen bonding between amides in proteins and nucleotide bases in DNA duplexes. Both the donor and the acceptor are generally heteroatoms such as nitrogen or oxygen. The strength of the bond is

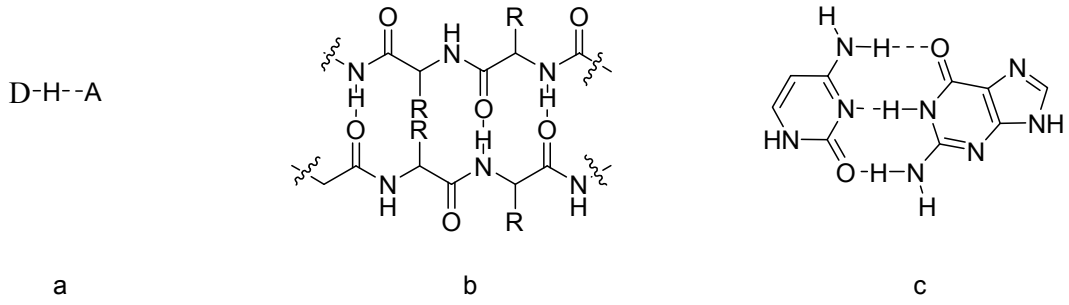


Figure 1.0 Examples of Hydrogen Bonding from Nature. a) Depiction of a generic hydrogen bond between a donor and an acceptor atom. b) Multiple hydrogen bonds are present between the peptide backbones of proteins and are responsible for the secondary structure of proteins. c) Nucleic acid bases are present in RNA and DNA duplexes, displaying specific hydrogen bonded interactions as shown in this cytosine-guanine pair.

dependent on the pK_a values of both the donor and the acceptor. In general, the strength of a hydrogen bond increases as the acceptor becomes more basic and the donor more acidic. A unique feature of the hydrogen bond is that of directionality, which arises from the presence of a dipole between interacting donor and acceptor atoms and their geometries; the optimum arrangement is a linear hydrogen bond. The linear arrangement allows for the best dipole alignment between the donor and acceptor. It is also argued that this maximizes the overlap of the interacting orbitals[1]. This unique feature of a hydrogen bond is responsible for the specificity observed in natural systems.

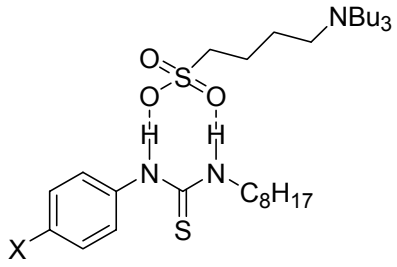
The strength of a single hydrogen bonding interaction is weak, averaging between 3-9 kcal/mol for charged hydrogen bond partners and 0.5-1.5 kcal/mol for uncharged partners in natural systems.[1] The strength in water is highly dependent on how favorable the equilibrium is for hydrogen bond formation between the donor and acceptor relative to the hydrogen bonded interactions between the donor and acceptor with the solvent:

$$D\text{-}H\cdots OH_2 \quad + \quad A\cdots HOH \quad \Leftrightarrow \quad D\text{-}H\cdots A \quad + \quad HOH\cdots OH_2$$

The thermodynamics of hydrogen bond formation relies on the participating donor and acceptor, and on the solvent. In aqueous media, they are generally thought to proceed with no net gain or loss in enthalpy, but with favorable entropy as water molecules are released into bulk solution. The free energy of intramolecular hydrogen bonding in hydroxyl ethers ranged from -2.3 kcal/mol in $CDCl_3$ to -0.5 kcal/mol in D_2O, with unfavorable entropy (-3.5 cal/mol·K) in $CDCl_3$.[2] Work by Rebek, using a synthetic host-guest model reveals that a single hydrogen bond in water has a free energy value of 0.2 kcal/mol, with an enthalpy change of 0.8 kcal/mol and an unfavorable entropy change of -5 cal/mol.[3] For this system the enthalpy just compensates for the unfavorable entropy.

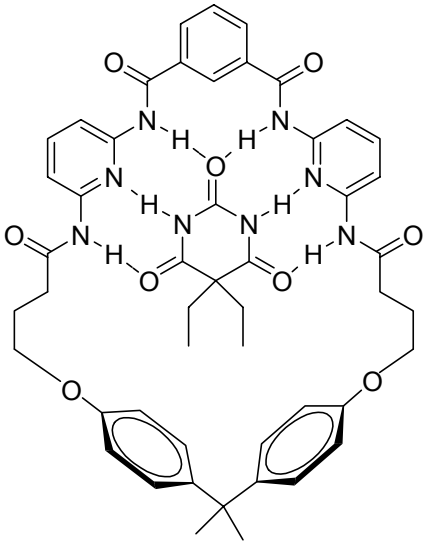
1.1.1.1 Hydrogen Bonding in Anion Binding

Molecular recognition chemists have been successful in modulating the physical properties of synthetic hosts as a means of effecting the complexation of a guest through hydrogen bonds.[4] Substituent effects and host preorganization are the primary means by which this has been accomplished. Wilcox has shown that the difference in binding energy between nitro-substituted host **1.1** and dimethyl amine substituted host **1.2** with a sulfonate guest is 3.8 kcal/mol ($CDCl_3$).[5] The electron withdrawing ability of the nitro group on **1.1** renders the host a better H-bond donor, thereby increasing the binding affinity to the guest relative to **1.2**.



1.1 X = NO_2
1.2 = NMe_2

The importance of preorganization of the host is exemplified by Hamilton[6] and co-workers in which host **1.3** binds a barbital guest with an affinity on the order of 10^6 in chloroform. The analogous freely rotating cleft (**1.4**) binds on the order of 10^4.



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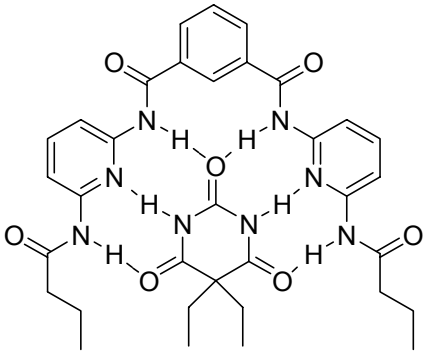
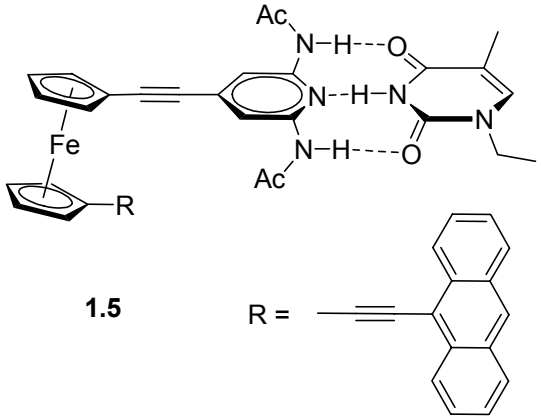


1.4

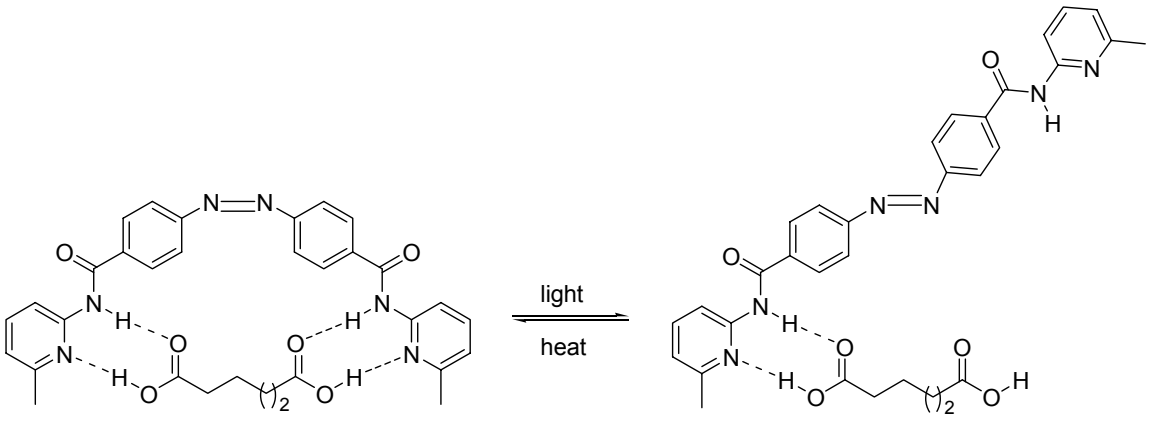
Another approach to modulating hydrogen bonded complexes involves the use of different intermolecular interactions to complement and promote hydrogen bonded arrays. An example of this comes from the work of Inouye[7] in which π-stacking interactions were used to appropriately organize the donor and acceptor groups for effective hydrogen bond formation with 1-butylthymine (**1.5**). The polyaromatic groups (R) used for the π-stacking were varied, and it was found that poorer overlap of the aromatic systems resulted in smaller binding affinities with the guest.



1.5

R =

The use of external stimuli to induce hydrogen-bonded motifs has also been explored. Goswani[8] employs a photo-chemical reaction to control the geometry of diazo host **1.6** to induce hydrogen bonding in host-guest complexation. In acetonitrile the complex on the left has a binding affinity of 1.81 x 10^4 M^{-1}, while that on the right binds with K_a = 5.16 x 10^3 M^{-1}. Other such work involves redox chemistry and charge transfer reactions.



1.6

1.1.2 Ion- Pairing

Ion-pairing is another example of an electrostatic interaction that can participate in complex formation. In the case of ion-pairing, oppositely charged functional groups approach through space to form non-bonded contacts (Figure 1.1). The simplest example is found in salts such as sodium chloride, potassium sulfate, or magnesium sulfate. In nature the more elegant examples are found in

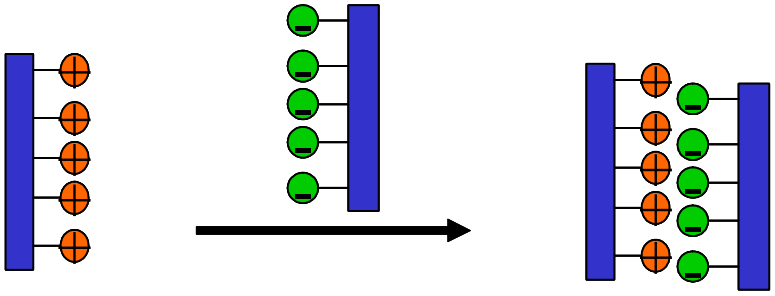


Figure 1.1 Schematic of Ion-Pairing. A molecule with positively charged groups on the surface will approach another having negatively charged groups to form a complex through non-bonded interactions.

the active site of enzymes where charged moieties on the peptide backbone interact with charged groups on the substrate with good affinity and high specificity. The active site of acetylcholinesterase possesses an anionic pocket for the quarternary ammonium center on its native substrate acetocholine. Uncharged analogues have reduced interactions with the enzyme active site.

In general, larger cations on ion-exchange resins form tighter ion pairs with larger monoatomic anions. The large anions are poorly solvated, therefore they shed their hydration shell more easily to form ion-pairs. In contrast, the smaller ions have a more tightly held solvation shell, and therefore form weaker contacts with cations. This is also true for poly-atomic anions, such as perchlorate, which forms tighter ion pairs with an ion-exchange resin than does phosphate, due to a diffuse charge density.[9]

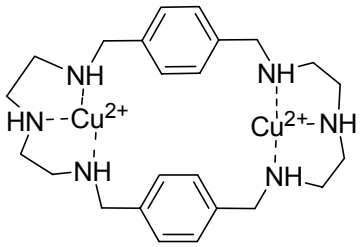
The energies of ion-pairing interactions are dependent on the solvent, the size of the ions, and the charge density of the ions. The formation of an ion pair from monoatomic partners in water is approximately 1 kcal/mol, and the solvation of a single ion falls within the range of -50 to -100 kcal/mol. This highlights the solvation ability of water which leads to rather weak ion-pairing interactions. ^{1}H NMR methods combined with computational methods have shown that a single anion-cation interaction in water contributes 5 kJ/mol of energy to the free energy of binding.[10, 11] Dougherty and co-workers have shown that the energy for the formation of a single salt bridge between a carboxylate group and an ammonium group in water ranges between 0-3 kcal/mol.[12]

Ion-pair formation has favorable enthalpy change as the charged moieties interact to attain a charge neutral state. This is accompanied by unfavorable entropy change as the disorder of the system is reduced. Solvent mediated ion-pairing in water is further complicated by the exchange of hydration spheres on each of the ions for an ion-pair contact. The formation of an ion-pair contact with release of solvent molecules leads to an increase in disorder of the water, with

concomitant local ordering of solvent molecules around the ion-pair. Though the free energy of ion-pairing is small, the contributions of the enthalpy and entropy can vary depending on the extent to which they compensate each other.

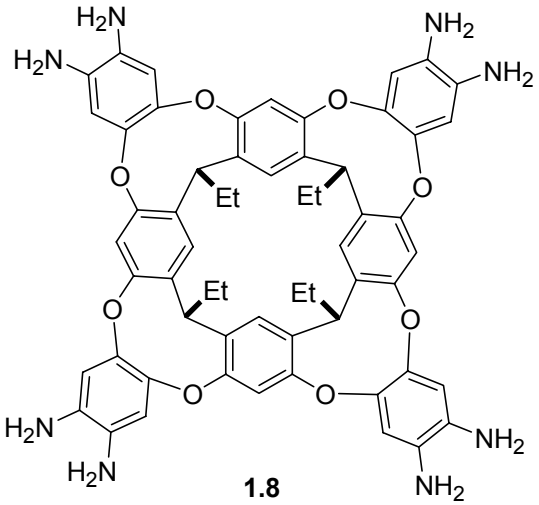
1.1.2.1 Ion-Pairing Molecular Recognition

The utility of ion-pairing interactions in molecular recognition chemistry has been demonstrated by the design of synthetic receptors bearing charged functional groups for the purpose of binding anions or cations. Fabbrizzi and co-workers[13] used di-copper(II) host **1.7** as a chemosensor for pyrophosphate in water at pH 7. He proposes that the interatomic distance between the Cu (II) centers is ideal for pyrophosphate, with the Cu (II) centers serving as binding sites for the anionic oxygen centers on the guest. In the strictest sense these ion-



1.7

pairing interactions involve cation-anion interactions, but other ion-pairing interactions are often utilized in molecular recognition. These include cation-π[14,15], π-π[16-18], dipole-dipole and metal-anion interactions. Rebek[19] reports studies on a 'vase'shaped cavity (**1.8**) which binds tetramethylammonium chloride in d_6-DMSO with an affinity of 2.2 x 10^4 M^{-1} as a result of cation- π interactions.



1.8

Electrostatic interactions cannot be fully characterized without recognizing the role of van der Waals forces that are present for each of these interactions. As atoms approach another in space there is an attractive force involved, but at a specific interatomic distance the atoms repulse each other. These are described as van der Waals forces. The distance of approach if favorable will contribute positively to the strength of binding, but if the distance of approach is too close, there will be an unfavorable contribution to the binding. These too, are weak interactions, contributing approximately 2.0 kcal/mol per interaction.

1.1.3 Hydrophobic Interactions

The individual electrostatic interactions described above are weak, yet combined they are partially responsible for the high affinities and selectivities seen in natural and synthetic systems. The apparent driving forces for the formation of salt bridges or hydrogen bonds between binding partners are not as dominant in aqueous media, indicating that there are other binding forces that

contribute to the high affinity complexes observed in nature. This additional binding force arises from hydrophobic interactions.

Hydrophobic interactions describe the proclivity of non-polar molecules (hydrocarbons) to interact with other non-polar molecules in water. The driving force derives from the strong hydrogen bonded interactions between water molecules. This contributes to the binding of molecules as the hydrophobic portions of a binding partner transfer to the hydrophobic interior of a binding cavity. To highlight the significance of hydrophobic interactions to binding energies, a study using the energies of the transfer of amino acid side chains from ethanol to water show that each methylene group increases the binding energy by 0.7 kcal/mol and each benzene or indole ring increases the binding energy by 2 kcal/mol.[20] A more descriptive analysis comes from the enthalpy (-2.7 kcal/mol) and entropy (-18 e.u.) associated with the transfer of methane from an inert solvent to water. The favorable enthalpy reflects the increase in the bonding between water molecules as they become more ordered (unfavorable entropy) around the methane unit.

The approach of two hydrophobic surfaces in water to form a hydrophobic bond is characterized by an increase in entropy as the water molecules on the surfaces of the interacting molecules are displaced into bulk solution upon binding (Figure 1.2). This may be accompanied by a near zero or positive enthalpy change as the number of hydrogen bonds between surrounding water molecules may decrease. Here, the driving force derives from the unique nature of water. The classical hydrophobic effect is often characterized as having a near zero enthalpy change and a positive entropy change.[21]

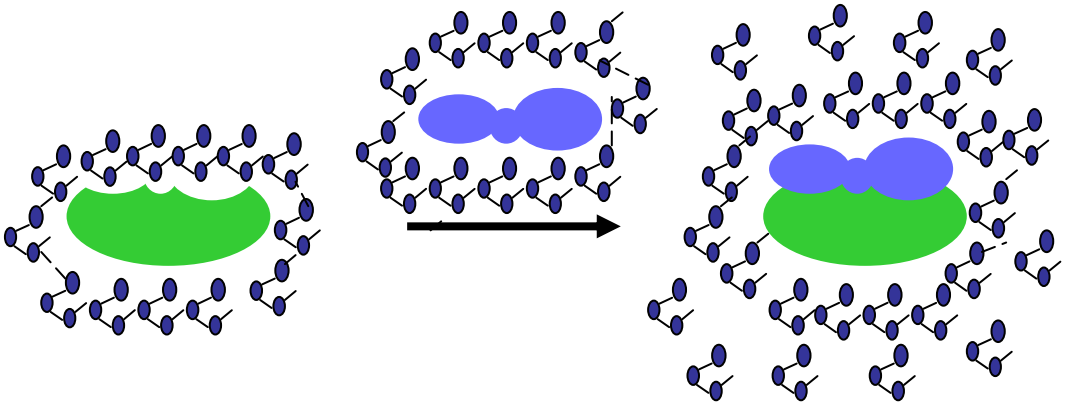
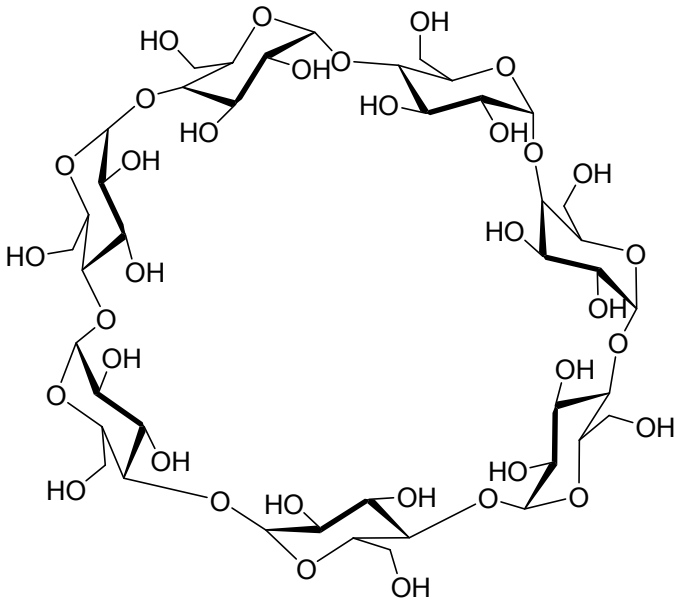


Figure 1.2 Hydrophobic Interactions. The interaction of hydrophobic surfaces in water leads to the release of solvent molecules into bulk solution thereby increasing the entropy of the system. The number of hydrogen bonds before and after binding may remain the same or decrease leading to a near zero or positive enthalpy change.

1.1.3.1 Hydrophobic Interactions in Molecular Recognition

Hydrophobic interactions have been incorporated into the design of synthetic host-guest systems employing cyclophanes or cyclodextrins. The interior of these receptors are hydrophobic, and in aqueous solvent encapsulate hydrophobic guests as a result of hydrophobic bonding.[22, 23] Inoue et. al. have studied the binding of several napthalenesulfonates to β-cyclodextrins (**1.9**) in water. The binding proceeds through hydrophobic interactions between the naphthalene ring of the guest and the hydrophobic interior of the cyclodextrin cavity. Several of the guests were characterized by favorable entropy changes and positive enthalpy changes. The favorable entropy is thought to arise from displacement of water molecules from the cavity upon binding the guest.[24]



1.9

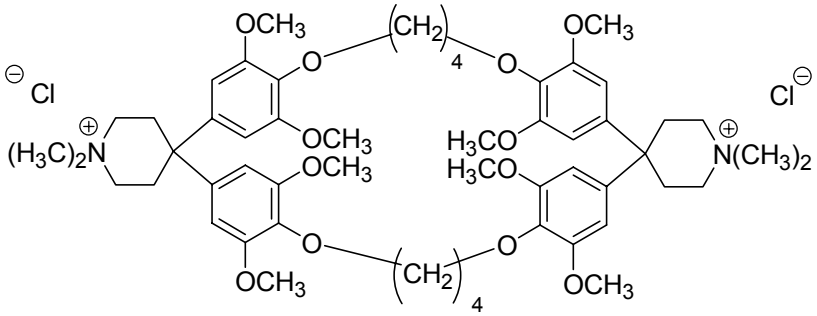
Although each of the predominant binding forces has been addressed individually, in reality they are all operative on some level, each influencing the strength of the other. In general the individual binding forces are relatively weak, yet substrate-enzyme and host-guest complexes can be rather robust. Some or all of the binding forces identified above act simultaneously in the binding of enzyme-substrate or host-guest, and their combined strengths are responsible for the high affinity complexes observed. With some knowledge of the binding forces at work in a binding event, the molecular recognition chemist seeks to appropriately match binding partners and combine them in such a way to arrive at tight associations of molecules such as those found in nature.

1.1.4 Solvent Effects

The strengths and thermodynamic profiles of binding interactions are highly dependent upon the properties (dielectric constant, protic, aprotic) of the medium in which they occur. In any molecular recognition study the solvent

choice can significantly change the dynamics of host-guest binding. Ideally, high affinity and selectivity complexes in water are desirable, as many of the applications to natural systems are more useful in water at neutral pH.

Solvent effects on hydrophobic binding interactions has been extensively studied by Diederich and co-workers.[25] A series of van't Hoff analyses on the inclusion complexes of several benzene guests with cyclophane **1.10** in water indicated the presence of an enthalpic driving force (range: -7.1 to -11.7 kcal/mol) accompanied by unfavorable entropy changes (range: -2.2 to -5.6 kcal/mol).[26] Similar studies in methanol indicated that the favorable enthalpy was decreased.

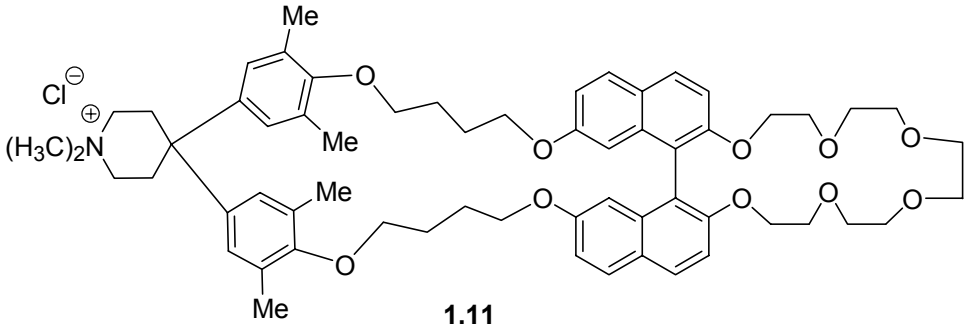


1.10

The authors propose that the geometry of the host-guest complex in water is similar to that in methanol, thereby indicating that the observed enthalpic differences are due to the solvent. The more exothermic ΔH° value in water is thought to arise from a combination of reduced water-water interactions and increased London dispersion interactions between the hydrophobic surfaces of the host and the guest. Subsequent studies on this host-guest association in twelve solvents, using isothermal titration calorimetry (ITC), confirmed that the binding affinities and the favorable enthalpy decreased as the solvent polarity decreased.[27] It was found that the Gibbs free energy of binding was similar in three of the solvents, but the differences were more apparent in the ΔH° values. One of the

interesting things to note about this work is that the data for binding in water is in contrast to the classic hydrophobic effect in which $\Delta H^\circ \sim 0$ and $T\Delta S^\circ$ is positive.

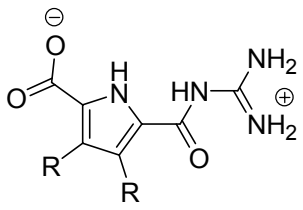
Diederich also demonstrated that cyclophane receptor **1.11** was effective at binding 2-cyano-6-methoxy-napthalene in a 60/40 water/methanol solvent



1.11

mixture (K_a = 4.5 x 10^3 M^{-1}).[28] The affinity decreased as the methanol content was increased. Conversely, the receptor was effective at binding potassium (K_a = 1.7 x 10^3 M^{-1}) in methanol, with decreasing affinity as more water was added. Remarkably, minor alterations in the solvent system can convert cyclophane **1.11** from being a receptor for aromatic guests to a potassium cation receptor.

Recent work by Schmuck demonstrates the shift in self assembly of a guanidiniocarbonyl pyrrole zwitterion resulting from solvent effects. Compound **1.12** was estimated to form dimers in d_6-DMSO with an affinity greater than 10^{12} M^{-1}.[29] The more water soluable analogue (**1.13**) was found to dimerize (170 M^{-1})



1.12 R = H
1.13 R = OTEG

TEG = triethyleneglycol

in 2.5% DMSO in water.[30] The use of water leads to more competitive solvation of the binding sites, therefore weakening the hydrogen bonds that facilitate dimerization.

The properties of various solvents can have marked effects on the binding propensities of host-guest complexes promoted by hydrophobic or electrostatic interactions. The role of the solvent does indeed add another consideration to the design of effective host-guest complexes.

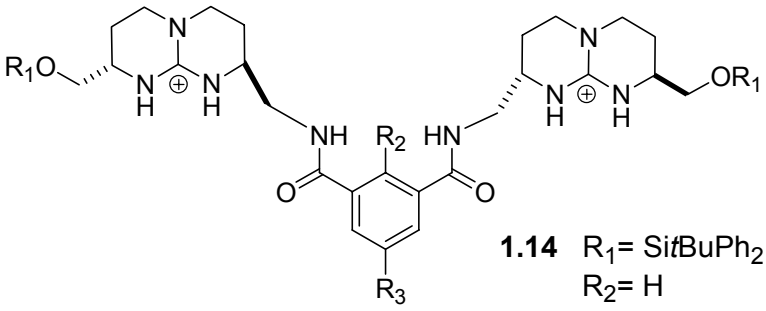
1.2 THERMODYNAMICS

The formation of a host-guest complex is a dynamic process that is not restricted to just the host and the guest, but rather the host, the guest, the solvent, and the counterions. Complex formation between a host and a guest with displacement of counterions and changes in the solvation shells are analogous to a reaction in which bonds are broken and formed. Just as a reaction has associated thermodynamic parameters, so too does complex formation between two entities in solution. A more comprehensive understanding of a binding system can be sought through quantification of the thermodynamic parameters such as the enthalpy changes and entropy changes of binding. Direct heat measurement of a binding event using isothermal titration calorimetry (ITC) permits such parameters to be quantified and has been shown to be amenable to host-guest chemistry. As alluded to in the introduction of this chapter thermodynamic investigations of synthetic receptor binding have provided insights into the fundamental energetics of molecular associations.

1.2.1 The Energetics of Binding

Calorimetric investigations by both Schmitdchen and Hamilton serve to highlight the power of using ΔH° and ΔS° values to decipher the roles of various

participants in a host-guest system. Schmidtchen reports the study of the binding of sulfate to ditopic host **1.14** in methanol. Binding of the guest to the host is on the order of 10^6 M^{-1}, with an unfavorable enthalpy change (+7.71 kcal/mol) and a highly favorable entropy change (+17.18 kcal/mol). Association of sulfate with analogous host **1.15** is also endothermic (+7.28 kcal/mol) and proceeds with a favorable entropy change (+15.10 kcal/mol), albeit to a lesser extent. The authors



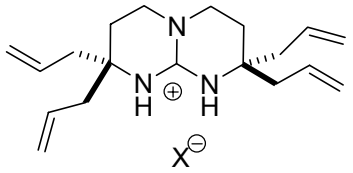
1.14 R_1= Si*t*BuPh$_2$, R_2= H, R_3=OBn

1.15 R_1= H, R_2= F, R_3=Br

propose that the guanidinium groups on the host and the charged sulfate guest are well solvated in methanol, so that upon binding the solvent reorganization is endothermic. Additionally, the host-guest complex is not as well solvated as the individual components, thereby accounting for increased entropy change as solvent is released into solution. The hydroxyl group of **1.15** leads to better solvation of the complex and this is reflected in the smaller entropy value determined by ITC experiments.

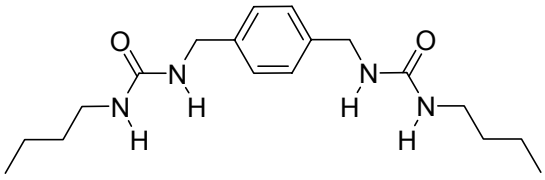
The counterion to a charged host molecule often influences the binding ability of the host to a guest. Schmidtchen recently reported a thermodynamic investigation to probe the effect of the counterion (Cl^-, Br^-, I^-, BF_3^-, PF_6^-) to bicyclic guanidinium **1.16** on the binding of tetraethylammonium benzoate in acetonitrile.[31] The data indicate that a strongly bound chloride anion has the

lowest exothermic (-2.93 kcal/mol) value upon exchange for benzoate. In contrast, the exchange of weakly bound hexafluorophosphate counterion for benzoate is more exothermic (-5.21 kcal/mol). Survey of the ΔG° values (-6.35 *vs* -7.73 kcal/mol) reveal a more subtle difference. In all cases the $T\Delta S^\circ$ term was positive, reflecting the release of solvent and/counter ions solvating the binding sites.



1.16

Binding that proceeds through the formation of multiple hydrogen bonds between functional groups on the host and the guest can be influenced by the solvent. While this is reflected in the binding affinities, the enthalpic and entropic contributions offer a more complete understanding of the bonding modes. This is exemplified in work by Hamilton[32] on the binding of di-carboxylates to a series of bis-functional hydrogen bonding receptors. Receptor **1.17** bound glutarate with



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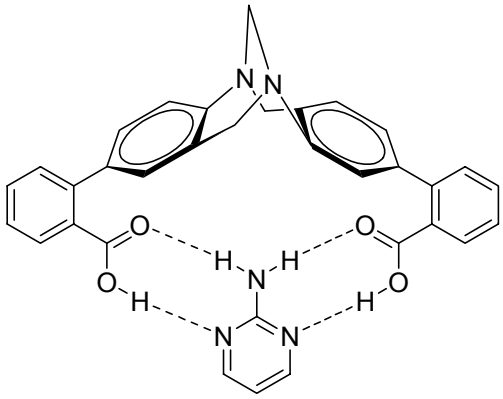
an affinity of 1.3 x 10^3 M^{-1} in DMSO, a ΔH° value of -2.5 kcal/mol and a ΔS° value of +5.9 cal/molK. Upon moving to a more competitive solvent such as methanol, the binding of glutarate to a similar host was 2.7 x 10^3 M^{-1} with a ΔH° of +3.7 kcal/mol and a ΔS° of 28 cal/molK. Although the values are not directly comparable, the authors postulate that competitive solvation of the host and the

guest in methanol results in endothermic enthalpy changes, and the complexation is driven by entropic factors.

Wilcox probes the effect of water on a hydrogen-bonded host-guest complex (**1.18**) using a thermodynamic analysis.[33] The complex forms in dry deuterated chloroform with ΔH° = -14.3 kcal/mol and ΔS° = -30 cal/mol deg. However, in wet deuterated chloroform the values become -9.3 kcal/mol and -16 cal/mol deg for the enthalpy and entropy changes respectively. Complex formation in the dry solvent results in stronger H-bonds as reflected in the greater enthalpy change relative to the wet solvent system. Though the entropy change is unfavorable in both cases, it is more favorable in water, due to the release of waters of solvation upon binding.



1.18

The investigations discussed above serve to exemplify the utility of thermodynamic parameters to identify differences or trends in host-guest binding that would otherwise appear rather subtle if the binding strength alone was used as the only criteria.

1.3 HOST DESIGN

The selective associations between molecules found within natural systems provide the inspiration for the rational design of synthetic hosts. Just as the molecular complexes in nature are 'exact' fits as a result of molecular evolution, the design of a synthetic host is fundamental to the function of the host in binding the intended guest. The implication here is that the guest often dictates the size, shape, and charge of the binding cavity. The first set of guidelines, of which there are eighteen, used in the design of a synthetic receptors originate in a review article by Cram.[34] Through the decades receptor designs have become more elaborate, yet molecular recognition chemists still apply some of the more fundamental design principles to their works.

1.3.1 Design Principles

In using synthetic receptors for the purpose of binding guests in solution it is desirable to maximize the number and the strengths of non-bonded interactions between the host and the guest. In molecular recognition we attempt to achieve this by incorporating the notions of the 'lock and key' design and preorganization into the receptor design. The 'lock and key' design approach is an adaptation of 'lock and key' model of enzyme-substrate binding proposed by Emil Fischer in 1894.[35] Here, the host (lock) is engineered to match the guest (key) such that the binding cavity of the host compliments the guest in terms of size, shape, and charge.

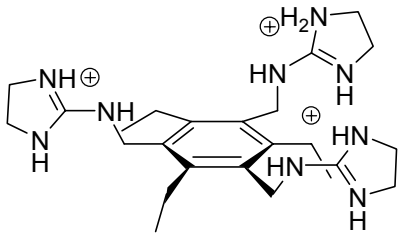
In the idealized host design the matched size, shape, and pairwise interactions of the host and guest should lead to a tight contact pair. Energetically, the unfavorable entropy change that may arise from conformational changes in the host as the guest binds can be minimized by incorporating the concept of preorganization.[34, 36-39] This design feature involves the use of a rigid

molecular scaffold which serves to lock the positions of the functional groups into a conformation and orientation suitable to guest binding. The incorporation of functional groups for the purpose of forming non-bonded interactions with the guest is often used to create an enthalpic advantage to the binding process.

The preliminary host design is often modeled with the aid of space filling molecular models to approximate a first guess at the desired cavity. In some cases this is subjected to molecular mechanics calculations using programs such as MacSpartan and Macromodel to simulate the guest bound to the host cavity.

Numerous molecular scaffolds have been used for molecular recognition purposes. One such scaffold used in the Anslyn group is the 1,3,5-substituted-2,4,6-triethylbenzene (**1.19)**. The alternating ethyl groups impart a steric bias of the functional groups to one face of the benzene ring, positioning them to participate in guest binding with little conformational change. The placement of the binding functionalities rendered the host selective for citrate binding in D_2O with an affinity of 6.9×10^3 M^{-1}.[40] A crystal structure of **1.19** with tricarballate bound to the cavity was reported, and verified the orientation of the guanidinium groups to one face of the plane. To further verify the effectiveness of pre-organization, the binding of citrate to a host lacking the ethyl groups resulted in a reduced affinity ($K_a = 2.4 \times 10^3$ M^{-1}).

Another motif derives from a cleft-type structure (**1.20**) in which a metal center is used to preorganize the binding groups as shown. This host was used in conjuction with a dye displacement assay for the detection of amino acids. Aspartate was found to have the highest affinity ($K_a = 1.5 \times 10^5$ M^{-1}) in a 1:1 methanol/water solvent system.[41] A crystal structure of the ligand without the Zn(II) present shows the appendant rings rotated away from the central ring, indicating that the Zn(II) does preorganize the cavity for binding guests.



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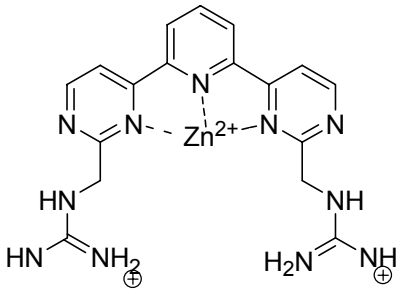


1.20

Other common scaffolds include a steroid backbone[42, 43], bicyclic guanidiniums[44-47], cages[48], and tweezers[49]. These strategies in host design enable the design of effective synthetic receptors with the appropriate size and shape for specific targets.

1.3.2 Functional Group Incorporation

Though the proper shape and size are necessary to a successful host design, the choice in functional groups to serve as binding sites is also crucial. The placement of appropriate functional groups can guide the association of host and guest in bulk solution. The pairwise interactions of the binding sites of the host with those of the guest can be matched by choosing groups that are complimentary in charge and geometry, and have some known affinity. The focus of the work herein is anion binding through ion-pairing interactions in aqueous media. Of the many functional groups used to bind anions, metal centers, ammonium groups, and guanidinium groups are highlighted below. Each of these is similar to the functional groups often found in the active sites of enzymes. Metal centers, such as zinc and copper, are present in enzyme active sites and can assist in the activation of the substrate and oxygen transport.[50-52] Ammonium groups are present in the side chain of the amino acid lysine, and the guanidinium group is present in the side chain of argenine.[53-55]

1.3.2.0 Ammonium Groups

Ammonium groups were first used in a synthetic receptor reported by Park and co-workers[56] to bind halide anions. These functional groups continue to be prevalent in the molecular recognition literature as binding sites for anions.[57-60] The ability of this group to maintain a charge at pH 7.4 makes it desirable for binding in aqueous media. The localization of the charge on the nitrogen center lends to the high charge density of the functional group, thereby making it an effective cation point charge for ion-pairing or hydrogen bonding. This can also serve as an effective hydrogen bond donor for interactions with anions or lone pair electrons.

1.3.2.1 The Guanidinium Group

The guanidinium group is often used as a binding site in synthetic receptors for anion binding through ion-pairing and hydrogen bonding interations.[61] The guanidinium group is amenable to binding applications due to its ability to maintain a charge over a wide pH range (pH 7 -13). Unlike the ammonium group, the charge on the guanidinium is delocalized across three nitrogen atoms which renders it an effective hydrogen bond donor. This group can participate in multiple hydrogen bonded interactions. The geometry of the guanidinium group imparts directionality to the hydrogen bonds with an anionic guest, resulting in three different hydrogen bonding motifs (Figure 1.3). The versatility of this functional group is exemplified by its incorporation into numerous synthetic receptors.[62, 63] This functional group has been used as a free

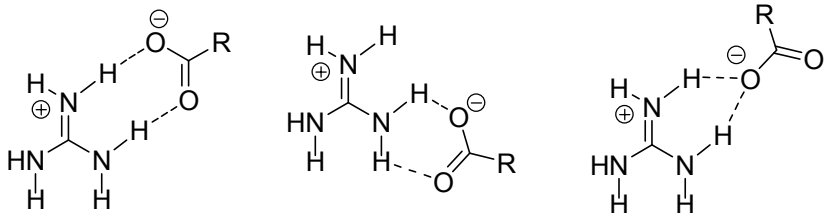
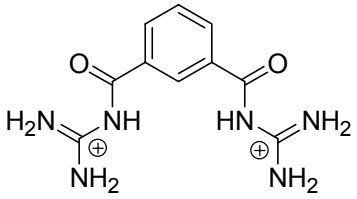


Figure 1.3 Hydrogen Bonding Motifs of a Guanidinium Group. The guanidinium groups can participate in bidentate hydrogen bonded complexes with anions such as carboxylates. The bonding motifs shown imply specific orientations and directionlalities of the hydrogen bonds.

guanidinium as in receptor **1.21**,[64] or embedded in an imidazoline as in receptor **1.22**,[65] or as a part of a bicyclic structural motif as in receptor **1.23**.[66] Both the imidazoline and bicyclic structures can participate in one interaction with a guest, where as the free guanidinium group has the potential to interact with more than one guest.



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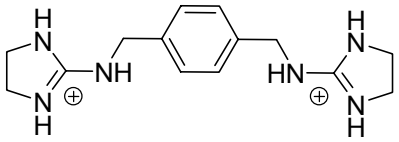

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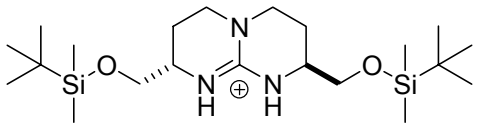

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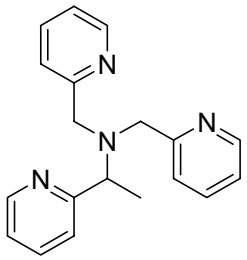
1.322 Metal Centers

Metal centers such as zinc and copper are particularly versatile as binding sites because of their strong coordination abilities with oxy-anions such as carboxylate and phosphate groups. Such strong binding interactions result from stabilized ligand field effects as a consequence of the d^9 electronic configuration of the metal center. The incorporation of metal centers as binding sites in synthetic hosts is commonly facilitated through coordination of the metal to a nitrogen rich host cavity. This anchors the metal and makes it available for binding. Recent work by Fabbrizzi[13, 67], Canary[68], and Kim[69] include metallo-receptors in which the metal centers serve as binding sites. In some instances the metal center serves both as a binding site and a signal transduction entity. As the properties of the metal are altered upon binding a guest, its colorimetric or fluorescent signals change. Receptor **1.24** complexes to the lanthanide metal



1.24

europium(II) in acetonitirle. Binding of anions to the metal center was observed through an enhancement of the lanthanide luminescence.[70]

1.4 BINDING STUDIES

The ability of a host to associate with a guest is commonly evaluated through the determination of a binding constant (K_a) and the binding stoichiometry (n). The analytical techniques that are often used in molecular

recognition to determine a binding affinity are absorption spectroscopy, nuclear magnetic resonance spectroscopy, and potentiometry. Also used, but less frequently, are solubility measurements, liquid-liquid partitioning, chromatography, and dialysis.[71] The utility of thermal methods in the study of host-guest binding has recently become more prevalent in the literature. Each of the techniques above permit the monitoring of an experimental observable as aliquots of a guest solution are added to a solution of the host. The data obtained from such changes in the observable, as the host and guest associate, can be used to generate a binding isotherm which can then be fit with a curve, from which K_a and n may be determined.[71-73]

Nuclear magnetic resonance (NMR) can be used to monitor the proton (and in some instances the phosphorous) signal of the host or the guest or the host-guest complex. One can follow the change in the chemical shift or the change in the integral of one peak as it disappears to form a new peak. These changes in the shift or the integral can be plotted versus the mole ratio of host to guest to produce a mole ratio plot. This raw data may then be fit with a curve generated from the binding equation to arrive at a K_a value, and the binding stoichiometry. In a very similar fashion UV/Vis or fluorescence spectroscopy data can be used to monitor a change in the absorbance or emission of the host, or the guest as it participates in the host-guest complex. Thermal methods can also be used to measure the heat absorbed or released upon host-guest complex formation. The heat change can be used to generate a mole ratio plot for curve fitting.

The equations used to fit the binding isotherm from the raw data are derived for the experimental observable unique to the technique. ^{1}H NMR, UV/Vis spectroscopy, and ITC have been used in the research discussed in the following chapters. In accord with this, the UV/Vis and ITC techniques are detailed below.

1.4.1 Binding Equations for UV/Vis

The binding equations that describe the binding of host and guest in a 1:1 stoichiometry detailed below through the application of the algorithms to UV/Vis data for the binding of molecule *M* to molecule *X* given by the equilibrium equation below:

$$M + X \leftrightarrow MX \qquad (1.1)$$

In a UV/Vis spectroscopy the absorbance of a solution of *M* behaves according to Beer's Law in which *A* is the absorbance, ε is the molar absoprtivity, and *b* is the path length of the light beam:

$$A_0 = \varepsilon b[M] \qquad (1.2)$$

Upon the addition of *X* to a solution of *M*, the properties of the solution change such that all components contribute to the overall absorbance as shown below:

$$A_0 = \varepsilon_X b[X] + \varepsilon_M b[M] + \varepsilon_{MX} b[MX] \qquad (1.3)$$

While this mathematically relates the concentrations of the species in solution to the experimental observable *A*, the equilibrium equation and mass balance equations need to be included. This establishes mathematical expressions for the concentration of each species in solution as they change with each addition of *X*. The subscript *i* is used to denote an unbound species and *t* denotes total.

$$K_a = \frac{[MX]}{[M_i][X_i]} \qquad (1.4)$$

$$X_t = [X_i] + [MX] \qquad (1.5)$$

$$M_t = [M_i] + [MX] \qquad (1.6)$$

Substitution of the mass balance relationships (1.5 and 1.6) into the equation 1.4 leads to an expression (see Equation 1.16) that can be substituted into equation

1.3. Once substituted the absorbance term for the guest ($\varepsilon_X b[X]$) is removed for simplification, because the guest does not have an absorbance at the particular wavelength being observed. Additionally, the [M_t] term is removed from the equation because this concentration remains constant throughout the titration. This then simplifies to the binding equation shown below:

$$\frac{\Delta A}{b} = \frac{\Delta \varepsilon K_a [M_i][X_t]}{1 + K_a [M_i]} \qquad (1.7)$$

The total concentration of X can be related to the concentration of M_i at a given aliquot addition, resulting in a quadratic equation. This can be solved for M_i, and substituted into equation 1.7. At this point the parameters K and $\Delta\varepsilon$ can be varied to produce a best fit curve to the raw data (Figure 1.4) which is plotted as the delta absorbance (ΔA) versus the concentration of the added component (X_i).

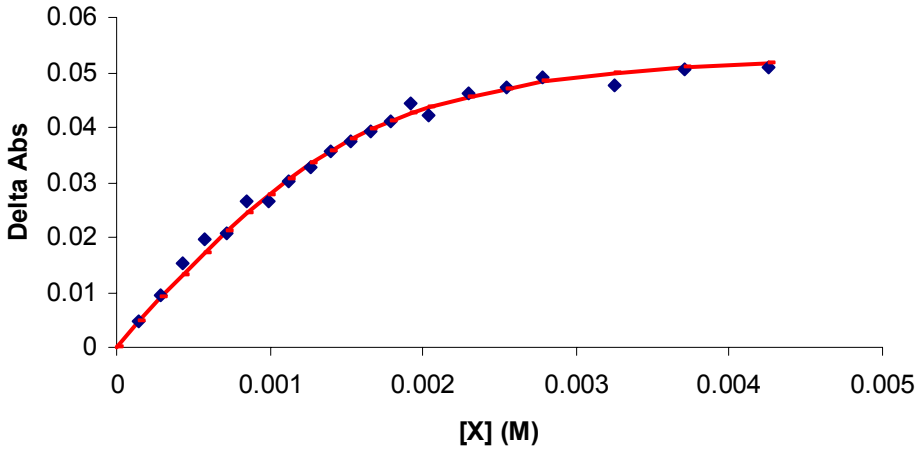


Figure 1.4 UV/Vis Binding Isotherm. This shows a typical binding isotherm derived from changes in the absobance of a host molecule as aliquots of guest X are added.

1.4.2Thermal Methods

The associations of molecules in solution as described above focus primarily on the binding interactions and the strengths of the interactions. Less often determined by experiment are the thermodynamic parameters that characterize the binding event. Such parameters are the Gibb's free energy of binding (ΔG°), the enthalpy of binding (ΔH°), and the entropy of binding (ΔS°). This aspect of host-guest chemistry is intriguing, as it treats the binding event as a dynamic process in which the solvent and the counterions are also involved. Molecular recognition chemists often use a van't Hoff analysis to determine the ΔH° and ΔS° values for the binding event. This requires the determination of the binding affinity at several different temperatures (necessitates multiple titrations) to produce a van't Hoff plot of ln$K$ vs $1/T$, from which the ΔH° and ΔS° values can be extracted. Interest in the thermodynamics of binding has grown in the last ten years, and new advancements in technology permits easier data acquisition.

The most recent developments in monitoring the formation of a host-guest complex involve isothermal titration calorimetry (ITC). This technique[74-76] has been primarily used to probe the thermodynamic profile of binding events such as enzyme-substrate, protein-protein, or antigen-antibody interactions. Only within the last five years has this technique been applied to molecular recognition studies. The study of the thermodynamics of host-guest chemistry in solution constitutes a substantial portion of this work, and therefore warrants some background on the instrumentation, the technique, and the algorithms that are commonly used.

The technique is unique in that not only can the K_a values be obtained, but thermodynamic parameters such as enthalpy changes, entropy changes and heat capacity changes can be obtained from a single titration. The experimental observable that is monitored is the heat absorbed or released upon complex formation. This is comparable to the van't Hoff approach, though it is more

precise since the heat quantity is directly measured, and it can be accomplished through performing only one titration. Binding constants on the order of 10^9 can be measured with this technique, which is more sensitive than other comparable spectroscopic methods. An isothermal titration calorimeter can respond to heat absorbed or released for multiple equilibrium processes or solvation/desolvation processes. This needs to be considered in the data collection and analyses.

1.4.2.1 The Instrument

The instrument consists of two cells, a reaction cell and a reference cell, both of which reside within an adiabatic jacket (Figure 1.5). The temperature difference between the cells is monitored and calibrated by power units. Access to both cells for filling and emptying is made possible through the stems. The syringe containing the sample to be injected to the reaction cell sits above the apparatus, and is fitted with a plunger, stirring mechanism, and sensors. The interfaced software uses the positions of the sensors to accurately measure the volume of each injection to the cell.

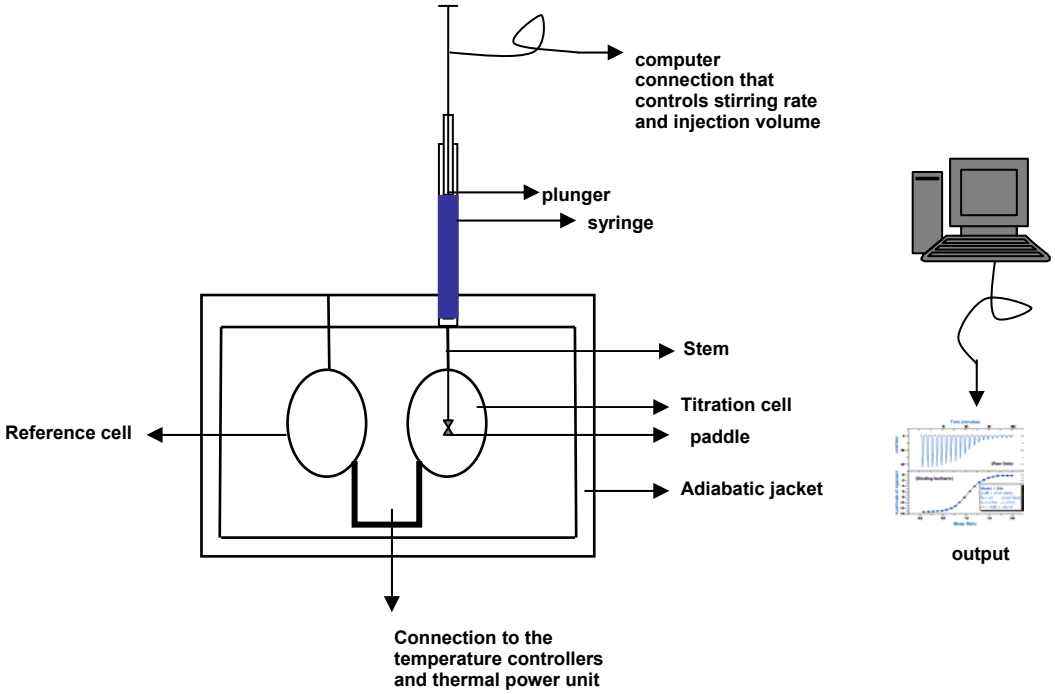


Figure 1.5 Microcalorimeter. A schematic representation of the microcalorimeter. The titration and reference cells are encased in an adiabatic jacket. The syringe is housed in a casing that is connected to the computer and the software controls the stirring rate and injection size. The temperature difference between the cells is monitored and adjusted through a thermal power unit which is monitored by the computer software. A sample output is depicted.

A typical isothermal titration involves filling the reference cell with a solution of the solvent, the reaction cell with a solution of the host, and the syringe with a solution of the guest. Small aliquots of the syringe contents are then introduced to the reaction cell. As the host-guest complex forms the absorbance or the release of heat associated with the binding alters the temperature of the reaction cell. The temperature difference between the cells is recorded by the computer, and the instrument compensates for this temperature difference through electrical input (thermal power). The thermal power necessary to re-establish a temperature difference of zero ($\Delta T = 0$) between the cells over time provides a direct measurement of the heat of binding for that injection. If the

binding is exothermic, negative peaks are recorded because the instrument does not need to supply heat to establish ΔT =0. For endothermic binding, positive peaks are recorded since the instrument has to supply heat to establish $\Delta T = 0$. At the end of the titration the same experiment is repeated with just the solvent in the titration cell. The solvation heats measured are used to correct the binding data prior to data analysis.

A series of similar injections provides a raw data plot of energy *vs* time. The software then performs integrations on each of the raw data peaks to yield a plot of $\Delta H°$ *vs* mole ratio of guest to host. The application of a binding algorithm using Origin 5.0 software provided by Microcal[77] proceeds with an iterative least squares analysis on the data using the binding isotherm equation to produce a curve fit. The curve fit is dependent on three parameters, namely the binding affinity (K_a), the binding stoichiometry (n), the enthalpy change of binding ($\Delta H°$). The values for these parameters derived from the curve fit can then be used to extrapolate the values for $\Delta G°$ and $\Delta S°$ (Figure 1.6).

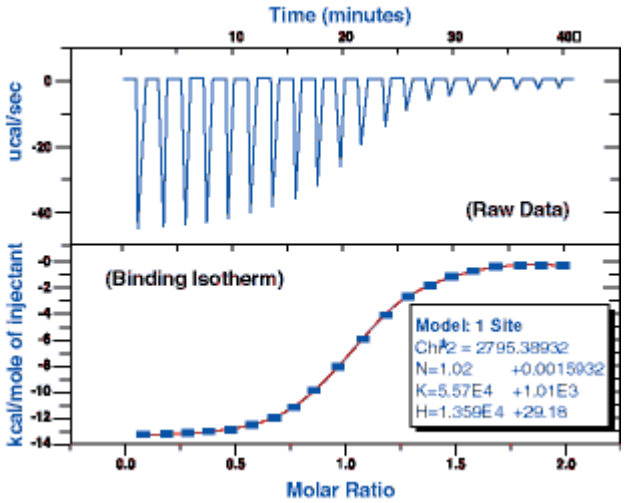


Figure 1.6 Sample ITC Data.[78] The upper graph shows an example of the endothermic binding of two molecules. Each 'spike' represents one injection from the syringe. The lower graph depicts the binding isotherm derived form

differentiation of the raw data. The solid line in the resulting curve fit from the data analysis.

1.4.2.2 The Derivation of the Binding Equation

To provide a more intricate understanding of the data analysis a discussion of the derivation of the binding algorithm is presented. Recall that the thermal power used to reestablish the temperature of the reaction cell to that of the reference cell following an injection provides a direct measurement of the heat of the reaction. This in turn is directly proportional to the enthalpy of the binding. This relationship originates from the first principles of thermodynamics. The first law of thermodynamics defines energy (U) as a combination of heat (q) and work (w). In the transfer of energy the following equation holds:

$$dU = dq + dw \tag{1.8}$$

This can be integrated over time to yield the following equation:

$$\Delta U = q + w \tag{1.9}$$

Rearrangement of equation 1.8 leaves us with the relationship in which heat is equal to the energy minus the work term:

$$q = \Delta U - w \tag{1.10}$$

The work term is defined as $\int_{v1}^{v2} -PdV$, so by substituting into the above equation, and integrating, the heat term becomes a summation of the change in energy and the change in volume multiplied by pressure:

$$q = \Delta U + P\int_{v1}^{v2} dV = \Delta U + P\Delta V \tag{1.11}$$

This is redefined as a state function for enthalpy H:

$$H = U + PV \tag{1.12}$$

By substituting the energy term from equation 1.11 we arrive at an expression for enthalpy:

$$\Delta H = \Delta U + P\Delta V \tag{1.13}$$

$$\Delta H = q - P\Delta V + P\Delta V \tag{1.14}$$

This simplifies such that the enthalpy is directly proportional to the heat of the reaction, which allows direct heat measurements using the calorimeter to be used to quantify the enthalpy of a binding interaction.

$$H = q \tag{1.15}$$

Having established the basis for the translation of heat to enthalpy we can proceed to relating the raw heat data with an equilibrium expression for 1:1 binding. The equations presented earlier show the binding equilibrium, the binding constant expression, and the mass balance relationships for a typical 1:1 binding of *M* and *X*. As aliquots of *X* are added to *M*, the complex *MX* is formed. An expression for both M_i and X_i can be derived from equations 1.5 and 1.6. These can then be substituted into equation 1.4 to arrive at an equation of the following form:

$$[MX] = K_a\left([MX]^2 + M_t X_t - M_t[MX] - X_t[MX]\right) \tag{1.16}$$

Grouping of the like terms and setting the resulting equation equal to zero yields an equation of the form:

$$[MX]^2 + M_t X_t - [MX]\left(M_t + X_t + \frac{1}{K_a}\right) = 0 \tag{1.17}$$

This is a quadratic equation for which the roots can be determined by application of the generic equation:

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \tag{1.18}$$

where $a = 1$, $b = \left(X_t + M_t + \frac{1}{K_a} \right)$, and $c = M_t X_t$. This yields a solution having only one root. This expression defines the concentration of the [*MX*] complex as a function of the concentrations of M_t and X_t, both of which are known at the start of the experiment.

$$[MX] = \frac{\left(X_t + M_t + \frac{1}{K_a} \right) - \sqrt{X_t^2 + M_t^2 + \frac{1}{K_a^2} - 4M_t X_t}}{2} \qquad (1.19)$$

The concentration of [*MX*] does not remain constant, but changes throughout the titration, therefore a more appropriate expression is one in which the change in [*MX*] is monitored with respect to X_t. This can be derived from differentiation of equation 1.19:

$$\frac{d(MX)}{dX_t} = \frac{1}{2} + \frac{1 - \frac{\left(1 + \frac{1}{K_a M_t} \right)}{2} - \frac{\left(\frac{X_t}{M_t} \right)}{2}}{\sqrt{\left(\frac{X_t}{M_T} \right)^2 - 2\frac{X_t}{M_t}\left(1 - \frac{1}{K_a M_t} \right) + \left(1 + \frac{1}{K_a M_t} \right)^2}} \qquad (1.20)$$

For every mole of *MX* that is formed there is an associated ΔH° value, therefore the change in heat can be related to a change in [*MX*] for each aliquot of *X* added. Thus, the observed change in heat is proportional to the enthalpy multiplied by the change in [*MX*] multiplied by the volume:

$$dQ = d(MX) \bullet \Delta H^\circ \bullet V^\circ \qquad (1.21)$$

The inclusion of a volume term relates the change in the heat to the change in the number of moles of *MX* formed. The expression for the change in *MX* can be substituted into equation 1.21 to yield the following expression:

$$\frac{1}{V^\circ}\frac{dQ}{dX_t} = \Delta H^\circ \left(\frac{1}{2} + \frac{1 - \frac{\left(1 + \frac{1}{K_a M_t}\right)}{2} - \frac{\left(\frac{X_t}{M_t}\right)}{2}}{\sqrt{\left(\frac{X_t}{M_T}\right)^2 - 2\left(\frac{X_t}{M_t}\right)\left(1 - \frac{1}{K_a M_t}\right) + \left(1 + \frac{1}{K_a M_t}\right)^2}} \right) \qquad (1.22)$$

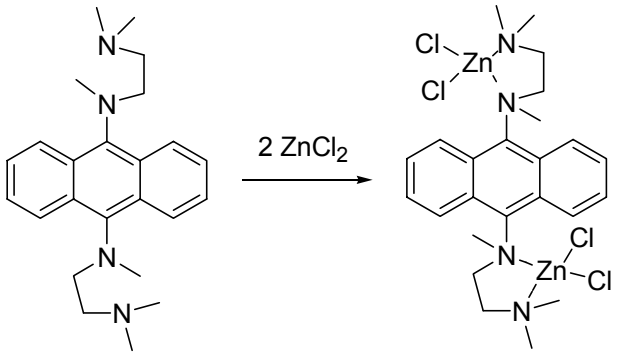
This represents the experimental observable, the differential heat, recorded by the calorimeter. This parameter is a function of M_t as it relates to X_t and K. The Origin software uses this equation in a linear least squares analysis to fit a curve to the binding isotherm. The area under the curve defined by this equation represents the enthalpy of the binding event, and can be quantified by integration of the binding isotherm. This curve fitting analysis results in values for K_a, n, and ΔH°, from which ΔG° and ΔS° values can be determined.

The Origin software contains additional algorithms for 2:1 binding and for molecules containing multiple binding domains (used for biological applications). The versatility of the technique and the software has and will continue to have a substantial impact on thermodynamic studies within the arena molecular recognition.

1.5 SENSING

In some cases a receptor having a reasonable affinity and high selectivity for a guest can be used in a sensing application to quantify a target guest in competitive media.[79] The use of a receptor as a sensor is termed a chemosensor. As defined by Czarnik[80], a chemosensor is comprised of a binding site and a signaling element such that when a guest binds (reversibly) to the binding site, there is 'communication' with the signaling element which results in a

colorimetric or fluorescent signal change. The mechanisms by which this signal may be transferred are numerous, including charge transfer excited states, photoinduced electron transfer, and electronic energy transfer.[81] Through this signal transduction mechanism a sensor reports the presence of the analyte. One such example reported by Czarnik was anthracene based receptor **1.25**, which upon binding 2 equivalents of Zn(II) resulted in enhancement of the fluorescence **1.25**.[82] The lone pairs on the N-centers quench the fluorescence of anthracene, but upon binding the metal through the nitrogen lone pairs the fluorescence is re-established. In essence receptor **1.25** is a chemosensor for Zn(II). This concept has had great utility in the field of molecular sensing.



1.25

1.5.1 Development of a Chemosensor

The transformation of a host to a chemosensor often entails the introduction of a chromophore or a fluorophore. The introduction of such a moiety through covalent bonds offers one solution to appending an optically active subunit. This is advantageous, for any spectroscopic modulation is directly correlated to the interaction with the guest. However, the disadvantage lies in the introduction of additional steps to the synthetic route of the host molecule. Additionally, the appended indicator often occupies a position on the host scaffold

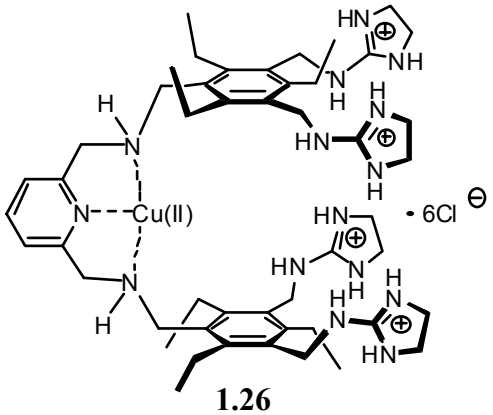
that could be used for the placement of a binding site. The implementation of a dye-displacement[83] assay can circumvent the additional synthetic steps and the potential loss in the number binding sites on the host.

A dye-displacement assay employs an indicator having similar functional groups to that of the guest to ensure some affinity with the host molecule. While free in solution the indicator has a signature absorbance or fluorescence. Upon introduction to the host, there are some binding interactions with the indicator that result in a change in the microenvironment of the indicator. This in turn modulates the properties of the indicator, often yielding a spectroscopic change. If the indicator and host are present in solution in a 1:1 ratio, the introduction of the guest, which has a higher affinity for the host, will displace the indicator into solution, thereby restoring the original color of the indicator free in solution.

1.5.2 Applications of Chemosensors

The application of dye-displacement assays to host–guest chemistry using fluorescent indicators was first introduced by Inouye[84] and Shinkai.[85] The Anslyn group has extended this to the use of dyes responsive to pH changes to produce a colorimetric response that can be monitored by UV/Vis spectroscopy. This method has been successfully used in our labs for the quantification of citrate[86] in juice, tartrate[87] in wines, gallic acid[88] in scotch whiskies. It has also been used by Fabbrizzi in the quantification of carbonate[67] and pyrophosphate detection.[13]

A receptor (**1.26**) recently developed in our labs was used in a dye displacement assay as a chemosensor for 2,3-bisphosphoglycerate (2,3-BPG) in a 1:1 MeOH/H_2O solvent system buffered at pH 7.4.[89] The binding of **1.24** to the


HN
NH
N
H
H
N
HN
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N
Cu(II)
N
HN
HN
N
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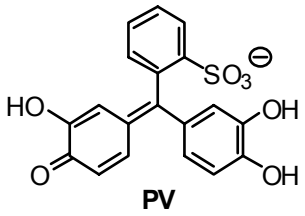
H
N
H
N
HN
• 6Cl

1.26


SO3
HO
OH
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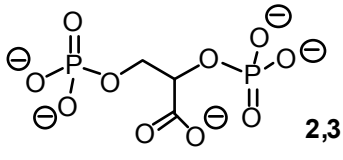
OH

PV


O
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2,3-BPG

dye, pyrocatechol violet (PV), resulted in a color change from yellow to blue. The change was followed by UV/Vis spectroscopy, showing a decrease in absorbance at 445nm with concomitant increase in absorbance at 590nm (Figure 1.7). Upon addition of the guest, 2,3-bisphosphoglycerate, the dye was displaced yielding a color change from blue to yellow. These data were used to determine a binding constant of 8 x 10^8 M^{-1} for BPG to the host. This assay has potential application in the determination of 2,3-BPG concentration in blood, as it is regulates the oxygenation level of hemoglobin.

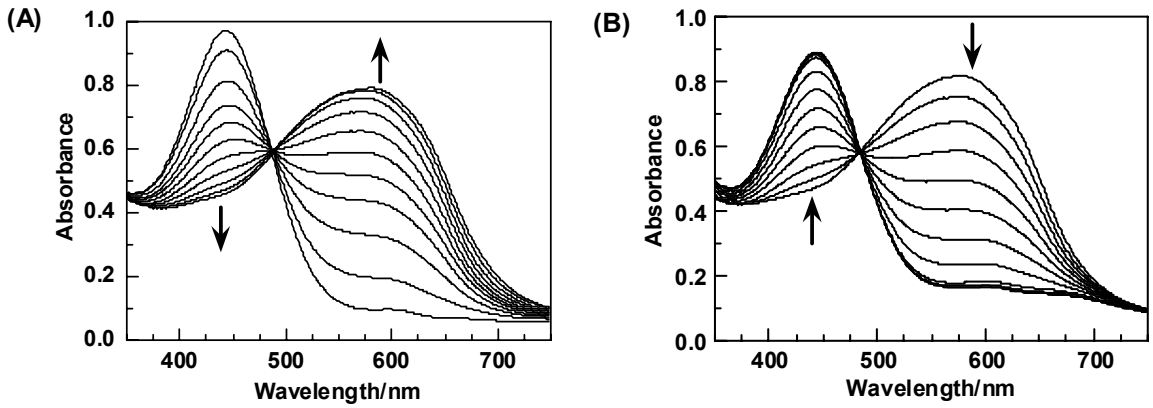

Figure 1.7 UV/Vis Spectra for a Dye-Disaplcement Assay. (A) The spectral response for the binding of the dye to the host with a decrease at 445 nm and an increase at 590nm. (B) The spectral response of 2,3-BPG displacing the dye from the host, showing a reversal in the absorbances at 445 nm and 590 nm.

1.7 SUMMARY AND OUTLOOK

The following chapters describe three research projects that have been completed for this thesis. Though different in their own right, these projects have a unifying goal, this being to investigate the thermodynamics of ion-pairing host-guest binding in water. Chapter 2 details the development of two metallo-hosts having high selectivity and affinity for phosphate in water. The energetics of the

binding assembly are described, as well as the incorporation of the receptor into a chemosensing ensemble for the quantification of phosphate in serum and saliva. Chapter 3 explores the thermodynamic origins of cooperative binding in the binding of poly-carboxylate guests to a metallo-receptor. Chapter 4 focuses on full characterization of an ion-pairing host-guest pair in water and the discovery of the formation of higher order complexes with a favorable entropic change.

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Chapter 2: Metalloreceptors for Binding Phosphate: Selectivity, Energetics, and Application

2.0 ANION BINDING

The first example involving the use of synthetic receptors for the binding of guests was reported in 1967 by Pederson.[1] In this work a crown ether was used to bind cations through interaction of the lone pairs on the oxygen centers with the charged cation. Subsequent work by Lehn, and Cram on cavitands and cryptands prompted the development of cation receptors.[2; 3] A year later Simmons reported one of the first anion receptors in which ammonium groups were used to bind halides.[4]

The challenge in designing a suitable synthetic receptor for anions derives from their variety in shape, size, and charge.[5] Unlike their cation counterparts that are primarily spherical with a localized charge density, anions are generally larger having smaller charge to radius ratios. This makes electrostatic interactions less effective for anion recognition. Additionally, anions have a variety of three dimensional shapes, namely spherical, linear, triganol planar, tetrahedral, octahedral, and amorphous (Figure 2.0). The variety of shapes inherently leads to charge localization as in fluoride, or charge delocalization as in the case of phosphate.

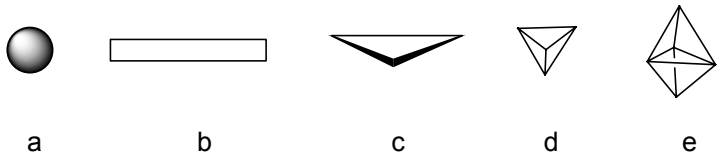


Figure 2.0 Shapes of Anions. This represents the various shapes of typical anions. a) spherical, b) linear, c) triganol planar, d) tetrahedral, e) octahedral.

Anions also have the ability to exist in multiply charged states depending upon the medium and the pH. These inherent differences between cations and anions often result in elaborate receptor designs for anion binding.

The goals of the work described here were 1) to design a metallo-receptor for binding phosphate in water at biological pH, 2) to explore the thermodynamic parameters of the binding event, and 3) to develop a sensing ensemble for phosphate in serum and saliva.

2.1 PHOSPHATE AS AN ANALYTE

Phosphate is ubiquitous in nature. Inorganic phosphate is present in blood, saliva, and urine. Although phosphate is naturally abundant in the body, the levels can be altered as it is a product of the hydrolysis of phosphodiester linkages present in biologically relevant molecules such as RNA, DNA, ATP, GTP, AMP. The consumption of foods and beverages throughout the day also serve as a source of phosphate for the body. Though it is desirable to quantify phosphate in the body, its quantification in waste water and beverages can have applications in waste management and quality control. Phosphate is notably one of the more challenging targets for molecular recognition, but is also one of the most important.

The tetrahedral shape of the anion dictates the necessity for a tetrahedral host cavity to effectively bind the anion. This anion can also exist in a mono-, di-,

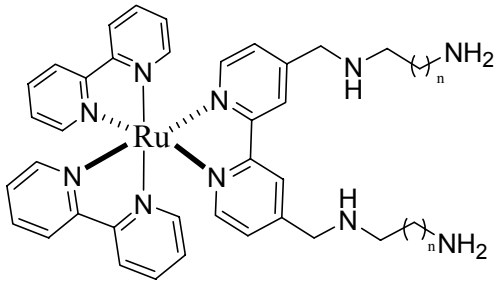
or tri- anionic state as a result of having three acidic protons with pH values of 2.15, 7.20, and 12.38. In aqueous media at pH 7.4 the predominant anion is hydrogen phosphate (HPO_4^{2-}). Since a phosphate receptor would have greater utility in aqueous media at pH 7.4, it would have to be highly charged and the cavity matched to a tetrahedral anion.

The application oriented goal of this research necessitated that the phosphate receptor be functional in competitive media. Ideally the receptor should be selective for phosphate over other tetrahedral anions such as sulfate, arsenate, and pertechnetate, as well as anions of other charges, sizes, and shapes. Therefore, an effective synthetic receptor design for tetrahedral oxy-anions like phosphate can lead to potential chemosensors or extractants for clinical or environmental applications.

2.2 PREVIOUS WORKS

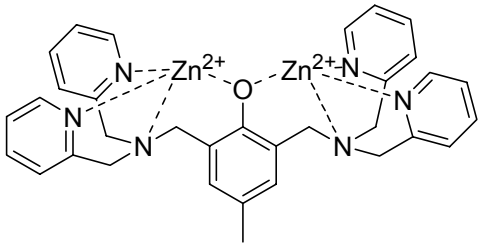
Several research groups have tackled the problem of designing receptors for inorganic phosphate. The approaches vary in the type of host cavity employed, which includes macrocycles, clefts, calixarenes, tripodal, and metallo receptors.[6-14] In addition to the use of various cavities, a variety of different functional groups were used to effect phosphate binding. These groups include amines, ureas, thioureas, guanines, and metal centers. A few of these investigations are reviewed here.

Beer and co-workers report a ruthenium based poly-aza receptor (**2.1**) that has affinity for phosphate on the order of 10^5 M^{-1} at low pH in water.[15] The binding is observed through the change in the fluorescent signal of the ruthenium



2.1

center as the guest binds to the charged amine groups. This is effective for phosphate binding at low pH, but no selectivities were reported. Recently Kim[16] reported the use of a bis-zinc complex (**2.2**) that was selective for phosphate over other anions in water a pH 7.4. The phosphate binding was reported as 11.2 x 10^4 M^{-1} as determined by ITC.



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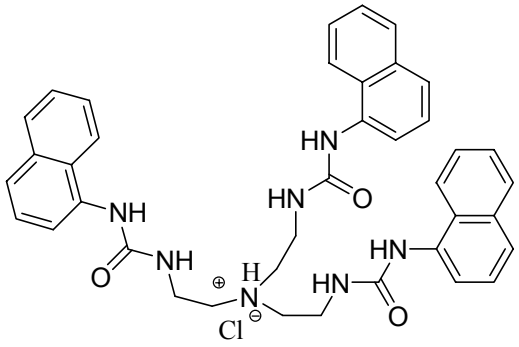
A tetrahedral arrangement of binding groups to compliment the shape of phosphate is a 'lock and key' design approach not present in the designs of **2.1** and **2.2**. Work by Xie incorporates a tripodal host design that provides a tetrahedral cavity upon binding.[17] The binding of hydrogen phosphate to host **2.3**



2.3

in *N,N'*-dimethylformamide was monitored using fluorescence and a binding constant of 4.0 x 10^4 M^{-1} was determined. Kim also used a tripodal host in which three imidazolium groups were placed in alternating positions around a benzene scaffold. This bound phosphate with K_a= 2.5 x10^3 M^{-1} in DMSO.[18]

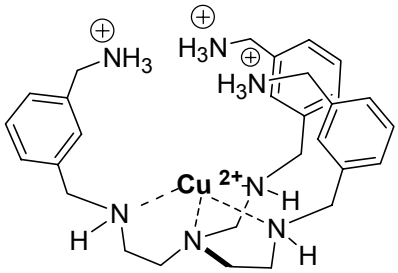
The numerous other receptors showing high affinity and in some cases high selectivity for phosphate are primarily functional in organic media, with little or no reported affinity in water. This attests to the success of the rational design approach to developing a receptor for binding phosphate, yet indicates that there is room for the development of receptors that have a high selectivity and affinity for phosphate in water at biological pH.

The approach described herein involves a "lock and key" design approach in which the cavity of the host is pre-organized such that the functional groups are arranged to compliment the shape, size, and charge of phosphate. Our goal was to design a receptor, complimentary to phosphate with high affinity and selectivity in aqueous media.

2.3 RECEPTOR DESIGN

A specific design approach to the development of a synthetic receptor for phosphate using metallo-receptors (**2.4** and **2.5**) is described. The design of each of the hosts takes advantage of a preorganized binding platform and the incorporation of effective functional groups as binding sites. The use of a preorganized[19-22] binding cavity serves to minimize any entropic losses that may arise from conformational changes resulting from host-guest binding. The use of functional groups as binding sites endows the receptor with the ability to form multiple non-bonded interactions with the guest. This design strategy is often used in order to establish an enthalpic advantage in the binding event.

The designs of receptors **2.4** and **2.5** feature a C_{3v} symmetric cavity pre-organized around a central Cu (II) center that can serve as an anion binding site. Three additional functional groups bearing positive charges are positioned to provide binding interactions with oxygens on phosphate. The shapes of the



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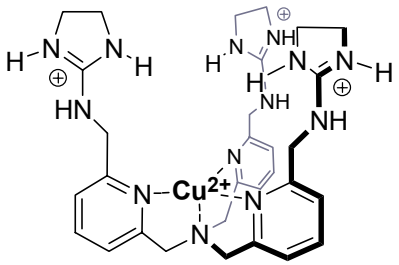
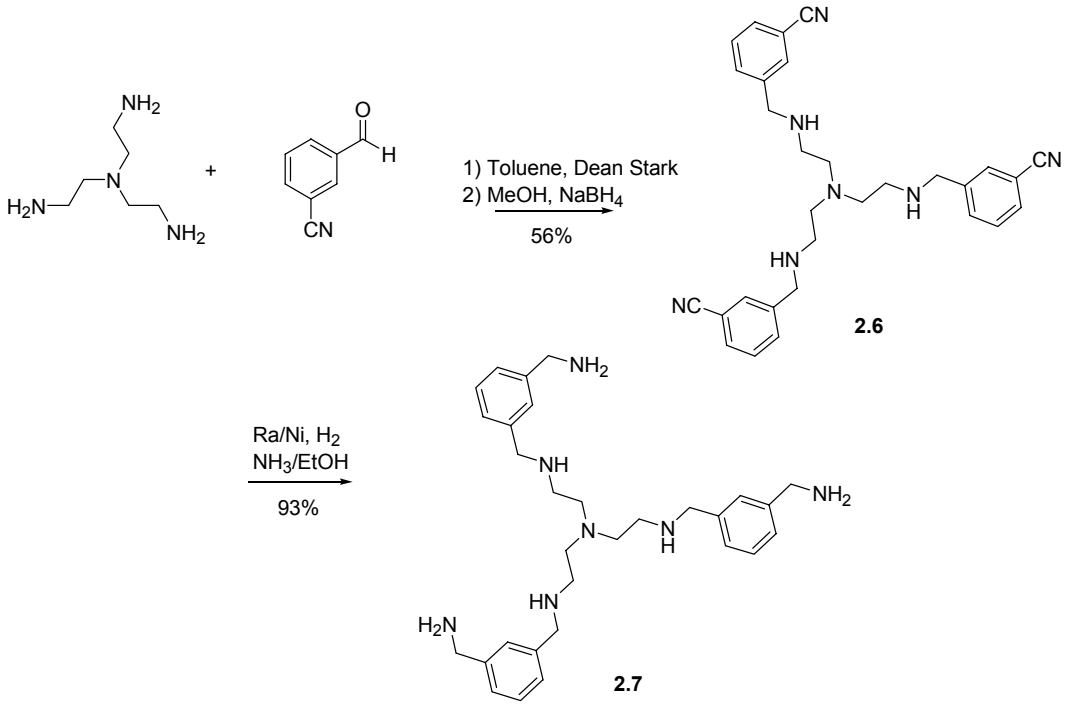


2.5

cavities are complementary to the three faces of a tetrahedron. Receptor **2.4** is derived from a tris(2-ethylamino)amine (TREN) unit with appended benzylamine groups, similar to the design exploited by Fabrizzi[23-27] and others[28, 29]. Similarly, receptor **2.5** is derived from a tris[(2-pyridyl)methyl]amine (TPA) subunit functionalized with appended guanidinium groups. This is analogous to compounds used by Canary[30-33], Karlin[34, 35], and others[36-39].

2.3.1 Synthesis

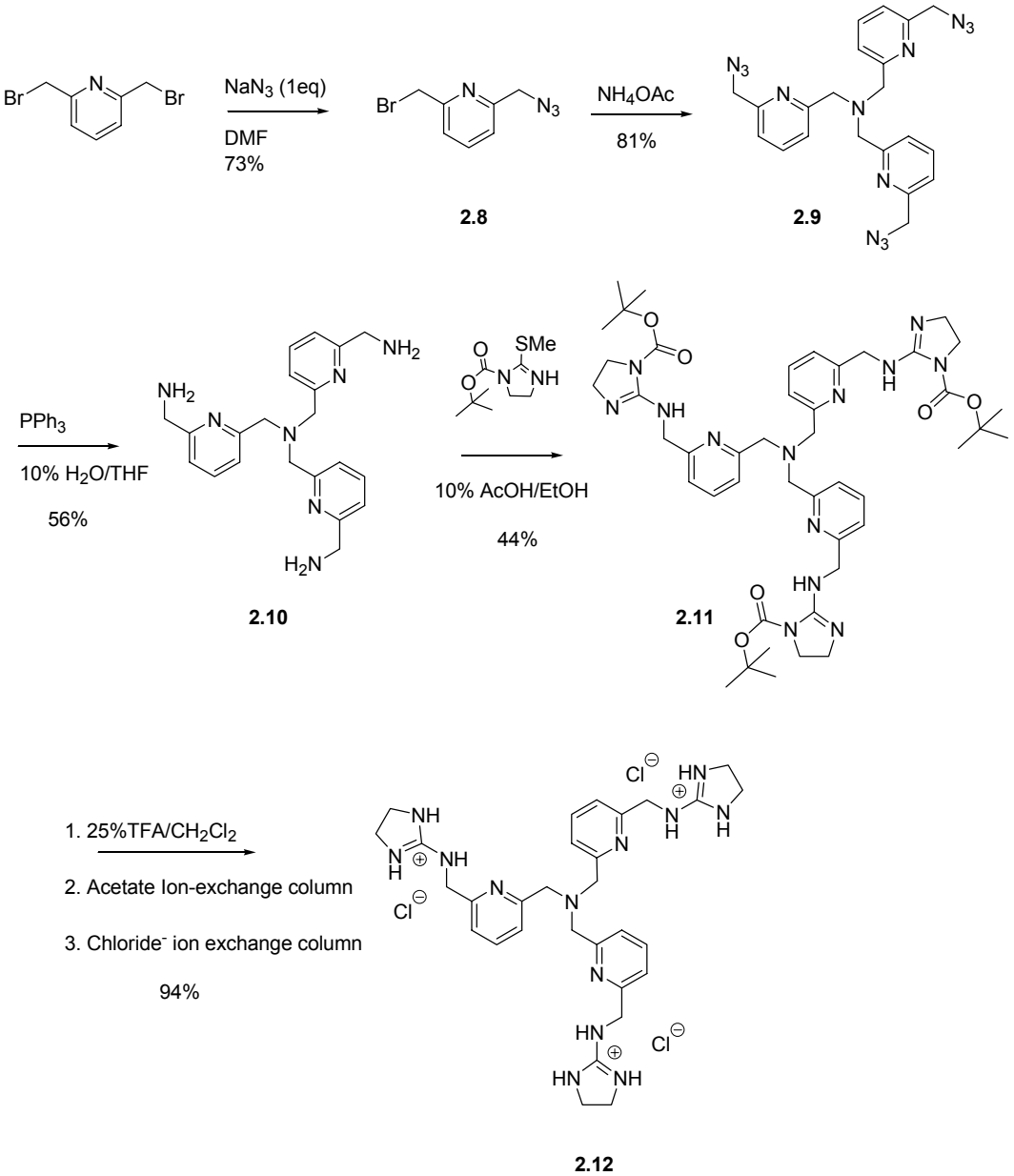
The synthesis of **2.4** begins a with reductive amination of 3-cyanobenzaldehyde and tris(2-ethylamino)amine. The resulting cyano compound **2.6** is reduced to the trisamine adduct (**2.7**) via hydrogenation with raney nickel catalyst in an ammonia saturated ethanol solution.



Scheme 2.1. Synthesis of TREN derived Receptor 2.4.

The synthesis of **2.5** commences with the reaction of 2,6-bisbromomethylpyridine with sodium azide in dimethylformamide. The resulting mono azidomethyl product **2.8** was used in a condensation reaction with ammonium acetate and dry potassium carbonate in acetonitrile, while recovered starting material was recycled. The trisazido adduct **2.9** is reduced to the trisamine under staugdinger conditions. The resulting amine (**2.10**) is combined with three equivalents of boc imidazoline[40] to yield **2.11**. Boc-protected

imidazoline was deprotected using trifluoroacetic acid, and the salt isolated. This was subjected to acetate anion exchange to ascertain the isolation of the triply charged salt. Subsequent chloride anion exchange yielded the tris chloride salt (**2.12**).



Scheme 2.2. Synthesis of TPA derived Receptor.

2.32 Copper Complex Formation

The introduction of a stoichiometric amount of copper(II) chloride pre-organizes the ligand to yield the desired receptor. UV/Vis spectroscopy was used to observe the absorbance at 900 nm as aliquots of copper(II) chloride were added to a solution of the ligand buffered at pH 7.4 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. The Cu(II)-tren stability constant has been reported to be approximately 10^{19} by Anderegg.[24, 41] The coordination[42] of Cu(II) to the buffer was explored by introducing copper(II) chloride to a solution of HEPES buffer under the same experimental conditions. The observed absorbance was negligible relative to that of compound **2.4**. The change in absorbance was used to generate a mole ratio plot and verified a 1:1 binding stoichiometry for ligand to metal binding.

A similar study was used to verify the coordination of the Cu(II) to ligand **2.12** by following the absorbance at 669 nm. This was carried out in an aqueous solution buffered at pH 7.4 with tris(hydroxymethyl)aminomethane (TRIS) buffer. The chelating ability of TRIS buffer to metals prompted investigations into the interaction of TRIS with Cu(II). Therefore a titration under identical experimental conditions, using a TRIS buffer solution with copper (II) chloride resulted in a linear increase in the absorbance of the Cu(II) center within the concentration range used for the ligand binding to Cu(II). The Cu(II) to receptor **2** titration absorbances were corrected for this, resulting in a mole ratio plot that verified 1:1 binding. The binding isotherm was not curve fit because the binding affinity was too high to generate a binding affinity with reasonable error. However, there is a binding constant of Cu(II) to a tris[(2-pyridyl)methyl]amine of 10^{17} reported in the literature[43], which further substantiates the Cu(II) binding

primarily to the TPA derived ligand. The TRIS-Cu(II) interaction was negligible for our purposes.

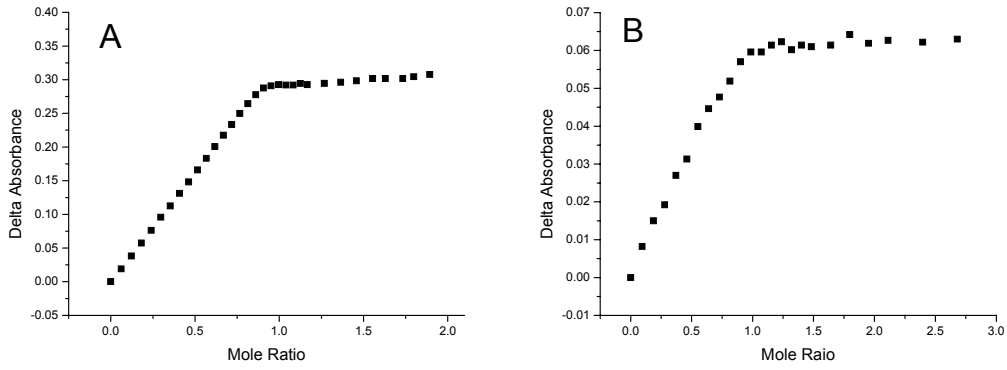


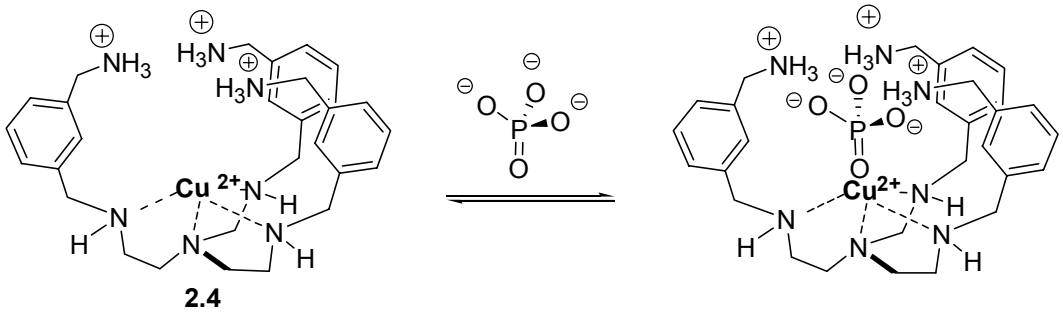
Figure 2.1 Mole Ratio Plots. A) Introduction of Cu(II) to a solution of the TREN derived ligand showed 1:1 binding stoichiometry. B) Titration of aliquots of Cu(II) to a solution of the TPA derived ligand yielded a mole ratio plot verifying a 1:1 stoichiometry of ligand to metal.

2.4 BINDING STUDIES

Having ascertained the Cu(II) complex formation to yield both **2.4** and **2.5**, binding studies were pursued to probe the selectivity and affinity for the intended guest, phosphate. Preliminary data indicated that the Cu(II) center served as a binding site as well as a signaling site. The interaction between phosphate and the Cu(II) center lead to an observed decrease in the absorbances of **2.4** and **2.5**, presumably due to ligand field effects.

2.4.1 Binding Affinities and Selectivities of Host 2.4

A 98:2 H_2O/MeOH solution of 1 (0.71 mM) was prepared for titration purposes and buffered at pH 7.4. The change in the absorbance of **2.4** (0.71 mM) at 800 nm was monitored by UV/Vis spectroscopy as aliquots of a phosphate solution (12.94 mM) were introduced. The resulting binding isotherm was fit[44] with a curve derived from a 1:1 binding algorithm to yield a binding affinity of 2.5 x 10^4 M^{-1}.



The species present in solution at this pH become important for interpreting the binding data. The data obtained for the pH titration of ligand **2.7**, ligand **2.7** in the presence of Cu(II), and receptor **2.4** in the presence of phosphate is shown below (Figure 2.2). By inspection the pK_a values of the free ligand (**2.7**) lie above 7, with potentially one pK_a as low as 2 or 3. In the presence of the Cu(II) there is an evident lowering of three pK values, presumably the secondary amines. This is reasonable because ligation of the ligand to Cu(II) is expected to lower the pK_A values, and this is observed by concomitant appearance of a blue color, an indication the Cu(II) is chelating the ligand. Once again, the remaining pK_a values lie above 7. The pH profile of the host/guest complex is similar, with a shift to the right by approximately one equivalent. This additional proton with a

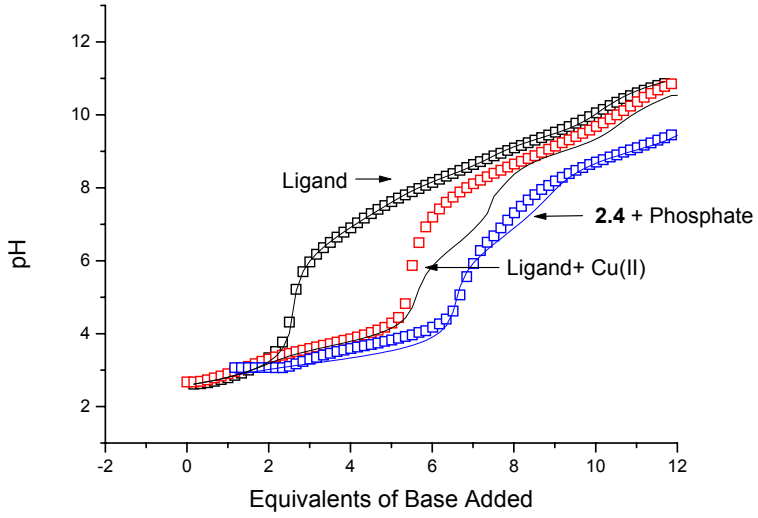


Figure 2.2 pH Titration Data. The three pH curves for the ligand, ligand and Cu(II) and the host-guest complex were obtained by addition of 10 μL aliquots of standardized NaOH to a solution of 0.005 mmole of the titre. The □ represent the raw data and the solid lines show the curve fits from HPERQUAD.

low pK_a value is consistent with the presence of phosphate, which has one pK_a at 2.15. The remaining pK_a values are above 8. A more comprehensive interpretation of the data using HYPERQUAD[45, 46] to fit the pH data is also included.

The curve fit to the titration of the ligand indicate that the following species are present: H_6L, H_5L, H_4L, H_3L, H_2L, HL. The calculated β values show there are at least three pK_a values between 6.95 and 9.93. In the case of the ligand and Cu(II) the lower and upper portions of the curve were successfully fit. However, a portion of the middle of the titration data proved difficult, regardless of the number of equilibria included in the curve fit analysis. Despite this, the species used to generate the curve fit are reasonable: H_7L, H_6LC, H_5LC, H_4LC, H_3LC, H_2LC, HLC, and LC. The pK_a values from the fit are not reliable, however inspection of the curves show that the higher pK_a values lie above 7. The pH data

for the host/guest titration necessitated inclusion of the three pK_a values for hydrogen phosphate. In addition to these species a reasonable curve fit was obtained by also including the free phosphate, H_7L, and the host guest species in the following protonation states: H_5LCuP and H_4LCuP. As calculated, the pK_a value for H_5LCuP is 7.24. We cannot distinguish whether the proton resides on an amine or on phosphate, though the second pK_a of phosphate would be expected to be depressed in the presence of the host.

The binding affinities of several other anions to **2.4** in aqueous media were determined by UV/Vis titrations (Table 2.1). The affinity of arsenate with **2.4** is the same as phosphate within our error margins, possibly due to their similarity in size and shape. Perrhenate, also tetrahedral in shape, has a binding constant on the order of 10^3, likely due both to the larger size and reduced charge of the anion (compared to phosphate). The addition of sulfate resulted in small spectral changes and a reproducible binding curve was not obtained. Anions of different geometries demonstrated much lower affinities to **2.4**, and in some cases the binding stoichiometry was 2:1, guest:host (Cl^- and HCO^-_3 , see Table 2.1). Acetate was determined to have a binding affinity below 900 M^{-1}. Therefore, the shape, size, and charge of **2.4** leads to a high affinity and high selectivity for phosphate relative to perrhenate, and spherical or triganol bipyramidal anions.

Receptor	**Anion**	**Stoichiometry** anion:**2.4**	**Binding Constants (M^{-1})**
2.4	HPO_4^{2-}	1:1	2.5×10^4 ($\pm 6 \times 10^2$)

2.4	$HAsO_4^{2-}$	1:1	$2.5 \times 10^4 (\pm 6 \times 10^2)$
2.4	ReO_4^-	1:1	$2.0 \times 10^3 (\pm 7 \times 10^2)$
2.4	HSO_4^{2-}	-	-
2.4	AcO^-	1:1	< 900
2.4	NO_3^-	1:1	< 20
2.4	HCO_3^-	2:1	n.d
2.4	Cl^-	2:1	n.d
2.13	HPO_4^{2-}	1:1	$8.0 \times 10^3 (\pm 7 \times 10^2)$
2.13	$HAsO_4^{2-}$	1:1	$9.0 \times 10^3 (\pm 7 \times 10^2)$
2.14	HPO_4^{2-}	1:1	$9.0 \times 10^2 (\pm 3 \times 10^2)$

Table 2.1 Binding Affinities. The binding of several anions to receptor 2.4 was investigated.

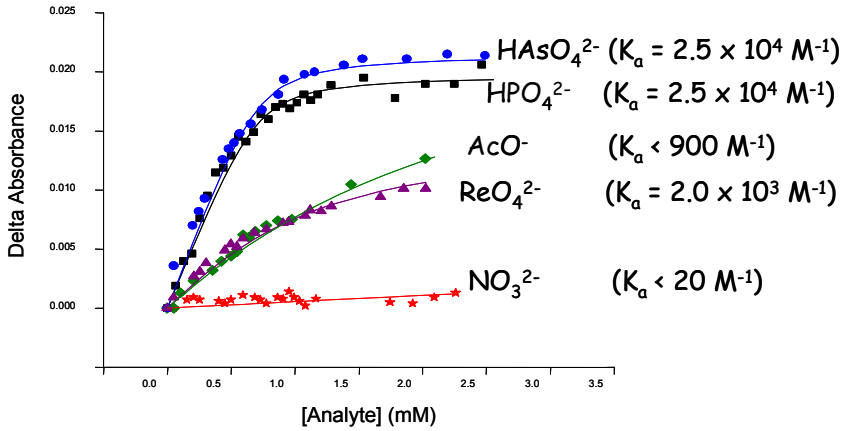
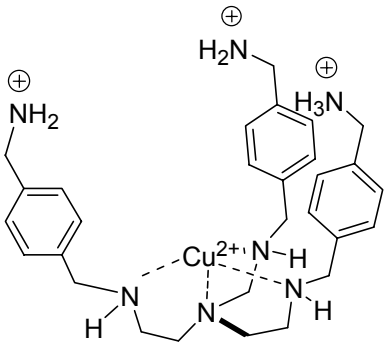


Figure 2.3 Binding Isotherms. A graphical representation of the binding selectivity of host 2.4 to phosphate over other anions.

To decipher the roles of various binding sites on **2.4**, hosts **2.13** and **2.14** were examined. The structure of **2.13** differs from that of **2.4** by the placement of the methyl amine moiety on the para position of the phenyl rings. While the affinity of phosphate with **2.13** is still comparable (3 times smaller), it serves to demonstrate that the shape of the cavity of **2.4** appears to be slightly better suited

for small tetrahedral anions. The K_a value of 900 M^{-1} for the binding of phosphate to **2.14** suggests that a large portion of the binding of phosphate to **2.4** is due to the metal center, a key feature in the host design. However, it also



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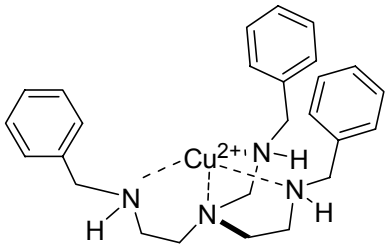
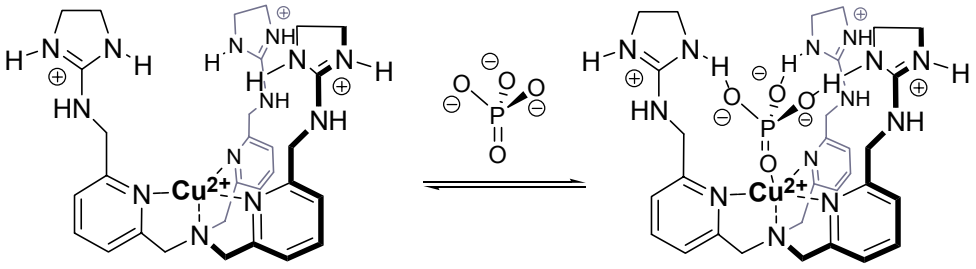


2.14

indicates that the ammonium groups are contributing significantly to the observed affinity constant for phosphate (~30 times), by possibly forming a better suited cavity and being predisposed to form tighter non-bonded interactions.

2.4.2 Binding Affinities and Selectivities of Host 2.5

Titrations of receptor **2.5** (1.16 mM) with different anions were also performed. Modulations in the UV/Vis absorbance at 790 nm was monitored as 5μL aliquots of a phosphate solution (19.8 mM), buffered at pH 7.4, were added. The resulting binding isotherm was fit with a 1:1 algorithm to yield a binding affinity of 1.5 x 10^4 M^{-1}. The titration with arsenate resulted in a



2.5

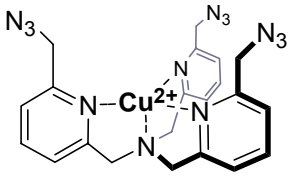
similar binding affinity (Table 2.2), much like in the case of **2.4**, as a result of their similarity in shape and size. Although the K_a for phosphate and **2.5** is slightly smaller than that of the **2.4**:phosphate complex, this is among the highest reported in the literature to date. Sulfate showed no shift in absorbance,

Receptor	Anion	Stoichiometry anion:**2.5**	Binding Constants (M^{-1})
2.5	HPO_4^{2-}	1:1	1.5×10^4 ($\pm 6 \times 10^2$)
2.5	$HAsO_4^{2-}$	1:1	1.7×10^4 ($\pm 6 \times 10^2$)
2.5	ReO_4^-	-	<100
2.5	HSO_4^{2-}	-	-
2.5	AcO^-	-	<100
2.5	NO_3^-	-	<100
2.5	HCO_3^-	-	<100
2.5	Cl^-	-	<100
2.15	HPO_4^{2-}	1:1	4.0×10^3 ($\pm 7 \times 10^2$)[a]
2.16	HPO_4^{2-}	1:1	1000 ($\pm 7 \times 10^2$)[a]

Table 2.2 Binding Affinities. Tabulated binding constants for the binding of anions to receptor 2.5. The poor binding ability of anions of different sizes and shapes renders the receptor selective for phosphate.

indicating no binding. Titrations with various other inorganic analytes were performed using identical experimental conditions, and no binding was observed. Host **2.5** therefore, has both a high affinity and excellent selectivity in water at neutral pH for tetrahedral oxyanions.

Again, to decipher the roles of the various binding sites in **2.5**, similar hosts were examined. Titrations of a phosphate solution (29.3 mM) to a solution of receptor **2.15** (1.5 mM), which lacks the guanidinium groups, demonstrates a binding affinity of 4.0×10^3 M^{-1}. This was measured in a solvent system having a higher percentage (15%) of methanol. The binding affinity of phosphate to control host **2.16**,[47] under the same experimental conditions as with **2.5**, was 300 M^{-1}. The lower affinity compared to **2.5**, verifies the cooperative effect of the guanidinium groups and the Cu(II) center as well as the effective match of the cavity to the size and shape of phosphate.



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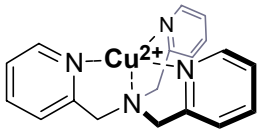


2.16

The selectivity for phosphate for both **2.4** and **2.5** is ascribed to the design of the cavities which provides excellent shape, size, and charge complimentarity to the tetrahedral oxyanion. The high affinities reported for phosphate to both **2.4** and **2.5** are attributed to the combined charge-pairing interactions of the ammonium/guanidinium groups and the Cu(II) center with the oxygens of the tetrahedral oxyanion.

The inherent flexibility of **2.4** compared to that of **2.5** decreases its selectivity for phosphate. In contrast the rigidity of **2.5** leads to a decrease in affinity for phosphate while increasing its selectivity for phosphate. To further

investigate this, we sought to determine the enthalpy and entropy origin of these differences in selectivity.

2.4 THERMODYNAMICS OF PHOSPHATE BINDING IN WATER

Having determined the selectivities and affinities of phosphate binding to both **2.4** and **2.5** it seemed appropriate to pursue a study of the thermodynamics of the binding systems. These complexes presented an opportunity to explore the contributions of ΔH° and ΔS° parameters to ion-pairing host-guest binding in water. Such a comparison was expected to offer insight into any differences between the **2.4:**phosphate and **2.5**:phosphate complexes that were not evident from the similarity in their binding energies. The ability to draw correlations between structure and thermodynamics has the potential of aiding the chemist in improved receptor designs.

2.4.1 Energetics of Phosphate Binding with Host 2.4

Isothermal titration calorimetry (ITC)[48-50] was used to probe the driving force for the large affinity of phosphate with **2.4** by quantifying the thermodynamic parameters (ΔG^0, ΔH^0, and ΔS^0) of binding. The titration proceeded with the addition of 5 μL aliquots of a 5.1 mM solution of complex of **2.4** to the titration cell containing a 0.32 mM solution of inorganic phosphate at 25°C. Less than 1kcal/mol of heat was generated per injection for the duration of the titration. The experiment was repeated using arsenate as the guest, only to yield a similar result. This, coupled with confirmation of binding from the UV/Vis data suggested that the binding event was essentially entirely driven by entropy. To verify the ITC data, a van't Hoff plot was generated using K_a values determined from UV/Vis titrations at 13.2°C, 20.1°C, and 27.0°C. The K_a values

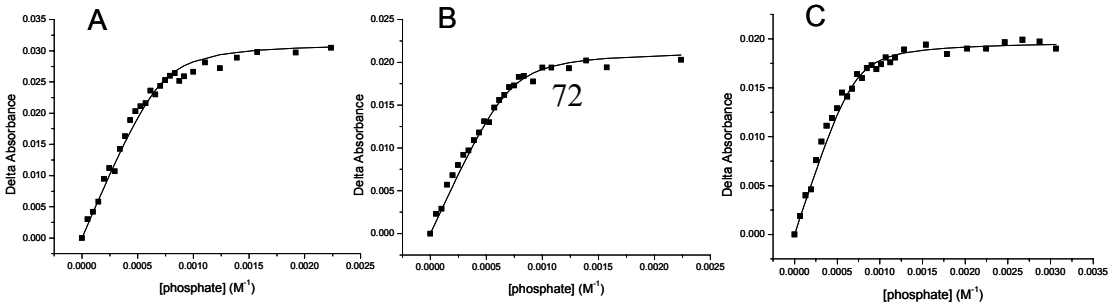


Figure 2.4 Binding Isotherms for the Titration of 2.4 with Phosphate. Aliquots of a phosphate solution buffered at 7.4 were added to a solution containing receptor 2.4. The raw data was fit with a 1:1 binding algorithm. A) Titration at 13.2°C, B) titration at 20.1°C, and C) titration at 27.0°C.

at each temperature were comparable within experimental error of the data, resulting in a van't Hoff plot with a slightly positive slope. The values determined from the plot were $T\Delta S^\circ$ = +6.6 (±1.0) kcal/mol and ΔH° = +0.6 (±0.5) kcal/mol, thus confirming the major contribution of the entropy changes to the overall strength of the binding. The complex formation of phosphate and control compound **2.14** was also analyzed using a van't Hoff approach, yielding ΔH° = -0.9 (±0.5) kcal/mol and $T\Delta S^\circ$ = +2.9 (±1.0) kcal/mol. This indicates that in the absence of ammonium groups for binding, the primary mode of phosphate binding to **2.14**, through ligation to the Cu(II) center, is slightly endothermic with an entropic driving force. The favorable entropy change is thought to arise form the release of solvent and/or counterions from the host and the guest upon complex formation. The ammonium groups in **2.4** enhance the free energy of phosphate binding by 2.2 kcal/mol relative to **2.14**, the source of this being primarily entropic. The more dominant entropy change for **2.4** possibly derives from interaction of the phosphate with not only the Cu(II) center, but from the additional interactions with the ammonium groups leading to more solvent and/or counterion release. Additionally, the cavity volume of **2.4** occupied by phosphate is larger than that of **2.14**, leading to more solvent release.

Receptor	Guest	ΔG° (kcal/mol)	ΔH° (kcal/mol) (± 0.5 kcal/mol)	TΔS° (kcal/mol) (± 1.0kcal/mol)
2.4	HPO_4^{2-}	-6.6	+0.6	+5.9
2.4	ReO_4^-	-3.7	-2.2	+1.5
2.4	AcO^-	-3.4	+0.7	+4.1
2.5	HPO_4^{2-}	-5.3	-3.8	+1.5
2.14	HPO_4^{2-}	-3.8	-0.9	+2.9
2.16	HPO_4^{2-}	-4.1	-0.8	+3.3

Table 2.3 Thermodynamic data for host-guest Complexes. Tabulated binding energies with the component enthalpy and entropy changes for the complex formations of anions with receptors **2.4** and **2.5**. The entropy change is favorable in all cases.

Thermodynamic studies were also used to examine the binding energetics of anions with differing affinities to **2.4** (Table 2.3). Arsenate showed almost identical thermodynamic parameters to those of phosphate. The binding of perrhenate to **2.4** was quantified (500 M^{-1}) using ITC techniques. The addition of 5 μL aliquots of a solution of **2.4** to a solution of perrhenate displayed exothermic heats of binding. The raw data, when fit with a binding isotherm, yielded a ΔH° value of -2.2 (±0.5) kcal/mol and a $T\Delta S^\circ$ value of +1.5 (±1.0) kcal/mol. Similar methods for acetate as the guest resulted in ΔH° = +0.71 (±0.5) kcal/mol and $T\Delta S^\circ$ = +4.12 (±1.0) kcal/mol.

In all cases the binding of the guest to **2.4** was accompanied by a favorable entropy change. Both phosphate and acetate have a near thermoneutral enthalpy change, and perrhenate shows an exothermic enthalpy change. This series of guests serve to add yet more examples to the literature in which ion-pairing interactions in water have a significant contribution to the binding energetics from favorable entropy changes. These favorable entropy changes often result from solvent/counterion release. Both the host and the guest are solvated with solvent molecules, and upon binding through ion-pairing interactions, solvent molecules are released into bulk solution, thereby increasing the entropy of the overall

system. This is well known from studies on both natural systems[51-54] and molecular recognition events involving synthetic receptors[55-62].

It is interesting to note that the host design renders **2.4** selective for phosphate, and in comparison to other anions, phosphate appears to have the largest entropy change associated with its binding. Perrhenate and acetate have lower binding affinities with **2.4**, as well as smaller associated entropy changes. Relative to phosphate, perrhenate occupies a larger molar volume and has a loosely held solvation shell due to its smaller charge density.[63] Therefore, in terms of entropy changes, the complexation of perrhenate displaces less solvent from the cavity. Additionally, there is less solvent and/or counter ion release for any non-bonded contacts between binding epitopes on the host and the guest, relative to phosphate. Overall, the entropy changes for perrhenate binding are less favorable compared to phosphate binding, and the data show that this is compensated by a favorable enthalpy change.

The smaller molar volume of acetate relative to phosphate indicates that acetate should displace less solvent from the binding cavity. In terms of solvation, acetate has a greater charge density compared to dihydrogenphosphate, and should therefore have a more ordered solvation shell.[63-65] However, at the working pH of these titrations hydrogen phosphate is present, which has a higher charge density, possibly increasing the ordering of the solvation shell relative to dihydrogenphosphate.[66] Indeed, the data show that acetate does bind with a significant entropy change as a result of disruption of the solvation shell, and displacement of solvent and/or counterions from the host. Correspondingly, phosphate has a significant entropy change, though larger than in the case of acetate. Relative to acetate this may arise from the increased occupied volume of the cavity, as well as additional non-bonded interactions between host and guest that are not present (phosphate has four contact points, acetate has only two) in the acetate:**2.**4complex.

2.4.2 Energetics of Phosphate Binding with Host 2.5

Thermodynamic investigations on the binding of phosphate to **2.5** were pursued using ITC techniques. 4.5 μL aliquots of the host solution (5.07 mM) were added to a solution of phosphate (0.21 mM) buffered at pH 7.4. The pattern of the heat peaks on the raw data plot indicated that the binding was exothermic. Though these titrations were reproducible, the data analysis was inconclusive. The application of a curve fit using the Origin software converged on a 2:1 guest:host stoichiometry for the complex. The resulting binding affinity and ΔH° value were unreasonable based on the associated errors. There appeared to be multiple equilibria present and a 2:1 stoichiometry may not even be correct for the concentrations used for the ITC studies (Chapter 4).

The suggestion of the presence of multiple equilibria lead to a Job plot analysis to verify the binding stoichiometry of the **2.5**:phosphate complex. The solutions used had a total concentration of 2.1 mM. The absorbance values of host:guest ratios between zero and one were recorded. The absorbance of the free host in solution for each mole ratio was subtracted from these values to provide an accurate absorbance of the host-guest complex. A plot of the absorbance of the percentage of the guest bound was plotted against the mole ratio of the host to yield a Job plot with a maximum at 0.5 mole ratio host (Figure 2.5). This is a clear indication that the host-guest stoichiometry is 1:1 at the concentrations used for UV/Vis analysis.

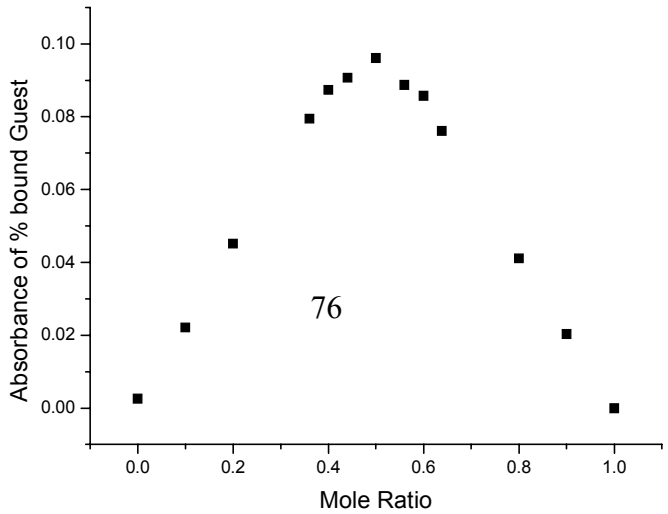


Figure 2.5 Job Plot. A maximum at 0.5 mole ratio indicates a 1:1 host-guest stoichiometry for binding.

The verification of a 1:1 binding stoichiometry allowed investigations to proceed using a van't Hoff analysis within a concentration range similar to that of the Job plot experiments. UV/Vis spectroscopy was used to determine the binding affinities of phosphate to **2.5** over a range of temperatures from 16°C to 55°C. These affinities were used to generate a van't Hoff plot (Figure 2.6). The resulting data was fit with a straight line from which a ΔH° value of -6.3 (±0.5) kcal/mol and a $T\Delta S^\circ$ value of -0.7 (±1.0) kcal/mol were determined. This indicated that there was a strong favorable enthalpy change associated with phosphate binding, with a slightly negative entropy change. This suggests that the guanidinium groups offer an enthalpic advantage of 1.5 kcal/mol in binding phosphate to **2.5** This is even more apparent when comparing the ITC data for the **2.16**:phosphate complex which has a slightly exothermic enthalpy change (ΔH° = -0.8 (±0.5) kcal/mol) and a favorable entropy change ($T\Delta S^\circ$ = +3.3 (±1.0) kcal/mol).

Although a linear fit to the data in the above van't Hoff analysis for **2.5** was achieved, the raw data plot showed curvature. A curved van't Hoff plot indicates that there is a heat capacity change in the system. This seemed reasonable for this host-guest system given the temperature range investigated. We felt it would be instructive to fit the data with a modified van't Hoff equation which incorporates the heat capacity change:

$$R \ln K = -\Delta H_0 \left(1/T\right) + \Delta C_p^0 \ln T + \left(\Delta S_0 - \Delta C_p^0\right) \qquad (2.1)$$

A plot of *RlnK* against *T* can be fit with equation 2.1, in which ΔH_0, ΔS_0, and ΔC_p° are the dependent variables. The resulting ΔH_0 and ΔS_0 values are then used in two additional equations that account for the temperature dependence of the enthalpy and entropy changes of the system to yield ΔH° and ΔS° values. The equations are:

$$\Delta H^0 = \Delta H_0 + T\Delta C_p \qquad (2.2)$$

$$\Delta S^0 = \Delta S_0 + \Delta C_p^0 \ln T \qquad (2.3)$$

Treatment of the binding data in this fashion yielded a ΔH° value of -3.8 (±0.5) kcal/mol and a $T\Delta S^\circ$ value of +1.5 (±1.0) kcal/mol for the binding of phosphate to **2.5**. The binding data was collected over a 40° temperature range, thus we are inclined to be more confident in the values obtained from the corrected van't Hoff plot. These data show that the binding is characterized by a favorable entropy change, but the complex formation is primarily driven by a favorable enthalpy change. The dominant enthalpy driving force contrasts to the binding of phosphate to receptor **2.4**, which is entropically driven.

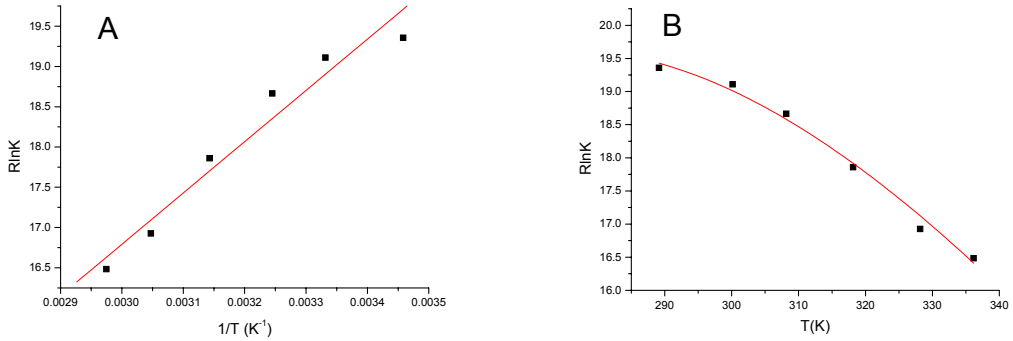


Figure 2.6 van't Hoff Plots. The binding data from UV/Vis titrations was used to generate van't Hoff plots. A) Linear fir to the data with an R^2 value of 0.95. There appears to be some curvature to the data. B) The data in A was replotted and fit with a corrected form of the van't Hoff equation. This demonstrates a better fit.

The van't Hoff analysis yielded a ΔC_p° value of -174 (±44) cal/molK. The heat capacity change is sensitive to changes in the structure upon complex formation. A negative heat capacity change is an indication that the solvation of the individual components is more structured than the solvation of the host-guest complex. The heat capacity changes (ΔC_p° = -12 to -190 cal/molK) for hydrophobic interactions have been reported for cyclophane and cyclodextrin systems in water.[67-70] The change in heat capacity for hydrogen bonding interactions in abiotic host-guest complexes (porphryn-mannoside, quinone-porphyrin, and diacid-aminopyrimidine) in polar solvents have been reported to be range from -30 to -560 cal/molK.[71-73]

We attribute the ΔC_p° value found with **2.5** to the solvation differences between the host and the guest versus the host-guest complex. The receptor system reported here is highly charged with solvent exposed functional groups, therefore it is reasonable to expect the host and guest to be highly solvated in aqueous media. Upon binding the solvent spheres are disrupted and water excluded. This increase in entropy observed and the negative heat capacity

change fits well with this scenario, which is very similar to the classical hydrophobic effect.[74]

This abiotic host-guest system differ from natural systems in that the heat capacity changes of binding in natural systems involve combined electrostatic and hydrophobic interactions. A theoretical approach to protein-ligand binding by Gallagher and Sharp conclude that the heat capacity change due to electrostatic interactions is small, however the overall heat capacity change is generally negative, with burial of non-polar groups and water exclusion.[75] Our binding complex has a significant heat capacity change.

2.4.3 Structure-Energetics Correlations

The results described above clearly indicate that the binding of phosphate to **2.4** and **2.5** have comparable Gibb's free energies of binding. Yet, the component enthalpy and entropy changes are quite different. Receptor **2.5** complexes phosphate with a dominant enthalpy driving force, whereas the binding of phosphate to receptor **2.4** is entropically driven. A discussion on the differences in the design of the hosts may offer some insight into the differences in their thermodynamic profiles.

The designs of both **2.4** and **2.5** incorporated a preorganized binding cavity to match the size and tetrahedral shape of oxyanions, specifically phosphate. Functional groups were placed on the periphery of the cavities of each of the receptors to promote electrostatic interactions, a design strategy often used to increase the favorable enthalpy change upon binding. Receptor **2.4** employs ammonium groups for this purpose, as they are known to be effective in binding epitopes. However, the ammonium groups in **2.4** do not appear to contribute to the overall enthalpy change, but their presence does lead to enhanced binding when compared to **2.14** due to entropy changes. This enhanced binding may be explained by solvation considerations. The inherent flexibility in receptor **2.4** and

the presence of ammonium groups renders it a well solvated and open binding cleft. Upon binding phosphate, solvent molecules and/or counterions are excluded from the cleft, leading to the dominant favorable entropy change observed. This favorable entropy change must overcome any restriction of the degrees of freedom upon organizing the structure of the host with phosphate bound. If the ammonium groups do offer an enthalpic advantage through electrostatic interactions, this must be countered by endothermic changes resulting from changes in the solvent structure.

In contrast, receptor **2.5** has a binding cleft that is less well solvated, but highly preorganized. Upon complex formation with phosphate, solvent and/or counterions are displaced, but to a lesser extent than seen with receptor **2.4**. Also, due to charge delocalization, guanidinium groups are less well solvated than ammonium groups. However, the guanidinium groups which have a delocalized charge and fixed geometry, rendering these groups a more effective hydrogen bonds donors. The use of guanidiunium groups for enhanced binding enthalpy is reflected in the dominant exothermic ΔH° of binding phosphate to **2.5**. This is further substantiated by the thermodynamic data for phosphate binding to **2.16** which shows a near zero enthalpy change in the absence of the guanidinium groups.

The entropy changes of phosphate binding with **2.4** relative to phosphate binding to **2.5** are more significant. This is possibly a consequence of the solvation differences between the smaller ammonium group relative to the guanidinium group. Smaller ions are known to be better solvated, thus upon losing its solvation shell when bound, the ammonium groups necessarily releases more solvent molecules into bulk solution than do guanidinium groups. This comparison of binding groups, as it applies to **2.4** and **2.5**, assumes that solvation of each receptor is identical in all other respects. This assumption seems reasonable based on the thermodynamic data for the binding of phosphate to both

control hosts **2.14** and **2.16**. They both interact weakly with phosphate through the Cu(II) center with a small enthalpy change and almost identical entropy changes. Therefore the differences of phosphate binding to **2.4** and **2.5** arise from the differences in the functional groups on the periphery of the cavities.

Differential solvation of the functional groups within our molecular complex formations show a trend similar to work reported by Smith and co-workers.[76] They report that guanidinium functionalized cyclodextrin hosts bound aryl phosphates with a more favorable enthalpy change than the analogous ammonium functionalized hosts. Additionally, the aryl phosphates complexed the ammonium functionalized hosts with more favorable entropy changes than those functionalized with guanidinium groups.

2.5 Determination of Phosphate in Serum and Saliva

Receptor **2.5** demonstrated not only high affinity for phosphate, but also a high selectivity over other types of anions. This presented a potential candidate for a phosphate chemosensor, with an eye toward analysis of biological samples such as blood and saliva.

Oxyanions such as phosphate and sulfate are inorganic analytes commonly found in biological samples, beverages, and waste water. Of particular relevance to this research are the concentration levels of phosphate in serum and saliva. In a clinical setting phosphate levels in serum are determined as part of a routine blood analysis. The typical phosphate concentrations in adult human serum range from 0.81 – 1.45 mM.[77] Persons with abnormally high blood phosphate levels are diagnosed with hyperphosphatemia, which manifests in acute or chronic renal failure. Those with low phosphate levels suffer from hypophosphatemia, which can be associated with rickets, hyperthyroidism, or Fanoci Syndrome. Phosphate is also prevalent in saliva and the concentrations are variable, ranging from 5 – 14 mM.[78; 79] The presence of phosphate ions in saliva serves to buffer the fluids

within the oral cavity as bacterial acids are present.[80] This facilitates the repair of enamel and dentine, thereby maintaining the health and integrity of the teeth.

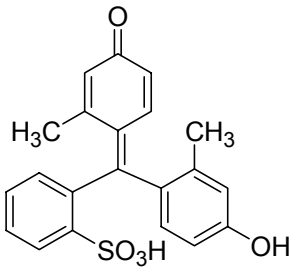
2.5.1 Methods of Phosphate Determination

There are several types of methods[81, 82] reported in the literature for the determination for phosphate concentrations in blood, however the methods most often used involve an ammonium molybdate reagent.[83-87] A typical serum sample is combined with an ammonium molybdate reagent under acidic conditions, and the inorganic phosphorous present reacts to form a phosphomolybdate complex. The absorbance of this complex at 340 nm is proportional to the phosphate concentration in the serum. Alternatively, the phosphomolybdate complex can be reduced to produce a color change, the absorbance of which is also proportional to the phosphate concentration in serum. Phosphate determinations in saliva are not as routine, but they are determined using the ammonium molybdate reagent[79; 88] as well as ion-selective electrode methods.[78; 79]

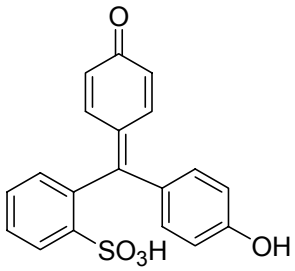
2.5.2 Dye-displacement Assay

The approach reported sought to quantify phosphate concentrations using a chemosensor. A chemosensor[89] often includes a molecular scaffold with a binding site and a signaling site. The binding of a guest 'communicates' with the signaling site to generate an optical signal by which to observe the binding event. There is significant interest in developing synthetic receptors[8,11,14,90-92] and chemosensors[10,16,93,94] for binding phosphate in medicinal and biological applications. Our interest here was to develop a dye-displacement assay employing **2.5** for phosphate sensing.

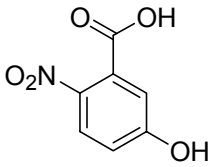
The complex formation of several commercially available indicators (dyes) with receptor **2.5**, was investigated as a potential receptor-dye pair for the sensing ensemble. Dyes such as alizarin complexone and pyrocatecholviolet demonstrated large color changes upon complexing the host. However, only small color changes were observed upon the introduction of a phosphate solution, indicating that the dye was not completely displaced from the cavity. The dyes are all highly functionalized with phenolic groups, and the hydroxyl-Cu(II) interactions are dominant, so any added phosphate may have interacted with the host-dye complex to produce a ter-molecular complex that resulted in the observed absorbance changes.



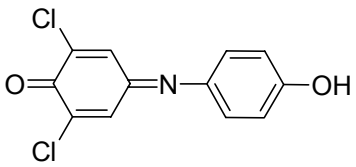
m-Cresol Purple



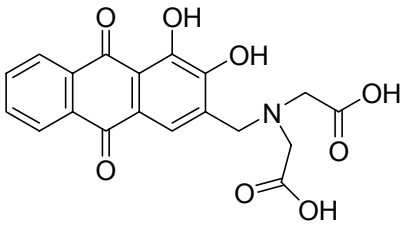
Phenol Red



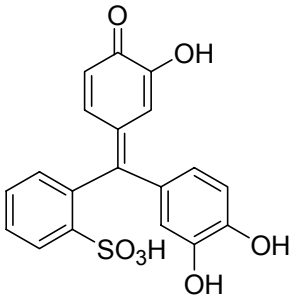
4-hydroxy-nitrobenzoic acid



2,6-dichlorophenol

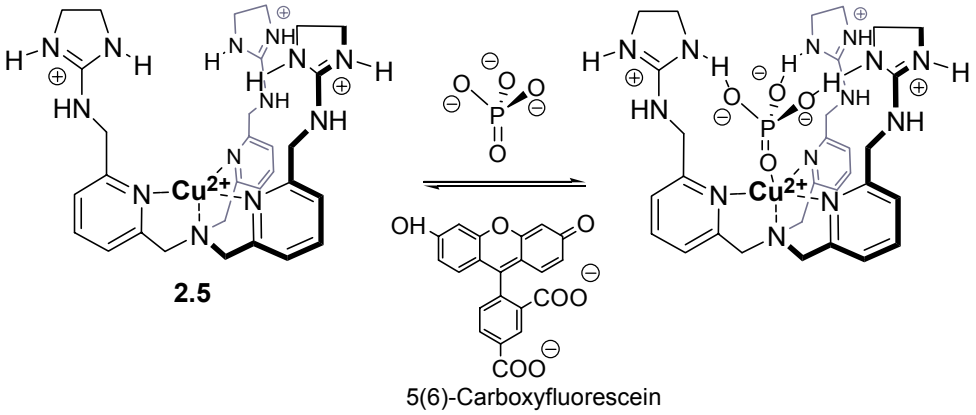


Alizarin Complexone



Pyrocatechol Violet

Previous work with the indicator carboxyfluorescein as part of a dye displacement assays with a guanidinium based receptor prompted us to pursue studies with the dye. This indicator is yellow in a 50:50 (v/v) water:methanol solution buffered at pH 7.4 with TRIS buffer. Upon addition of aliquots of **2.5** to a solution of 5-(and 6)-carboxyfluorescein, binding proceeds in a 1:1



stoichiometry with a color change from yellow to light orange (Figure 2.7A), with an increase of absorbance at 495 nm. This colorimetric response derives from a change in the microenvironment of the dye as it binds to **2.5**. As aliquots of a phosphate solution are introduced to the host-dye complex, the light orange color reverts to the yellow color of free dye in solution (Figure 2.7B). This indicates that the phosphate displaces the dye from the host cavity, thereby creating a sensing ensemble for phosphate. This signaling motif is advantageous over the traditional methods of covalently attaching a signaling unit directly to the host scaffold, in that no extra synthetic steps are necessary, and that the signaling unit does not occupy a position on the scaffold that could potentially serve as an additional binding site for the guest.

The dye-displacement assay was used to generate a calibration curve for phosphate using UV/Vis spectroscopy. Protein free samples of horse serum and saliva were analyzed using this sensing ensemble and compared to the calibration curve (λ_{max} = 500 nm). The phosphate concentration in the serum was determined

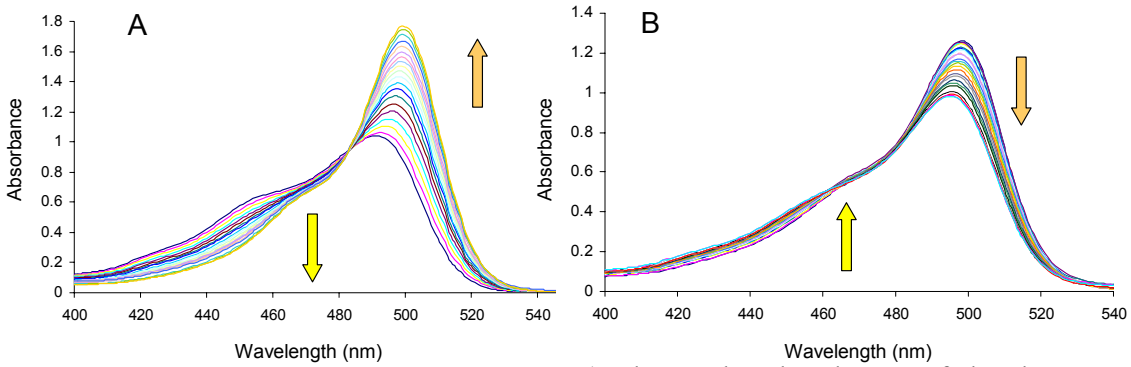


Figure 2.7 Dye-Dispalcement Assay. A) Shows the absorbance of the dye at 495 nm with an increase as host is added; color change from yellow to orange. B) Addition of a phosphate solution to the host-dye complex results in a decrease of the absorbance at 495 nm as the color changes from orange to yellow.

to be 1.6 (±0.2) mM and that of the saliva was 5.5 (±0.5) mM. The phosphate concentrations in the same samples were measured using a commercially available inorganic phosphorous kit from Diagnostic Chemicals Limited, which yielded comparable values of 1.8 (±0.2) mM and 5.1 (±0.6) mM for serum and saliva respectively. The literature values compare well, with horse serum ranging from 1.3 – 1.7 mM[95] and saliva ranging from 5 -14 mM.

In summary, metallo-receptor **2.5** when combined with 5(and 6)-carboxyfluorescein makes an effective chemosensor for inorganic phosphate in complex biological fluids. The results of the assay are comparable with clinically approved methods of phosphate determination. The success of using a chemosensor approach for a medicinal application highlights the

2.6 EXPERIMENTAL

General Considerations

The chemicals used were obtained from Aldrich and were used without further purification, except where noted. Methanol was refluxed over magnesium and

distilled. Flash chromatography was performed on Whatman 60 Å 230-400 mesh silica gel. ^{1}H (300 MHz) and ^{13}C (75 MHz) spectra were measured by Varian Unity Plus spectrometer. Mass spectra were recorded on a Finnigan VG analytical ZAB2-E spectrometer. UV/Vis spectra were collected on a Beckman DU-640 at 25°C unless noted otherwise.

UV/Vis Titrations

The titrations were performed on a Beckman DU-640 UV/Vis instrument. A typical titration is described below, though concentrations varied from experiment to experiment. A solution of the receptor (5.12 mM) was prepared and buffered with TRIS (10 mM) at pH 7.4. A similar solution of the guest (52.29 mM) was prepared. A cuvette was then filled with 770 μL of a TRIS (10 mM) solution and scanned as the blank reading. 230 μL of the host solution was introduced to the cuvette (total host concentration of 1.17 mM) and the absorbance recorded. Aliquots of a stock solution were then added to the cuvette and the absorbance recorded after each addition. The stock solution contained the host (1.17 mM) and guest (19.86 mM) in TRIS buffer (10 mM). The absorbances for each addition, at a chosen wavelength, were used to calculate the delta absorbances relative to the first absorbance reading. These values were then plotted versus the concentration of the added guest for each aliquot. The binding isotherm from this raw data was curve fit using the 1:1 binding equation (either done manually in Excel or done iteratively in Origin).

Isothermal Titration Calorimetry

The titration apparatus was purchased from Microcal Inc. The VP-ITC instrument is interfaced with Origin (version 5) software for both data collection and data analysis. A typical titration is described, though concentrations, buffers, and

parameters varied from experiment to experiment. The reference cell was filled with a buffer solution (HEPES 5 mM) identical to that in the titration cell. The titration cell was filled with a HEPES buffered (5 mM, pH 7.4) solution of the host (0.82 mM). The syringe was filled with approximately 250 μL of a solution of the guest (19.94 mM) buffered with HEPES (5 mM, pH 7.4). The concentration of the syringe contents is typically 20 times that of the concentration of the cell contents. The syringe was fitted above the cell and the following parameters set: Injection size: 5 μL, Number of injections: 43, Temperature: 25°C, Injection Interval: 300 sec, Cell Feedback: 20 μcal. Following data collection the Origin software was used to apply a 1:1 binding algorithm to the data, the fit of which yields a binding affinity, enthalpy change, entropy change and binding stoichiometry for the titration.

pH Titrations

The titration data were collected using a DOS based program (Iassist) interfaced with an Orion 720A pH meter and a Harvard Apparatus 55-2222 syringe pump. The pH electrode was purchased from Orion, PerpHecT Ross Electrode Model: 8203BN. The concentrations of the NaOH (83.5 mM) and HCl (130.24 mM) solutions were determined by titrations with potassium hydrogenphtalate using phenolphthalein as the indicator. The substrate (0.005 mmol) was added to an aqueous solution (5 mL) containing NaCl (20 mM) and HCl (0.065 mmol). Aliquots (10 μL) of NaOH were added and pH readings were taken 60 seconds after addition. On average 80 -100 data points were recorded. The collected data were fit using HYPERQUAD.

Phosphate determinations for serum and saliva

The preparation of the serum and saliva samples for analysis was identical. An aliquot of serum (500 μL) was added to a solution of MeOH (500 μL). The sample was placed in a centrifuge at 4° C and spun at 9000 rpm. This was repeated until precipitates were no longer observed.

All titration solutions contained 10 mM TRIS buffer in a 50/50 v:v water:methanol solution. Prior to analysis a calibration curve was determined using the dye-displacement assay. A solution of receptor **2.5** (25.2 mM) and 5(and 6) caboxyfluorescein (25.2 mM) buffered at pH 7.4 with TRIS buffer (10 mM) was placed in a quartz cuvette. Aliquots of a stock solution containing receptor **2.5** (25.2 mM), 5(and 6) caboxyfluorescein (25.2 mM), and phosphate (494 mM) buffered at pH 7.4 were added. The absorbance values plotted versus the [phosphate] yielded the calibration curve.

A cuvette containing a solution of **2.5** (25.2 mM) and 5(and 6) caboxyfluorescein (25.2 mM) buffered at pH 7.4 with TRIS buffer (10 mM) was prepared in a similar fashion for the serum (saliva) analysis. To this was added a stock solution containing **2.5** (25.2 mM), 5(and 6) caboxyfluorescein (25.2 mM), and serum (140 μL). The change in the absorbance of the host:dye complex as phosphate in the samples bound the host were compared to the linear portion of the calibration curve for the determination of the phosphate concentration present in the stock solution. This value was then used to determine the phosphate concentration in the serum sample.

N-(3-cyanol-benzyl)N'-[2-(3-aminomethyl-benzylamino)-ethyl]N'-[(3-cyano-benzylamino)-methyl]-ethane-1,2-diamine (2.6)

To a flask fitted with a Dean-Stark apparatus containing toluene (150 mL) was added 3-cyanobenzaldehyde (1.9 g, 15.2 mmol). Tris- (2-aminoethyl)amine (0.76 g, 5.1 mmol) was added via syringe. The reaction mixture was heated to reflux (solution was yellow in color) for 2 hours to ensure removal of water. The contents of the reaction flask were cooled and the toluene removed by rotary evaporation. The crude oil was dissolved in dry MeOH (150 mL) and stirred under an inert atmosphere. To the solution was added sodium borohydride (0.57 g, 15.2 mmol) as a solid portion. The reaction mixture was stirred for 1 hour. Water was added dropwise to quench any remaining $NaBH_4$, and then concentrated en vacuo without further workup. The crude mixture was purified by silica gel chromatography, using 2% NH_3 sat'd. MeOH in CH_2Cl_2 as the eluent. The desired product was isolated as a yellow oil in a 56% yield (1.4 g, 2.9 mmol).

^{1}H NMR (CD_3CN): δ 7.63 (s, 3H), 7.55 (d, 6H, J = 7.8 Hz), 7.39 (t, 3H, J = 7.8 Hz), 3.71 (s, 6H), 2.54 (m, 12H). ^{13}C NMR (CD_3CN): δ143.9, 133.5, 132.3, 131.3, 130.1, 119.9, 112.7, 55.0, 53.4, 47.8. HRMS (CI+) *m/z* 492.2871; calcd. 492.2875. IR (2224 cm^{-1})

N-(3-aminomethyl-benzyl)N'-[2-(3-aminomethyl-benzylamino)-ethyl]N'-[(3-aminomethyl-benzylamino)-methyl]-ethane-1,2-diamine (2.7)

To an ethanolic solution (20 mL) of **3** (375 mg, 0.76 mmol) saturated with NH_3(g) was added Raney Nickel catalyst (pipette tip). The reaction mixture was sealed in a high pressure apparatus. H_2(g) was introduced to the reaction flask at 250 psi for 24 hours. The crude mixture was filtered over celite, dried over Na_2SO_4, and concentrated by rotary evaporation to yield a yellow oil in 93% yield (353 mg, 0.70 mmol).

^{1}H NMR (CD_3CN): δ7.17 (m, 12H), 3.69 (s, 6H), 3.66 (s, 6H), 2.54 (m, 12H). ^{13}C NMR δ 144.0, 141.1, 128.5, 127.1, 126.5, 125.7, 54.3, 53.5, 46.9, 45.9. HRMS (CI+) *m/z* 504.3817; calcd. 504.3814.

N-(4--benzyl)N'-[2-(3-aminomethyl-benzylamino)-ethyl]N'-[(4-cyano-benzylamino)-methyl]-ethane-1,2-diamine

The procedure delineated above for the meta ligand was used to synthesize the para analogue in a 55% yield.

^{1}H NMR (CD_3CN): δ7.58 (d, 6H), 7.530(d, 6H), 3.80 (s, 6H), 2.67 (m, 12H). ^{13}C NMR δ 146.29, 133.75, 130.74, 120.33, 112.14, 54.58, 53.50, 47.9,. HRMS (CI+) *m/z* 492.2863; calcd. 492.2875. IR (2222 cm^{-1})

N-(4-aminomethyl-benzyl)N'-[2-(3-aminomethyl-benzylamino)-ethyl]N'-[(4-aminomethyl-benzylamino)-methyl]-ethane-1,2-diamine (2.13)

The procedure delineated above for the meta ligand was used to synthesize the para analogue in a 84% yield.

^{1}H NMR (CD_3CN+ CD_3OD): δ7.22(m, 12H), 3.71 (s, 6H), 3.64 (s, 6H), 2.61 (m, 12H). ^{13}C NMR δ 142.9, 138.1, 129.6, 128.5, 54.0, 53.2, 47.3, 46.1. HRMS (CI+) *m/z* 504.3810; calcd. 504.3814.

N-Benzyl-N'-(2-benzylamino-ethyl)-N'-(benzylamino-methyl)-ethane-1,2-diamine (2.14)

The procedure delineated above for the meta ligand was used to synthesize control ligand for compound **3** in a 97% yield.

^{1}H NMR (CD_3CN): δ7.26(s, 12H), 3.71 (s, 6H), 3.67 (s, 6H), 2.55 (m, 12H). ^{13}C NMR δ 142.21, 129.15, 128.94, 127.49, 55.20, 54.34, 48.05. HRMS (CI+) *m/z* 417.3006; calcd. 417.3018

2,6,-monobromomethylazidomethypyridine (2.8)

To a solution of 2,6-bisbromomethylpyridine (3.67g, 13.8 mmol) in dry N,N-dimethyformamide (80 mL) was added NaN_3 (0.89g, 13.8 mmol). The reaction mixture was stirred under argon for five days at room temperature. The solvent was removed en vaccuo, and the residue dissolved in CH_2Cl_2. The suspension was filtered through celite, dried and concentrated to yield an oil. After flash chromatography on silica gel, eluting with CH_2Cl_2, as a clear colorless oil was obtained (0.64 g, 2.8 mmol) in a 67% yield. The starting material was recovered. ^{1}H NMR ($CDCl_3$): δ7.69 (t, 1H, *J*=8.1 Hz), 7.35(d, 1H, *J* = 7.8 Hz), 7.24 (d, 1H, *J* = 7.8 Hz), 4.50 (s, 2H), 4.44(s, 2H). ^{13}C NMR δ 156.78, 155.66, 138.01, 122.62, 121.08, 55.29, 33.44. HRMS (CI+) *m/z* 226.9938; calcd. 226.9932. IR (2104 cm^{-1})

Tris-(6-azidomethyl-pyridin-2-ylmethyl)amine (2.9)

To a solution of 2,6,-monobromomethylazidomethypyridine (0.83 g, 3.65 mmol) in dry CH_3CN (30 mL) was added NH_4OAc (84 mg, 1.1 mmol) and K_2CO_3 (0.30 g, 2.2 mmol) as solid portions. The suspension was stirred under argon for seven days at room temperature. The reaction mixture was filtered through a pad of celite, dried, and concentrated to yield an

oil. Purification using flash chromatography on silica gel (eluent CH_2Cl_2 to 5% NH_3 sat'd MeOH in CH_2Cl_2) yielded the product as an oil (0.40 g, 0.89 mmol) in an 81% yield.

^{1}H NMR ($CDCl_3$): δ7.64 (t, 3H, *J* = 7.8 Hz), 7.53(d, 3H, *J* = 7.8 Hz), 7.16 (d, 3H, *J* = 7.5 Hz), 4.40 (s, 6H), 3.87(s, 6H). ^{13}C NMR δ 159.01, 155.25, 137.34, 122.13, 120.18, 59.93, 55.48. HRMS (CI+) *m/z* 456.2120; calcd. 456.2121. IR (2102 cm^{-1})

Tris-(6-aminomethyl-pyridin-2-ylmethyl)-amine (2.10)

To a solution of tris-(6-azidomethyl-pyridin-2-ylmethyl)amine (0.28 g, 0.61 mmol) in tetrahydrofuran (4.8 mL) was added triphenylphosphine (0.56 g, 2.13 mmol) and water (0.3 mL). The reaction mixture was stirred at room temperature for 4 hrs. The reaction mixture was concentrated, and the residue dissolved in water (10mL) and CH_2Cl_2 (10ml). The pH was adjusted to 1 using 2M HCl and the organic layer separated. The aqueous layer was washed with CH_2Cl_2 (3 x 10mL). The pH of the aqueous layer was adjusted to 10 using 2M NaOH and extracted several times with CH_2Cl_2. The organic layer was concentrated to a yellow oil (0.13 g, 0.34 mmol) in a 56% yield.

^{1}H NMR (CD_3OD): δ7.72 (t, 3H, *J*= 5.7 Hz), 7.53(d, 3H, *J*= 5.7 Hz), 7.24 (d, 3H, *J*= 5.4 Hz), 3.86 (s, 6H), 3.85(s, 6H). ^{13}C NMR δ 161.55, 159.74, 138.65, 122.41, 120.95, 61.00, 47.62. HRMS (CI+) *m/z* 378.2413; calcd. 378.2406. IR (3355 cm^{-1})

Tris-(6-[[(1,1-dimethylmethoxy)carbonyl]methylamino]-pyridin-2-ylmethyl)-amine (2.12)

To a solution of tris-(6-aminomethyl-pyridin-2-ylmethyl)-amine (0.23 g, 0.61 mmol) in ethanol 92.7 mL) was added N-(t-butoxycarbonyl)-2-thiomethyl-2-imidazoline (0.45 g, 2.2 mmol) and acetic acid (0.3 mL). The reaction mixture was stirred at 50 C for 4 hrs. The reaction mixture was concentrated and purified by flash column chromatography (silica) with a gradient of 1-5% NH_3 saturated

methanol in CH_2Cl_2. The product was isolated as a brown oil (0.24 g, 0.26 mmol) in a 44% yield.

^{1}H NMR ($CDCl_3$): δ7.52 (t, 3H, , *J*= 7.5 Hz), 7.44(d, 3H, *J*= 7.8 Hz), 7.09 (d, 3H, *J*= 7.5 Hz), 4.51 (s, 6H), 3.81(s, 6H), 3.69(m, 6H), 3.59(m, 6H), 1.42(s, 27H). ^{13}C NMR δ 159.10, 156.45, 136.89, 121.17, 119.57, 81.88, 59.96, 53.34, 28.26. HRMS (CI+) *m/z* 882.5102; calcd. 882.5102.

Tris-(6[(4,5-dihydro-1H-imidazol-2-yl)methylamino]-pyridin-2-ylmethyl)-amine

To a solution of tris-(6-[[(1,1-dimethylmethoxy)carbonyl]methylamino]-pyridin-2-ylmethyl)-amine (0.11 g, 0.124 mmol) in CH_2Cl_2 (1 mL) was added trifluoroacetic acid (0.3mL). This was stirred at ambient temperature for 12 hours. The reaction mixture was concentrated and toluene (2 mL) was added. The mixture was then concentrated under reduced pressure. The residue was dissolved in water an liopholized to yield a cream colored sold. This was passed through an acetate anion exchange column. The eluent was liopholized and observed by ^{1}H NMR to ensure the quantitative exchange of the trifluoroacetate ions. The acetate salt was then passed through a chloride anion exchange column to yield a solid (80 mg, 0.11 mmol) in a 94% yield.

^{1}H NMR (CD_3OD): δ7.79 (t, 3H, *J*= 6.0 Hz), 7.56(d, 3H, *J*= 5.4 Hz), 7.28 (d, 3H, *J*= 5.4 Hz), 4.51 (s, 6H), 3.90 (s, 6H), 3.72(s, 12H). ^{13}C NMR δ 159.38, 155.15, 138.53, 138.41, 122.85, 120.60, 65.20, 60.24, 48.11, 44.01. HRMS (CI+) *m/z* 688.26.32; calcd. 688.26.

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Chapter 3: The Thermodynamic Origin of Cooperative Binding in Host-Guest Complex Formation

3.0 INTRODUCTION

Complex formation between a guest molecule and a host molecule is analogous in many ways to an organic reaction in which two starting materials react to form a new molecule. The bond breaking and bond forming processes of the reaction have associated thermodynamic parameters in the form of enthalpy and entropy changes. In much the same way enzyme-substrate and host-guest associations have thermodynamic parameters associated with them. Comprehensive thermodynamic studies of host-guest binding are attracting much interest within the field of molecular recognition.

The formation of a host-guest complex rarely results from one binding interaction, but from a collection of binding interactions acting together. The binding strength (ΔG°) of a host-guest complex is often estimated based on a summation of the strengths of the individual interactions that contribute to the overall binding event. Though this is successful in some cases, this does not hold for the majority of host-guest systems. The dissection of binding energies into individual interactions does not result in a good approximation of an overall binding energy because the proximity of one interaction to another necessarily imposes an influence on neighboring binding interactions.

Generally, it is accepted that the individual binding forces that promote host-guest association do not act independently i.e. one binding interaction can influence another, leading to cooperative binding. The thermodynamic driving force for cooperative binding is poorly understood, and the work reported here is

directed at deciphering the component ΔH° and ΔS° values for cooperative binding of ion-pairing interactions in water.

3.1 THEORY OF COOPERATIVITY

There are two fundamental approaches to the concept of cooperativity that need to be distinguished for the purposes of the research presented here. The most familiar type of cooperativity is that of allosteric cooperativity, which is best exemplified by the binding of molecular oxygen to hemoglobin. The binding of one oxygen molecule to one sub-unit of hemoglobin induces a conformational change. Thus, the binding affinity of a second O_2 molecule is stronger than the first. This proceeds with the third and fourth binding of O_2 to hemoglobin being stronger than the previous. The enhanced binding of each O_2 has its origins in allosterics. This is quite different from the type of cooperativity we focus on here.

The fundamental difference lies in the nature of the interacting species. The cooperativity addressed here derives from the presence of multiple interactions working together, but unlike the hemoglobin-O_2 complex, the interacting moieties on both the host and the guest are tethered. This aspect of cooperative binding is present in several natural systems.[1] The theoretical and mathematical definitions for this type of cooperative binding have been addressed by both Jencks and Williams.

The thermodynamics of cooperativity is discussed in a theoretical analysis by Jencks, in which he argues that entropy is the driving force for positive coopertivity.[2] The basis of Jencks's proposal relies on an analysis of a protein containing two binding pockets that are complimentary to A and B moieties. In his analysis, both A and B bind to their respective pockets. However, when A is tethered to B (A-B), the binding of A will assist the binding of B by increasing the effective molarity of B, thereby imposing an entropic gain on the binding pair (Figure 3.0). The price for unfavorable entropy derived from the association of

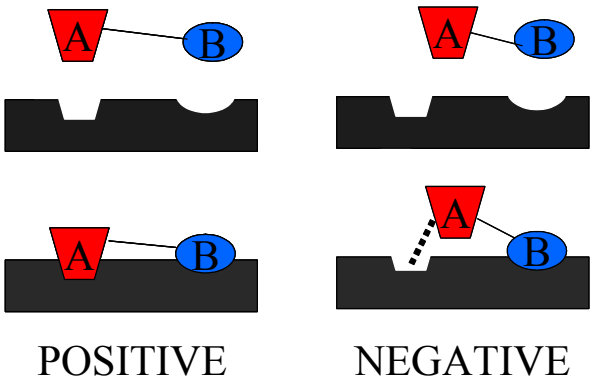


Figure 3.0 Jencks' Model of Cooperativity. The introduction of a tether of sufficient length between A and B provides an entropic advantage to binding A-B vs A and B individually; thereby demonstrating positive cooperativity. A tether of insufficient length hinders the A-B guest from binding achieving its full enthalpic potential; leading to negative cooperativity.

the binding partners is paid once in the case of the tethered moieties. Jencks proposes that the Gibb's free energy of binding A-B (ΔG°_{AB}) is a summation of the free energies of binding for the individual parts A (ΔG°_{A}) and B (ΔG°_{B}) plus an additional term, the Gibb's free energy of connection (ΔG°_{s}) that arises from the presence of the tether (Eq. 3.1). A positive ΔG°_{S} represents positive cooperativity and a negative ΔG°_{S} represents negative cooperativity. In Jencks' analysis negative cooperativity can arise from decreased enthalpy, for example, if the tether is of insufficient length to allow the binding moieties to realize their full enthalpic potential.

$$\Delta G^\circ_{S} = \Delta G^\circ_{A} + \Delta G^\circ_{B} - \Delta G^\circ_{AB} \qquad (3.1)$$

Recent work by Williams and Westwell[3,4] provides another approach to defining the thermodynamic parameters that characterize cooperativity. Their analysis relies on enthalpy-entropy compensation effects[5-7], in which high affinity complexes display larger exothermicities and less residual motion. Williams proposes that a complex held together by non-covalent interactions has less residual motion than that of a complex held together by a less extensive network of similar interactions. The enhanced binding derives from the increased enthalpy of the interactions that are enforced by the presence of the tether. The increased enthalpy is a consequence of shorter contacts between the binding groups in A-B

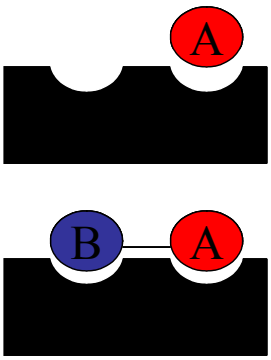


Figure 3.1. Williams' Model of Cooperativity. In this analysis the tether leads to increased binding interactions compared to just A or B. The increased binding interactions lead to a tighter host-guest complex. Positive cooperativity should have a significant enthalpic contribution.

with the host *vs* A and B with the host individually (Figure 3.2). Williams proposes that positive cooperativity will have a significant enthalpic component, and that negative cooperativity can arise from either enthalpy or entropy, depending on the extent to which they compensate each other.

Experimentally, Willimas[8] showed that the binding energy of glycopeptide antibiotics increased with increasing number of binding interactions. He also reported that the higher binding complexes were characterized by a tighter binding complex having shorter contact distances between funtional groups as determined by ^{1}H NMR studies.

3.2 LITERATURE SURVEY

Investigations addressing the concept of cooperativity, as defined by Jencks, with synthetic host-guest systems represent a small portion of the molecular recognition literature. Two such examples can be found in work by Anslyn[9] and Scheeren[10]. Anslyn describes the binding of cyclohexanediol to a polyaza cleft, and Scheeren details the binding of resorcinol guests to a "clip-like" receptor. The Jencks equation is used to determine the type of cooperativity present in their respective host-guest systems. The authors quantify the strength of the host-guest complex formation, as well as that of the binding of the guest to different portions of the host molecule. In both cases they conclude that positive cooperativity is present. These studies demonstrate how the Jencks equation is used, but the studies do not attempt to determine the thermodynamic origin of the measured cooperativity.

Inquiries into the dominant thermodynamic parameter of cooperative binding can be found in work by both Breslow and Whitesides. Breslow and co-workers reported a study on the binding of a series of *bis*-β-cyclodextrin receptors with guests containing two hydrophobic regions (Figure 3.2).[11] The binding energies of the guests were reported to be smaller than the sum of binding energies of the individual hydrophobic regions to the cyclodextrin cavity; the system displayed negative cooperativity. Analyses of the entropy and enthalpy

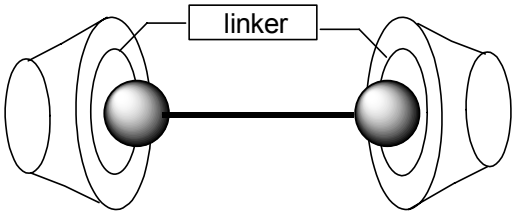


Figure 3.2 Binding Scheme for Breslow's Work. The cone shaped structures represent the cyclodextrin cavities tethered by a linker. The guests used in the study comprised of two hydrophobic moieties adjoined by a linker. The thermodynamic parameters of the host-guest complex shown were compared to those of the monovalent analogue.

changes in binding indicate that there was a loss in entropy upon binding the guest relative to the parts. There was also an increase in enthalpy for guest binding relative to that of one cyclodextrin-guest interaction, indicating that enthalpy-entropy compensation was operative.

Whitesides[12] investigated the binding of a trimeric host-guest system. The host consisted of three vancomyacin units adjoined by a tether, and the guest contained three peptidic arms tethered in a similar fashion (Figure 3.3). The

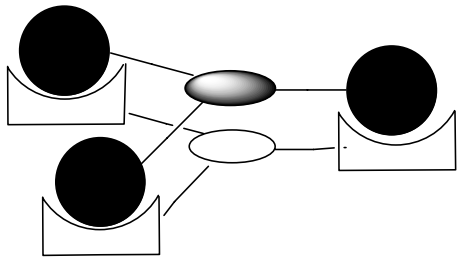


Figure 3.3 Whiteside's Trimeric System. The individual vancomyacin units (unshaded pockets) are tethered at a central point. The interacting guest consists of three peptide units (shaded circles) adjoined by a tether.

binding studies indicated that overall binding was less than a summation of the individual interactions. Further analysis identified entropy as the thermodynamic origin of the observed negative cooperativity. The authors proposed that the loss in entropy was due to conformational changes upon binding.

These studies represent a growing interest in attempting to understand the fundamental thermodynamic parameters that characterize cooperative binding in simple host guest systems. The research introduced here specifically characterizes the cooperative effects obtained from increasing the number of electrostatic interactions between host and guest in aqueous media.

3.3 EXPERIMENTAL APPROACH

The research presented here employs a rationally designed synthetic receptor to explore the thermodynamic parameters of cooperative binding. The approach to deciphering the thermodynamic origin of cooperativity introduced herein relies on the quantification of the ΔH° and ΔS° values for A-B, A, and B as guests. We propose to dissect the ΔH° and $T\Delta S^\circ$ values, analogous to Jencks' analysis, to give ΔH_s° and $T\Delta S_s^\circ$ terms (Eq 3.2 and Eq 3.3). As with the Jencks'

$$\Delta H_s^\circ = \Delta H^\circ_A + \Delta H^\circ_B - \Delta H^\circ_{AB} \quad (3.2)$$

$$T\Delta S_s^\circ = T\Delta S^\circ_A + T\Delta S^\circ_B - T\Delta S^\circ_{AB} \quad (3.3)$$

analysis, the signs of the resulting ΔH_s° and $T\Delta S_s^\circ$ values are important to our analysis. A positive ΔH_s° value indicates that the binding enthalpy change for A-B is more favorable that the sum of the enthalpy changes for A and B. Conversely, negative ΔH_s° values tell us that the binding enthalpy change for A-B is less favorable than the combined enthalpy changes of A and B. A positive sign with regard to the $T\Delta S_s^\circ$ value indicates that the binding entropy change for A-B

is not as favorable as the combined entropy changes for A and B. It follows that a negative $T\Delta S_s^\circ$ means that the binding entropy change of A-B is more favorable than the sum of the entropy changes for A and B.

Having established a mathematical basis for our studies we sought to investigate whether increased ion-pairing interactions would lead to increased binding, and whether this lead to increased enthalpy or entropy changes. Based on prior studies (discussed above) we expected entropy to dominate the Gibbs free energy of binding, but it was not clear if the entropy or enthalpy would increase as the number of ion-pairs increased. Secondly, we wanted to discover if the increased binding affinities were indicative of positive or negative cooperativity as defined by Jencks. We expected to find negative cooperativity. Lastly, we intended to discover whether the cooperativity, positive or negative, was a result of favorable or unfavorable enthalpies and entropies of connection.

The host was chosen to provide a total of four binding sites to complement guests having negatively charged functional groups (carboxylates). The energetics of the binding of guests, having one to four functional groups, to the host were quantified and used to dissect the contributions of the thermodynamic parameters of the "parts" to those of the "whole".

3.3.1 Design Criteria

Receptor **2.4** was studied in order to explore the origins of cooperativity on a synthetic host/guest system that relies primarily on ionic interactions. The host as described previously, features a C_{3v} symmetric cavity derived from the pre-organization of a tripodal ligand around a Cu(II) center, reminiscent of receptors from Fabbrizzi and others. The Cu(II) center and the three ammonium groups on the periphery of the cavity were intended to provide a total of four binding sites for anionic guests such as oligo-carboxylate guests. Host-guest binding for this study was expected to occur through the action of multiple

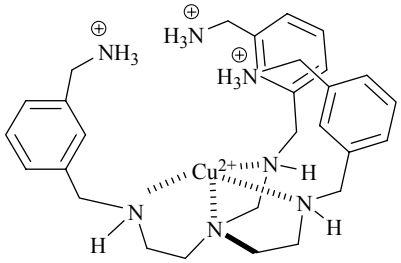
complementary electrostatic interactions between functional groups of each of the binding partners.

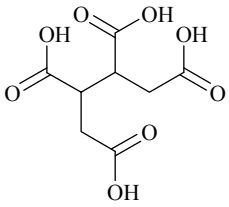
3.3.2 Synthesis

The synthesis of the receptor was described previously in Chapter 2.

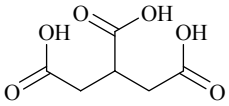
3.3.3 Binding Studies

A series of carboxylates were used as guests for **2.4**. The binding of 1,2,3,4-butanetetracarboxylate, tricaballate, glutarate, and acetate to **2.4** were quantified by observing the change in the UV/Vis spectra of **2.4** as aliquots of a guest solution were added to a HEPES buffered (5 mM) solution of **2.4**. The binding curves generated for each guest were fit with a 1:1 binding algorithm[13] to yield binding constants (Table 3.1). These data reveal that the tetracarboxylate and tricarballate complexes with **2.4** have binding affinities on the same order of magnitude (both near $K_a = 10^5\ M^{-1}$, near -7 kcal/mol), but the binding of the tetracarboxylate is stronger by around 0.5 kcal/mol. Conversely, the binding constant for glutarate to **2.4** is almost two orders of magnitude smaller ($K_a = 2 \times 10^3\ M^{-1}$, -4.5 kcal/mol) than for the tetra- and tricarboxylate. Acetate has a

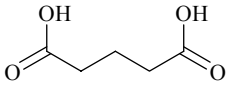




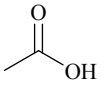
1,2,3,4-butanetetracarboxylic acid



tricarballic acid



glutaric acid



acetatic acid

smaller binding constant (K_a = 9.0 x 10^2 M^{-1}, -4.1 kcal/mol) than glutarate, as it can only interact with one of the four binding sites on **2.4**, the most likely site being the Cu(II) center. This affinity is reasonable because it is comparable to a binding affinity of 50 M^{-1} determined for the binding of acetate to phenanthroline bound Cu(II) center in water.[14] Yet, the increase observed for glutarate over aetate is not large, differing by 0.5 kcal/mol.

A Guest	K_a (M^{-1})	
	UV/Vis[a]	ITC[b]
1,2,3,4-butanetetracarboxylate	2.0×10^5 ($\pm 2 \times 10^4$)	1.8×10^4 ($\pm 1 \times 10^3$)
tricarballate	9.0×10^4 ($\pm 4 \times 10^3$)	1.9×10^4 ($\pm 3 \times 10^3$)
glutarate	2.0×10^3 ($\pm 2 \times 10^2$)	4.6×10^2 ($\pm 1 \times 10^2$)
acetate	9.0×10^2 ($\pm 2 \times 10^2$)	3.3×10^2 ($\pm 1 \times 10^2$)

B Guest	ΔG° (kcal/mol)		ΔH° (kcal/mol)	TΔS° (kcal/mol)
	UV/Vis	ITC		
1,2,3,4-butanetetracarboxylate	-7.30	-5.80	-0.29 (±0.01)	+5.39
tricarballate	-6.82	-5.82	-0.47 (±0.01)	+5.37
glutarate	-4.54	-3.60	+3.28 (±0.5)	+6.83
acetate	-4.10	-3.43	+0.71 (±0.5)	+4.12

Table 3.0 Binding Affinities and Thermodyanmic Parameters of Anions to 2.4. aThe UV/Vis data obtained form the addition of 5μL aliquots of a 15.0 mM solution of guest to a solution buffered with HEPES (5 mM) of 1 (0.69 mM) at pH 7.4. aThe ITC data was obtained for a binding isotherm generated from 40 injections of a 20.0 mM solution of 1 to a 1.18 mM solution of guest solution buffered with HEPES (10 mM) at pH 7.4. NOTE: The values obtained from the ITC data were corrected for the heat generated from dilution of the host. For the tetracarboxylate and the tricarballate the points at the end of the titration curve were slightly less exothermic than the parallel dilution titration, therefore the values were adjusted accordingly to provide a more realistic curve fit.

Inspection of the binding constants indicates that there is indeed cooperativity between the binding groups on the host for the complex formation, for the binding affinities increase with various guests when proceeding from the acetate to the glutarate to the tricarballate, and the tetracarboxylate. However, it is clear that the addition of another carboxylate to tricarballate, giving a "tetracarboxylate", does not result in a significant increase in affinity; therefore cooepprativity is expected to be strongly negative. Similarly, the addition of a

carboxylate to acetate, giving "glutarate" does not significantly increase the binding. In contrast, a relatively large increase in affinity is observed when one or two carboxylates are added to glutarate (giving tri- or tetra-carbalylate). Less obvious is whether the cooperativity in comparing glutarate to the tri- and the tetracarboxylate is positive or negative, and whether the associated increase in affinity has an enthalpic or entropic origin.

The binding of the analytes to **2.4** were also characterized by ITC to further probe the enthalpic/entropic origin of the cooepprativity. In these studies, 5μL aliquots of a solution of **2.4** were added to a HEPES buffered (10 mM) solution of guest. The data show the same general trend of binding affinities throughout the series of carboxylate guests (Table 3.0). The binding constants were found to range from 1.8 to 1.9 $x10^4$ M^{-1} (-5.8 kcal/mol) at the high end for the tetracarboxylate/tricarballate, and as low as 3.3 x 10^2 M^{-1} (-3.4 kcal/mol) for acetate. These values are slightly depressed compared to those from the UV/Vis titrations, possibly due to competition from the buffer, which was used at a higher concentration in the ITC experiments. Importantly, the affinity constants, and therefore the Gibbs free energies of binding, from both the ITC and UV/Vis data are comparable. The trend is identical: the acetate and glutarate binding affinities are comparable, and the tricarballate and 1,2,3,4-butanetetracarboxylate binding affinities are on the same order of magnitude. Additionally, there is an increase in affinity when comparing acetate or glutarate to either the tricarballate or 1,2,3,4-butatnetetracarboxylate.

We are confident that a carboxylate-Cu(II) ligation is the primary interaction for the bidning of any oligocarboxylate to **2.4** based on the Gibbs free energy of binding for acetate, which is between 3.5 to 4.0 kcal/mol. The ammonium groups play an important role, as they add 2.5 to 3.2 kcal/mol in binding energy for potentially three ammonium-carboxylate interactions. This total is less than a single Cu(II)-carboxylate interaction.

It is likely that only two ammonium-carboxylate interactions are gained when comparing acetate to 1,2,3,4-butanetetracarboxylate, because the binding of the tetracarboxylate is almost identical to that of the tricarballate. Tricarballate can only form two ammonium-carboxylate interactions since one of the carboxylate interacts with the Cu(II) center. Our determination of 2.5 – 3.2 kcal/mol for two, potentially three ammonium-carboxylate interactions is consistent with literature values. Schneider has reported that on average an ammonium-carboxylate interaction is worth 1.2 kcal/mol in water.[15] Fersht has determined 3-9 kcal/mol for hydrogen bond formation between charged partners within hydrophobic enzyme active sites.[16, 17] Additionally, Fersht estimated that neutral hydrogen bonds are worth 0.5 to 1.8 kcal/mol in water in natural systems.[16, 17] We are not aware of many examples in which the enthalpy and entropy changes associated with carboxylate-ammonium interactions in water. However, Rebek has reported that a hydrogen bond in water is worth 0.2 kcal/mol in Gibbs free energy of binding, with an average ΔH° value of -0.8 kcal/mol and an average $T\Delta S^\circ$ value of -1.5 kcal/mol.[18]

3.4 ANALYSIS OF COOPERATIVITY

The series of carboxylate guests provides an opportunity to quantitatively analyze the presence of cooperativity in the binding of the guests using Jencks' and Williams' A-B *versus* A and B approach. The tetra-carboxylate (the whole) can be thought of as a combination of the tricarballate and the acetate (the parts) or as a combination of two glutarates (the parts). Similarly, tricarballate can be derived from glutarate and acetate. This "whole" *versus* the "parts" analysis explores the additivity of the ΔG° values of the parts (ΔG°_A, ΔG°_B) compared to the overall ΔG°_{AB} (Table 3.1), giving the Gibbs free energy of connection.

The analysis can be done using either the UV/Vis or ITC data. The trends are the same, and to simplify the discussion the UV/Vis data is discussed and the ITC data is given here in parentheses. The tabulated values indicate that $\Delta G°_S$ for both the tetracarboxylate are -3.62 kcal/mol (-3.43 kcal/mol from ITC)) and -1.78 kcal/mol (-1.40 kcal/mol from ITC) when the guest is treated as a combination of tricarballate and acetate or two glutarate molecules respectively. Negative cooperativity is indicated for either analysis. The largest negative cooperativity arises when the acetate-tricarballate pair is compared to the tetracarboxylate binding. We indicated above that this is expected, as the affinities of the tricarballate and 1,2,3,4-tetracarboxylate are the same within experimental error for both the UV/Vis and ITC techniques. It is interesting to note that the cooperativity obtained when analyzing the increase in affinity when going from glutarate to the tetracarboxylate or tricarboxylate is negative. When the tricarballate is treated as a combination of glutarate and acetate, negative cooperativity is again found, with a magnitude of -1.78 kcal.mol (-1.23 kcal/mol from ITC).

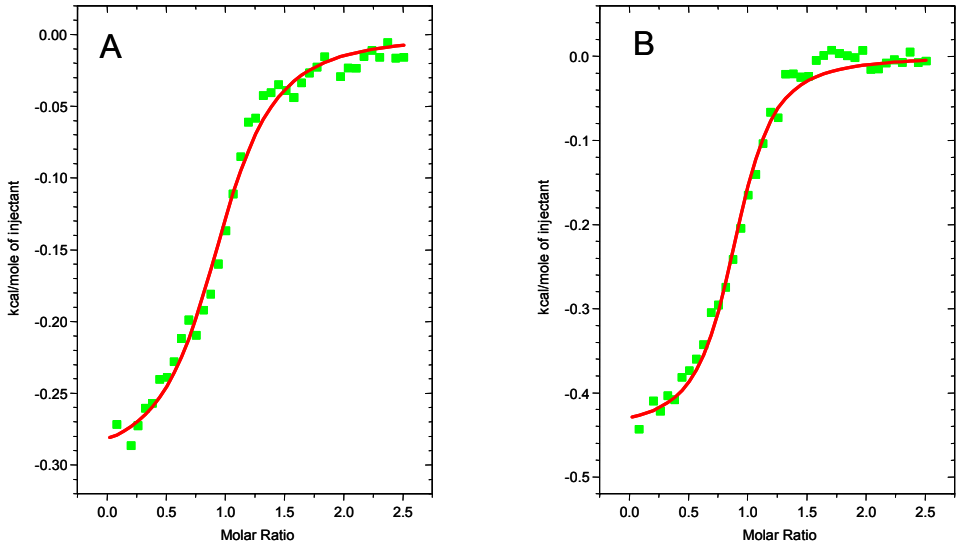


Figure 3.4 Binding Isotherms. Binding isotherms generated from ITC titrations of 1,2,3,4-butanetetracarboxylate and tricarballate. A) Aliquots of a buffered (HEPES 10mM) solution containing receptor 2.4 (20.0 mM) were added to a buffered solution of 1,2,3,4-butatnetetracarboxylate (1.18 mM). The data was fit with a 1:1 binding algorithm. B) Aliquots of a buffered (HEPES 10mM) solution containing receptor 2.4 (20.0 mM) were added to a buffered solution of tricarabllate (1.18 mM). The data was fit with a 1:1 binding algorithm.

3.4.1 Validity of A-B vs A and B analysis

The manner in which we approach the 'whole' *versus* the 'parts' analysis is not identical to Jencks' analysis. For example, let's consider the dissection of the tetracarboxylate into acetate and tricarballate. The ΔG° value for the acetate component reflects the binding of a carboxylate to the Cu(II) center, whereas the tricarballate ΔG° value reflects the binding of one carboxylate group to the Cu(II) center and two carboxylate groups to two ammonium groups. Therefore, the analysis includes a Cu(II)-carboxylate interaction for both the A and B fragments. A comparison more appropriate to Jencks' A-B *versus* A and B dissection would

be the combination of only one Cu(II)-carboxylate interaction with three ammonium-carboxylate interactions.

Therefore, the manner in which the A-B guests are cut into their A and B parts in this study is not exactly as that defined by Jencks. The justification of our dissection procedure relies on the treatment of all the components simply as ion-pairing interactions. In this approach we correlate cooperativity to the number of ion-pairs formed; we are not concerning ourselves with the specific nature of the ion pairs. This is an important caveat to this study.

The strength of ion-pairs vary, and the data demonstrate that a Cu(II)-carboxylate pair has a more favorable free energy of binding than an ammonium-carbaoxlyate pair. As described below, the thermodynamic driving force for a Cu(II)-carboxylate interaction derives from an increase in entropy. The binding does not result from stronger electrostatic interactions between the Cu(II) and the carboxylate relative to the solvation of the Cu(II) and the carboxyalte (it is actually slightly endothermic). Further, our study is consistent with literature precedent which reports that Cu(II)-carboxylate binding is driven by entropy, not enthalpy.[19,20] This is analogous to recent literature which indicates that ion-pairing interactions between organic functional groups in water are primarily entropy driven; therefore we expect an ammonium-carboxylate interaction to result in an increase in entropy.[21-24] The important thing to note here it that for our analysis the 'parts' have the same fundamental driving force: entropy.

The forthcoming analysis addresses two issues: 1) will the tethering entropy driven interactions give rise to a favorable enthalpy? and 2) will the entropy of connection be positive or negative?

3.4.2 Interpretation of Enthalpy and Entropy Changes as Related to Cooperativity

Before examining the 'whole' *versus* the 'parts' analysis, we describe the most obvious interpretation of the enthalpy and entropy changes of binding (Table 3.1) The $\Delta H°$ values are exothermic for the tetra- and tri-carboxylate guests (-0.29 kcal/mol and -0.47 kcal/mol). However, the $\Delta H°$ values are endothermic for both the glutarate (+3.2 kcal/mol) and acetate guests (+0.71 kcal/mol). Additionally, the binding of all four guests is characterized by a positive $T\Delta S°$ term.

The endothermic binding of glutarate and acetate to **2.4** indicates that the primary mode of binding to the metal center has an unfavorable enthalpy change as the guest exchanges for counterions and/or solvent on the Cu(II) center, but their release still drives the binding. The endothermic binding of a carboxylate to a Cu(II) center has been described previously, and was attributed to reorganization of solvent molecules.[19,20] While the binding of the tetra- and tricarboxylate guests is primarily entropic, the additional carboxylates relative to glutarate and acetate offer favorable enthalpy to enhance the association through increased electrostatic interactions. With increasing ammonium-carboxylate interactions, the endothermic binding of acetate and glutarate becomes exothermic. Williams postulates that as the interactions increase between a host and a guest, they become increasingly exothermic because there is less residual motion and their contacts are tighter. The data reported here supports this.

The favorable entropy change observed for ion-pairing interactions is postulated to arise from the displacement of waters of solvation and/or counter ions from both the host and the guest into solution, thereby increasing the entropy of the system as a whole.[25-28] One would expect the solvent displacement by the tetra- and tri- carboxylate to be greater relative to glutarate and acetate, due to increased ion-pairing. However, the $T\Delta S°$ values are similar for the tetra-,tri-

carboxylates and glutarate, but indeed lower for acetate. It is likely that the increase in favorable entropy changes derived from solvent release with the larger anions is in part opposed by a decrease entropy due to decreased residual motions in the complex as more binding contacts are formed. This makes the tetra- and tri- carboxylate show a decreased $T\Delta S°$ value relative to glutarate, with a concomitant increase in favorable $\Delta H°$ as reflected in the data.

3.4.3 Origin of Negative Cooperativity

The enthalpy and entropy values can be used to further characterize the thermodynamics of the "whole" *versus* the "parts" using equations 3.2 and 3.3 (Table 3.2). Treatment of the tetra-carboxylate as the combination of tricarballate and acetate gives a positive $\Delta H_s°$ value (+0.53 kcal/mol) and a positive $T\Delta S_s°$ value (+4.12 kcal/mol). The data show that there is a gain in enthalpy when binding A-B (tetracarboxylate), i.e. the enthalpy change of binding A-B to **2.4** is more exothermic (-0.29 kcal/mol) than the combined enthalpy changes of A (tricarballate) and B (acetate) individually (+0.24 kcal/mol). This arises from having more ion-pairing interactions and/or the presence of shorter contact distances between binding functionalities. However, the $T\Delta S°$ values show that the binding of A-B is favorable; i.e. positive (+5.39 kcal/mol), but to a lesser extent than simple summation of the $T\Delta S°$ values of binding of A and B separately (+9.51 kcal/mol).

This difference in $T\Delta S°$ values for binding A-B relative to A and B needs to be explained as having three contributing factors: 1) the presence of the tether, 2) the residual motion of the guest, and 3) the role of the solvent and/or counterions. As Jencks' discussed[2], the loss in translational entropy paid once in the case of A-B versus twice in the case of A and B offers an entropic gain to the binding of A-B. Conversely, the binding of A-B places more restrictions on the residual motions (vibrational and rotational) compared to those of A and B

separately, thereby introducing an entropic loss to the binding of A-B *versus* A and B. Further, the associations of A-B, A, and B to the host all displace solvent and/or counter ion molecules from the binding pocket, leading to an increase in the entropy of the system. It appears that the enthalpy gain of binding A-B is outweighed by the contribution of the loss of residual motion and/or decreased solvent release, thereby identifying entropy as the thermodynamic origin for the observed negative cooperativity.

The tetracarboxylate (the whole) can also be treated as the combination of two glutarate molecules (the parts). The thermodynamic data for this analysis indicates that the enthalpy change for the association of A-B (tetracarboxylate) is more exothermic (-0.29 kcal/mol) than summation of the $\Delta H°$ values (+6.57 kcal/mol) for A (glutarate) and B (glutarate) separately. This gain in enthalpy is analogous to the tricarballate-acetate pair. However, it is interesting to note that the gain in enthalpy for binding A-B *versus* A and B for the glutarate-glutarate pair is greater than that of the tricarballate-acetate pair. The values for the glutarate-glutarate pair reveals a positive $T\Delta S°$ (+5.39 kcal/mol) for the binding of the A-B system, but it is again less positive that the summation of the $T\Delta S°$ values (+13.67 kcal/mol) for A and B. The glutarate-glutarate combination demonstrates a greater loss in entropy for A-B *versus* A and B than the tricarballate-acetate pair. As discussed above the apparent gain in enthalpy for the A-B system is outweighed by the loss in residual motion and/or decreased solvent release, which highlights entropy as being the origin of the negative cooperativity. The glutarate-glutarate pair has a greater gain in enthalpy, which compensates for the

A-B	A + B	$\Delta G°_{AB}$ (kcal/mol)	$\Delta G°_{A} + \Delta G°_{B}$ (kcal/mol)	$\Delta G°_{S}$ (kcal/mol)
UV/Vis				
1,2,3,4-butanetetracarboxylate	Tricarballate + acetate	-7.30	-10.92	-3.62
Tricarballate	Glutarate + acetate	-6.82	-8.60	-1.78
1,2,3,4-butanetetracarboxylate	Glutarate + glutarate	-7.30	-9.08	-1.78
ITC				
1,2,3,4-butanetetracarboxylate	Tricarballate + acetate	-5.80	-9.25	-3.43
Tricarballate	Glutarate + acetate	-5.82	-7.05	-1.23
1,2,3,4-butanetetracarboxylate	Glutarate + glutarate	-5.80	-7.20	-1.40

A-B	A + B	$\Delta H°_{AB}$ (kcal/mol)	$\Delta H°_{A} + \Delta H°_{B}$ (kcal/mol)	$\Delta H_{s}°$ (kcal/mol)
1,2,3,4-butanetetracarboxylate	Tricarballate + acetate	-0.29	+0.24	+0.53
Tricarballate	Glutarate + acetate	-0.47	+4.00	+4.47
1,2,3,4-butanetetracarboxylate	Glutarate + glutarate	-0.29	+6.57	+6.86

		$T\Delta S°_{AB}$ (kcal/mol)	$T\Delta S_{A} + T\Delta S_{B}$ (kcal/mol)	$T\Delta S_{s}°$ (kcal/mol)
1,2,3,4-butanetetracarboxylate	Tricarballate + acetate	+5.39	+9.51	+4.12
Tricarballate	Glutarate + acetate	+5.37	+10.98	+5.61
1,2,3,4-butanetetracarboxylate	Glutarate + glutarate	+5.39	+13.67	+8.28

Table 3.1 Enthalpy and Entropy of Connection. The values tabulated above show the Gibbs free energy of connection calculated from both the UV/Vis and ITC data. Additionally, the analyses using the mathematical definitions outlined above were used to determine the enthalpy and entropy of connection.

greater loss in entropy when compared to the tricarballate-acetate pair. This is reflected in the $\Delta G°_{S}$ terms, in which the glutarate-glutarate pair is less negatively cooperative than the tricarballate-acetate pair.

Lastly, we examine the tricarballate guest as a combination of glutarate and acetate. As in the case of the other two A-B pairs, tricarballate binding is more exothermic (-0.47 kcal/mol) than the summation of the enthalpy changes (+4.00 kcal/mol) for the individual parts. Further, as found for the other two A-B

pairs, the $T\Delta S°$ value (+5.37 kcal/mol) for binding tricarballate is positive, albeit smaller than the summation (+10.98 kcal/mol) of the $T\Delta S°$ terms for the glutarate and the acetate. This again indicates that the gain in enthalpy for binding A-B is outweighed by the loss in residual motion and/or lower solvent release, pointing to entropy as the source for the negative cooperativity.

Of the three contributions to the entropy of binding, two factors contribute to the reduced entropy of binding A-B *versus* A and B: loss of residual motion and decreased solvent and/or counterion release in binding A-B *versus* A and B. In all three analyses above, we postulate that the smaller release of solvent/counterions is the major contributor. The experimental data show that the $T\Delta S_s°$ term is highly unfavorable, and it seems unlikely that this is primarily a result of decreased residual motions in binding A-B *versus* A or B individually. Thus, decreased solvent and/or counterion release upon binding A-B *versus* A and B must contribute significantly.

The introduction of a tether or covalent bond inherently leads to the occupation of smaller volume within the host cavity. Additionally, individual A and B molecules have a larger solvation sphere than an A-B molecule, therefore upon binding A-B fewer solvent and/or counterion molecules are released to bulk solution.

3.5 CONCLUSIONS

In summary, the experimental approach reported herein demonstrates the efficacy of using a synthetic receptor to explore the thermodynamic origin of cooperativity through binding a series of carboxylate containing guests. The data suggests that there is an entropic component that contributes to the negative cooperativity of the host/guest complexes in water. This entropic contribution may arise from loss in residual motions and/or attenuated solvent or counter ion

release. The data is in accord with Williams' model of cooperativity. We propose that reduced solvent/counter ion release in binding A-B *versus* A and B individually is dominant. This is reasonable when considering the occupied volumes of the guests and the relative amounts of solvent release.

Although isolated to a specific host-guest system, these results provide a first look into the thermodynamic origin of cooperativity in ion-pairing molecular recognition using a synthetic receptor in aqueous media. The study highlights the strength and value of the experimental approach, as it can be used to explore the cooperativity of different binding interactions within a single host design. Insights into the thermodynamic profile of binding and cooperativity can advance the field of molecular recognition to yet another level as we attempt to understand the energetics of the binding forces that promote host-guest complexation.

3.6 EXPERIMENTAL

General Considerations

The chemicals used were obtained from Aldrich and were used without further purification, except where noted. The water used for the titrations was obtained from a millipure.

UV/Vis Titrations

The titrations were performed on a Beckman DU-640 UV/Vis instrument. A typical titration is described below, though concentrations varied from experiment to experiment. A solution of the receptor (4.87 mM) was prepared and buffered with HEPES (5mM) at pH 7.4. A similar solution of the guest (19.94 mM) was prepared. A cuvette was then filled with 860 μL of a HEPES (5mM) solution and scanned as the blank reading. 140 μL of the host solution was introduced to the

cuvette (total host concentration of 0.68 mM) and the absorbance recorded. Aliquots of a stock solution were then added to the cuvette and the absorbance recorded after each addition. The stock solution contained the host (0.68 mM) and guest (12.61 mM) in HEPES buffer (5mM). The absorbances for each addition, at a chosen wavelength, were used to calculate the delta absorbances relative to the first absorbance reading. These values were then plotted versus the concentration of the added guest for each aliquot. The binding isotherm from this raw data was curve fit using the 1:1 binding equation (either done manually in Excel or done iteratively in Origin).

Isothermal Titration Calorimetry

The titration apparatus was purchased from Microcal Inc. The VP-ITC instrument is interfaced with Origin (version 5) software for both data collection and data analysis. A typical titration is described, though concentrations and parameters varied from experiment to experiment. The reference cell was filled with a buffer solution (HEPES 10 mM) identical to that in the titration cell. The titration cell was filled with a HEPES buffered (10 mM, pH 7.4) solution of the guest (1.18 mM). The syringe was filled with approximately 250 μL of a solution of the host (20.0 mM) buffered with HEPES (10 mM, pH 7.4). The concentration of the syringe contents is typically 20 times that of the concentration of the cell contents. The syringe was fitted above the cell and the following parameters set: Injection size: 5 μL, Number of injections: 43, Temperature: 25°C, Injection Interval: 300 sec, Cell Feedback: 20 μcal. Following data collection the Origin software was used to apply a 1:1 binding algorithm to the data, the fit of which yields a binding affinity, enthalpy change, entropy change and binding stoichiometry for the titration.

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Chapter 4: Thermodynamic Studies on Higher Ordered Complex Formation for Host-Guest Pair

4.0 INTRODUCTION

A host guest complex that proceeds through the action of multiple ion-pairing interactions in aqueous media has an associated thermodynamic profile. In general predictions can be made based on analogous complexations in natural systems.

When two species are bound together by intermolecular forces, inherently they dissociate upon dilution to increase the entropy of mixing, which is related to an increase in translational and rotational entropy of the individual components. Nature counteracts the unfavorable entropy associated with holding complexes together by using a series of weak interactions that are favorable enthalpically. Hence, a large series of these weak interactions is required to counteract the unfavorable entropy. This is a general phenomenon. Additionally, solvation/desolvation processes can give rise to favorable entropy effects which also counteract the unfavorable change in translational and rotational entropy that result from the association process. In the final analysis, irrespective of how large and favorable the total enthalpy, or how large and favorable the total entropy resulting from desolvation, that holds two components of a complex together, continued dilution results in dissociation, due to the entropy of mixing, and the creation of simpler structures.

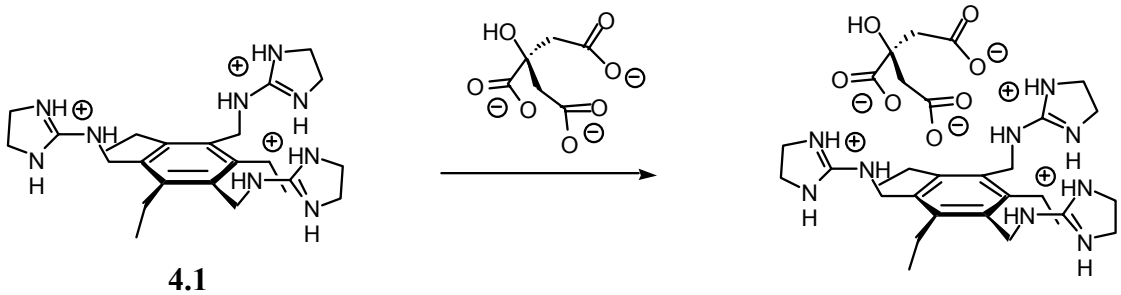
The goal of this research was to probe the energetics of ion-paring host guest formation in water. The initial results indicated that binding equilibria other

that 1:1 was present. This prompted a more detailed study into the energetics of higher order complex formation.

4.1 EXPERIMENT DESIGN

As implied in the discussion above, the relatively small enthalpy changes associated with weak non-covalent interactions, by themselves, are often not sufficient to render many supramolecular systems thermodynamically stable.[1] One usually introduces additional preorganization and/or functional group(s) to the monomeric components. The functional groups are included to increase the number of hydrogen bonds, ion-pairs, or other weak attractive forces. However, using built-in structure/function is inevitably accompanied by further loss of entropy, which often cancels the enthalpic gain obtained.[2-5]

We sought to use an already well studied host guest system from the Anslyn lab.[6] Compound **4.1** was reported to be selective for citrate in water with a binding affinity of $K_a = 6.8 \times 10^3\ M^{-1}$. The guanidinium groups are preorganized



4.1

on the hexasubstituted benzene scaffold. Their orientation to one face of the ring derives from the alternating ethyl groups which impart a facial bias. This allows citrate binding to proceed through ion-pairing interactions between the carboxylates and guanidinium functional groups.

4.2 AGGREGATION STATE OF COMPONENTS IN SOLUTION

Prior to investigating complex formation between **4.1** and citrate we sought to delineate the aggregation state of these structures under the experimental conditions to be employed. Dilution studies, mass-spectrometry and UV/Vis techniques were used.

4.2.1 Isothermal Titration Calorimetry Dilution Studies

The dilution of citrate in aqueous buffer solutions was studied using isothermal titration microcalorimetry. The heat patterns recorded were similar to a typical dilution of simple electrolytes[7] This supports the assumption that citrate (-3) does not self-associate under the experimental conditions employed.

Host **4.1** possesses a compact hydrophobic face consisting of a phenyl ring and three ethyl groups, and therefore has the potential to aggregate in water through the stacking of two aromatic rings and/or through micelle-like interactions of six ethyl groups from two molecules of **4.1**. Dilution of **4.1** was monitored as aliquots of a 75 mM solution of **4.1** in a 5 mM phosphate buffer (pH 7.4) were introduced to the reaction microcalorimetric cell containing with the same buffer solution. This exhibited relatively small endothermic heat effects which are consistent with the range of heat effects observed for the dilution of a simple electrolyte.[7] Though the heat effects were different for each of the components (heat patterns can differ depending on the nature of the salt), the patterns suggest that there is no significant complexation of **4.1** with the phosphate buffer.

On the slight chance that aggregation was occurring, it would be enhanced with increased salt concentration. Therefore, dilution of **4.1** (77.3 mM) was repeated under the same experimental conditions, but using an increased phosphate buffer concentration (103 mM). The heat of dilution of **4.1** is less endothermic compared to the corresponding experiment at lower buffer

concentration. Also, the change in heat absorbed at each titration at the higher salt concentration is lower than at low salt concentration (5 mM phosphate buffer; pH 7.4). Both features (less absolute magnitude of heat effect and the less steepness) are common for the dilution of conventional electrolytes.[7] The decreased steepness is readily attributed to the lower relative effect on the overall ionic strength of the solution with each injection at the high salt concentration versus those at low salt concentration (103 mM vs 5 mM phosphate buffer pH 7.4). The curves also show that even at the higher phosphate buffer concentrations, no significant complexation of **4.1** with phosphate is evident.

Thus, the results of our dilution microcalorimetric experiments give no indication of significant aggregation of **4.1** in the solution, even at the concentrations as high as 100 mM. The results also indicate that there is little to no direct competition for binding by the buffer, although affinity constants of near 50 M^{-1} or lower between phosphate and **4.1** likely would not be detected. This is consistent with a previous report of little to no binding between phosphate and **4.1** in water.[6]

4.2.2 Mass-Spectrometry Studies

A small degree of the aggregation of **4.1** in the range of several percent cannot be ruled out based on the dilution experiments, therefore electrospray ionization (ESI) mass-spectra were obtained to identify all species present in a solution of **4.1**. ESI measurements performed on a 100 mM solution of **4.1** using standard conditions indicate that the predominant species is monomeric, with some dimer, in a 97:3 ratio. Such data is not sufficient to conclusively state that 3% of dimer corresponds to the thermodynamically equilibrated monomer-dimer ratio in the solution. It is possible that a significant amount of the dimer existed in the original solution and then decomposed upon its transfer from solution into the gas phase. The ESI measurements were repeated using milder conditions.

The masses determined for the monomer species under milder ESI conditions correspond to the transfer of chloride ions from the solution phase into the gas phase together with **4.1**, indicating more ion-pairing under milder conditions. However, no increase in the dimer concentrations was observed. Therefore, the milder conditions did stabilize interactions with chloride in the gas phase, but does not support higher levels of dimerization.

4.2.3 Absorbance Aggregation Studies

In addition to the above experimental evidence, a Beer's law plot of UV-absorbance (270 nm) versus concentration of **4.1** shows no curvature, which is consistent with the existence of **4.1** in the solution in a monomeric form.[8] Therefore, based on microcalorimetric, ESI, and spectrophotometric data, both **4.1** and citrate are considered to exist as monomers in aqueous phosphate buffer solutions.

4.3 MICROCALORIMETRY INVESTIGATIONS

Having verified the propensity of both **4.1** and citrate to exist as monomers in a phosphate buffered solution, ITC experiments were pursued to evaluate the host-guest complex formation. Three types of titration conditions were evaluated: 1) low concentrations of host at low ionic strength, 2) high concentrations of **4.1** at low ionic strength, and 3) reverse titrations (host added to guest).

4.3.1 Low concentrations of 4.1 and low ionic strength.

The titration data using ITC techniques was recorded for the addition of citrate to a solution of **4.1** at different concentrations (Figure 4.0). The shapes of and inflections of each of the binding isotherms indicate that binding events other that simple 1:1 complexation events occurr as citrate and **4.1** interact. The

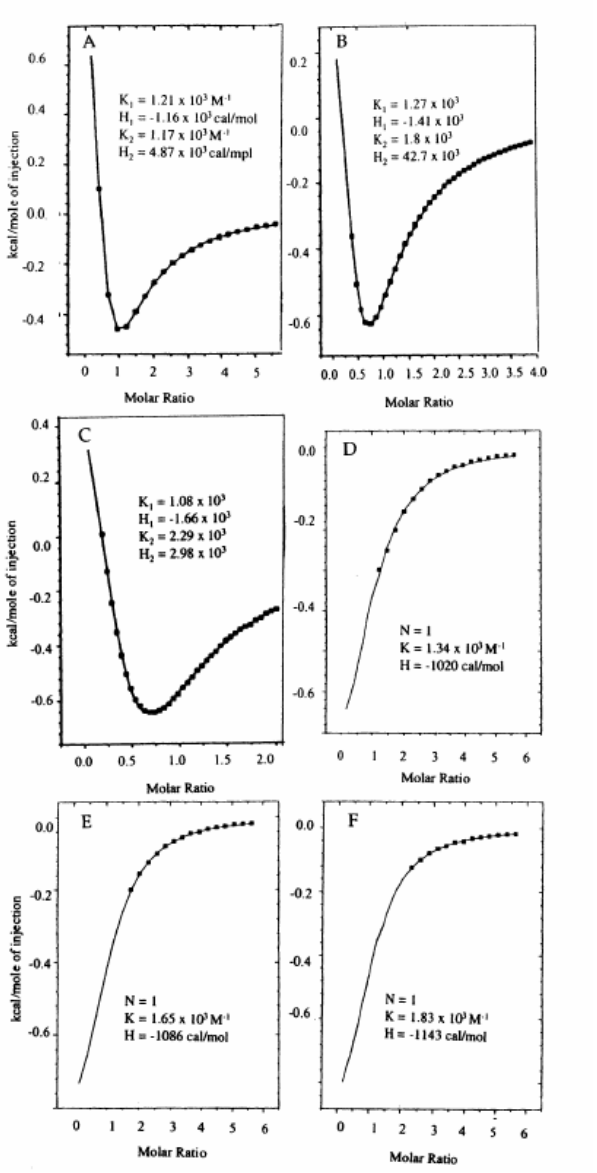


Figure 4.0 Binding isotherms. Each titration was performed using a 5 mM phosohate buffer at pH 7.4. A) Addition of aliquots of citrate (99.9 mM) to a solution of 4.1 (1.41 mM). B) Addition of 100.3 mM citrate to a 1.98 mM solution of 4.1. C) Addition of 51.7 mM citrate to a 1.98 mM solution of 4.1. The curve fit to the raw data plots are a result of using an identical interacting sites model with n = 2. D-F) These represent a gradual deletion of data points from the initial portion of the binding isotherm A. The curve fits are derived form using a 1:1 model.

complexation reaction that dominates at low citrate concentration (initial part of titration curves) is characterized partially by an endothermic (positive y-values and increasing negative values) heat effect. In contrast, the complexation reaction that predominates at higher citrate concentrations (beyond the inflection point) is exothermic (negative y-values). Because the first inflection occurs near 0.5 equivalents, a (citrate•**4.1**$_2$) complex is likely.

The simplest and the most reasonable theoretical model to fit titration curves presented in Figures 4.1a-c is a stepwise 2:1 complexation (Eqs. 4.1 and 4.2) (see experimental section). In light of the experimental data presented in Figures 4.1a-c, one should consider the following equilibria to occur in the reaction mixture of citrate and **4.1**:

$$\text{Citrate}^{-3} + \mathbf{4.1} = (\text{Citrate}\bullet\mathbf{4.1})^{0} \quad (4.1)$$

$$(\text{Citrate}\bullet\mathbf{4.1})^{0} + \mathbf{4.1} = (\text{Citrate}\bullet\mathbf{4.1}_2)^{+3} \quad (4.2)$$

The equilibrium constants reported for all fitting procedures (Figure 4.1a-c) are intrinsic equilibrium constants[9; 10](K_1^0, K_2^0) that result from applying the identical interacting sites model deconvolution method with n=2. These intrinsic equilibrium constants were recalculated to give K_1 and K_2 (Eqs. 4.3 and 4.4) using the relationship shown in equation 4.5. These K values are representative of the binding constants with the degeneracies removed, i.e. affinities assuming no interactions between binding groups. These K values were then used to determine ΔG° values (Table 4.0).

$$K_1 = [(\text{Citrate}\bullet\mathbf{4.1})^{0}] / [\text{Citrate}^{3-}][\mathbf{4.1}^{3+}] \quad (4.3)$$

$$K_2 = [(\text{Citrate}\bullet\mathbf{4.1}_2)^{3+}] / [(\text{Citrate}\bullet\mathbf{4.1})^{0}][\mathbf{4.1}^{3+}] \quad (4.4)$$

$$K_i = [(n-i+1)/i] \bullet K_i^o \qquad (4.5)$$

One must caution that the value of the uncertainty given by the ORIGIN program in the stepwise 2:1 complexation model is not a good criterion for judging the accuracy of the thermodynamic parameters obtained.[11] Several considerably different sets of parameters (K_1, ΔH^o_1; K_2, ΔH^o_2) can often result in good fits to the experimental curve. Therefore, the scattering of the experimental data points does not allow the ORIGIN program to find a single solution.[9; 12]

The majority of the experimental data points (Figures 4.0a-c) are located on the portion of the curve that exponentially decreases to zero. These data points correspond to the region where the formation of 1:1 species (exothermic enthalpy of formation) is predominant. Here, the thermodynamic parameters for formation of (Citrate•**4.1**)^0 complex from monomeric citrate and **4.1** are well defined. Indeed, the values of K_1 (Table 4.0) range from 2200 M^{-1} to 2580 M^{-1} (less than 10% from the average) and values of ΔH^o_1 are in the range from –4.85 kJ mol^{-1} to –6.95 kJ mol^{-1} (they deviate about 20% from the average). The quantitative estimations of the K_1 and ΔH^o_1 values were verified by applying a simple 1:1 model to the final part of the titration curves (Figures 4.0d-f). It is reasonable to assume that deletion of data points from the initial part of titration curve, endothermic formation of (Citrate•**4.1**$_2$)$^{3+}$, has little influence on experimental data for the later portion of the curve. Further, because several combinations of the four parameters (K_1, K_2, ΔH_1^o, ΔH_2^o) can fit the curve, one finds that there is only one set of parameters (K_1 and ΔH_1^o) that fits the later points in the titration. Also, because citrate is in excess at the final portion of the titration curve, formation of (Citrate•**4.1**$_2$)$^{3+}$ with excess citrate present becomes highly unlikely. As expected, a 1:1 curve fit improves with the continuous deletion of the early data points, and the K_1 and ΔH_1^0 values become more closely matched to the

range of the values obtained by the stepwise 2:1 complexation model (Figures 4.0a-c). Deletion of about one third of the data points (Figure 4.1f) followed by curve fitting with the 1:1 model results in K_1 equal to 1830 M^{-1} and ΔH^o_1 equal to –4.8 kJ mol^{-1}. This indicates that at large enough citrate/**4.1** ratios, the values obtained by both fitting procedures (simple 1:1 model and stepwise 2:1 complexation model) are comparable.

Given the above discussion, the estimation for K_1 and ΔH^o_1 values are reliable. However, the complexity of a stepwise 2:1 binding algorithm does not allow the same conclusion for K_2 and ΔH_2^0 values. Further, the magnitudes of these values vary depending on the experimental conditions, as discussed below.

4.3.2 Higher concentrations of 4.1 and low ionic strength

The above titrations were repeated using higher initial concentrations of **4.1** in the reaction cell. There were two reasons for doing this. First, the higher concentration of **4.1** increases the probability of higher order aggregation.[13] Second, it provides more data points with which to characterize the initial part of the curve where the endothermic processes dominate.

The results of the titrations using 100 mM citrate solution (in the syringe) into the reaction cell charged with 6.3 mM **4.1** in 5 mM phosphate buffer (pH=7.4) are presented in Figures 4.2a-c. In contrast to the experimental data obtained at the lower concentrations of **4.1,** the experimental data obtained at higher concentrations of **4.1** (Figure 4.1a) cannot be satisfactorily fit using a stepwise 2:1 complexation model. This result offers evidence for the existence of higher order complex species other than (Citrate•**4.1**)^0 and (Citrate•**4.1**$_2$)$^{3+}$ in solution, when **4.1** is at higher concentration. Large deviations from the stepwise 2:1 complexation model are observed only at very large excess of **4.1** in the solution (Figure 4.1a), but all the data points at the molar ratios larger than 0.5 are

well characterized by a stepwise 2:1 complexation model (Figure 4.1b). For completeness, we examined all the data using a stepwise 3:1 complexation model. This gives a good fit to all experimental data points because there are six parameters (K_1, ΔH^o_1; K_2, ΔH^o_2; K_3, ΔH^o_3) (Figure 4.1c). Furthermore, results of titration experiments using a higher concentration of **4.1** (11.2 mM) (Figure 4.1d) can also be described by stepwise 3:1 complexation model with a comparable set of parameters.

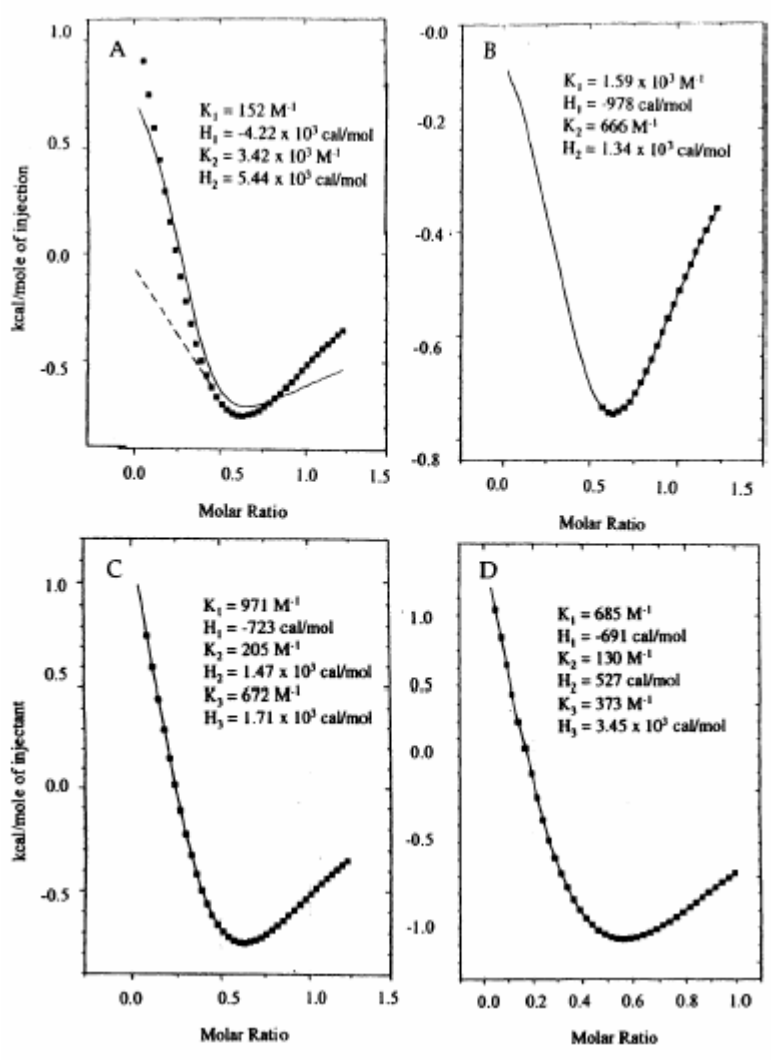


Figure 4.1 Binding Isotherms. Binding isotherm represents the titration of a 100.3 mM citrate solution into a solution of 4.1 (6.26 mM) using a phosphate buffer (5 mM pH 7.4). A) The solid line represents the resuting curve fit using a four parametric fit. The otted line is the four parametric durve fit from graph B. B) The curve fit is a four parametric fit for citrate:4.1 ratios greater than 0.5. C) This is a six parametric curve fit to all the data points. D) A six parametric curve fit to all data points resulting from a titration of a 145 mM solution of citrate into a solution of 4.1 911.3 mM).

An excellent curve fit using a stepwise 3:1 complexation does not verify the existence of (Citrate•**4.1**$_3$)$^{6+}$ in the solution, and indeed such an aggregate seems implausible. Intuitively, the existence of such a species is rather unlikely. A (Citrate•**4.1**$_3$) species having a +6 charge should possess a high affinity towards a second negatively charged (-3) citrate anion. Thus, it would be more logical to assume high order aggregation species with empirical stoichiometries near 1:1 such as (Citrate$_2$•**4.1**$_3$)$^{3+}$, (Citrate$_3$•**4.1**$_4$)$^{3+}$, etc. Note that these higher order aggregations occur at the lower concentrations of citrate and their formation is endothermic. This means the higher order aggregation is entropy driven.

4.3.3 Titration of citrate by a solution of 4.1.

We wanted to confirm the aggregation state and endothermic peaks, so we performed reverse titrations. The results of this titration, 77-107 mM **4.1** (in the syringe) into the reaction cell charged with 1.7-2.3 mM citrate solution in 5 mM phosphate buffer (pH=7.4), are presented in Figures 4.2a,b. As expected, excess **4.1** in the reaction cell (molar ratio > 1) during the latter part of titration experiment increases the probability of the formation of a (Citrate•**4.1**$_2$)$^{3+}$ species and endothermic heat effects again dominate. In principle, the predominant formation of (Citrate•**4.1**$_2$)$^{3+}$ species in the reaction mixture should facilitate

reliable determination of complexation thermodynamic parameters for these species.

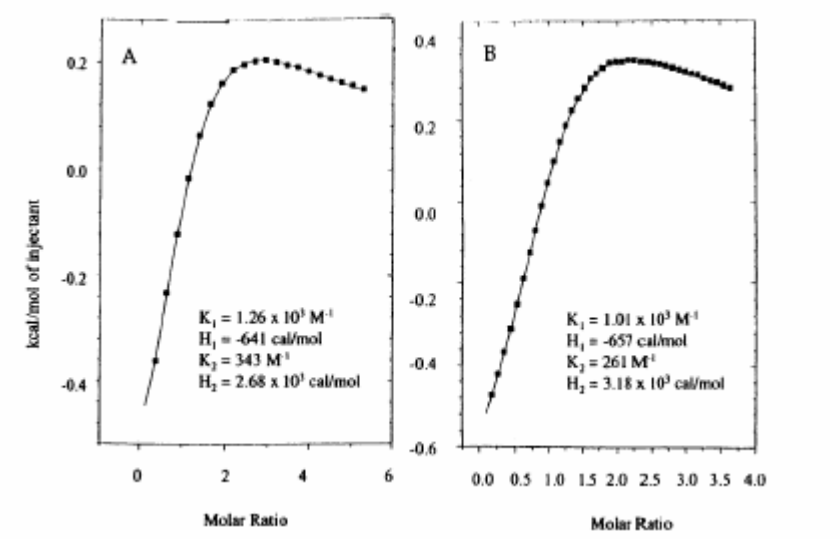


Figure 4.2 Reverse Titration. A) Binding isotherm for the addition of a 77.3 mM solution of 4.1 to citrate (1.16 mM). B) Titration of a 107.7 mM solution of 4.1 into citrate (2.27 mM). Both titrations were done in a 5 mM phosphate buffer at pH 7.4.

It is interesting that the measurement of K_1 for the formation of the 1:1 species could be obtained under these experimental conditions where **4.1** is in large excess. In contrast, the K_2 values presented in Figures 4.0a-c and Figures 4.2a-b are vastly different. This experimental observation indicates that the reaction mixture is more complicated than merely having the co-existence of two complex species, i.e. (Citrate•**4.1**)^0 and (Citrate•**4.1**$_2$)$^{3+}$. Again, the results indicate the existence of more complex aggregates with excess **4.1** in solution.

4.4 BINDING STOICHIOMETRY OF 4.1 AND CITRATE

The potential for the existence of multiple equilibria, including 1:1 binding stoichiometry, in solution warranted a more thorough understanding of

the binding stoichimetry. We approached this through NMR, mass-spectometry, and circular dichroism experiments.

4.4.1 Job Plot Analyses

To better understand the stoichiometry of the complexes in solution at the high concentrations of **4.1** Job plots using ^{1}H NMR data were obtained.[14-17] Job plots obtained at two different total **4.1**/citrate concentrations, namely 10 mM and 4.01 mM (Figure 4.3), reveal the existence of only one maximum at molar ratio 0.5. These results are counter to the suggestion that multiple complexes such as $(\text{Citrate}\bullet\mathbf{4.1}_2)^{3+}$ exist. There are two possible reasons for the disagreement between the stepwise 2:1 complexation model curve fit (Figures 4.0a-b and 4.3a-b) and the Job plot. One is aggregation in which the species maintain an empirical stoichiometry close to 1:1, such as $(\text{Citrate}_2\bullet\mathbf{4.1}_3)^{3+}$, $(\text{Citrate}_3\bullet\mathbf{4.1}_4)^{3+}$, etc. Another is that the chemical shifts of the aggregates and the 1:1 complex are all very similar.

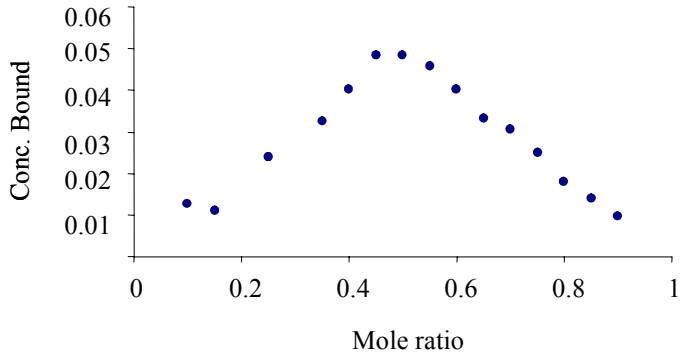


Figure 4.3 Job Plot. Job plot generate for citrate/4.1 in water buffed at pH 7.4 with a phsophate buffer. The total concentration of the samples was 4.01 mM. The plot shows a maximum at 0.5 mole ratio, indicating 1:1 binding.

4.4.2 Circular Dichroism

Job plots will also give a maximum at molar ratio 0.5, not only in the case of 1:1, but also for 2:2 complexation. To investigate the possibility of the formation of a complex dimer (Citrate•**4.1**)$_2$, circular dichroic (CD) spectra of the solutions containing chiral isocitrate and **4.1** at various concentrations were obtained (Figure 4.4). A two fold increase of the isocitrate concentration (from 9.74 mM to 17.1 mM) does not lead to an enhancement of the CD signal. This shows that a four fold excess of isocitrate (9.74 mM) over **4.1** is enough for the

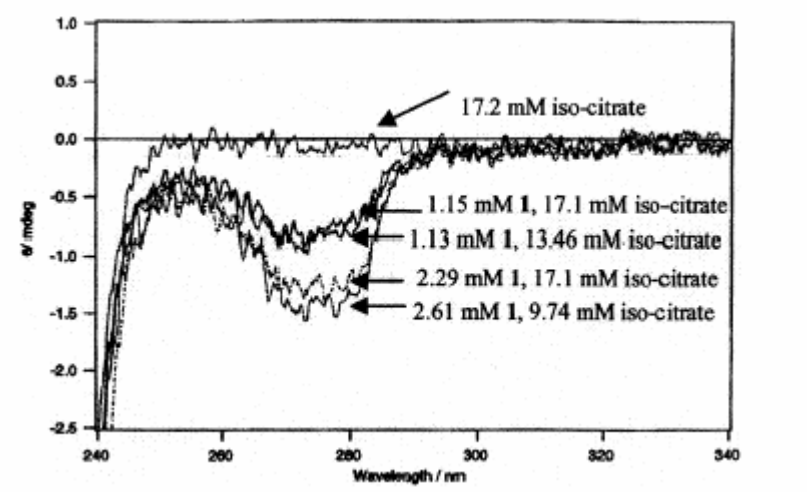


Figure 4.4 Circular Dichroism Spectra. The results of the circular dicrhoism experiments are shown.

complete saturation of **4.1**. A two fold reduction in concentration of **4.1** decreases the CD signal by only two fold. This result was obtained under conditions where **4.1** is saturated by chiral isocitrate. This gives a preliminary indication for the formation of 1:1 species rather than 2:2. One would anticipate a larger drop in a 2:2 complex dissociated upon dilution. This supports the existence of a Citrate•**4.1** complex rather than a complex dimer (Citrate•**4.1**)$_2$.

4.4.3 Mass- Spectrometry

To further identify the species present in the reaction mixtures during the ITC analyses, various mass-spectrometric techniques, e.g. ESI, MALDI and CI were applied.[18, 19] The most logical set of results comes from the ESI data in which (Citrate•**4.1**$_2$) dominates at low citrate concentrations and high host concentration. As the citrate concentration is increased (Citrate•**4.1**) (MW 646) begins to dominate the spectra. This observation rules out the possibility of (Citrate•**4.1**) dimerization. If dimerization occurs to form (Citrate•**4.1**)$_2$, it should dominate as the citrate concentration is increased. Indeed as the citrate concentration is increased the concentration of 1:1 complex is also increased according to reaction Citrate +**4.1** = (Citrate•**4.1**), and consequently the concentration of the dimers should also increase based on the equilibrium (Citrate•**4.1**) + (Citrate•**4.1**) = (Citrate•**4.1**)$_2$. Yet, (Citrate•**4.1**)$_2$ is not observed at any of the concentrations.

In summary, the simplest and the most reasonable model that describes all molecular events at the low concentrations of **4.1** (1-2 mM) is a stepwise 2:1 complexation. The co-existence of only two complex species, namely (Citrate•**4.1**) and (Citrate•**4.1**$_2$) is supported by various experimental and theoretical approaches. These two species are necessary and sufficient to explain and characterize quantitatively all the data obtained. Yet, with higher concentration of **4.1**, higher order aggregates exist.

4.4.4 Data Analysis

^{1}H NMR titration in previous work[20, 21] resulted in K_1 = 120 M^{-1} at 100 mM phosphate buffer and K_1 = 6900 M^{-1} in pure D_2O. Thus, there is a large ionic strength effect, as would be anticipated for an ion-pairing driven molecular recognition event. However, titration of phosphate with **4.1**, or vice versa, shows little to no complex formation in water.[6]

The affinity constant of a 1:1 complexation for **4.1** with citrate determined from the ITC data in this study is in the range of 2 x10^3 - 3 x 10^3 M^{-1} using a 5 mM phosphate buffer. In dealing with the association of oppositely charged poly-electrolytes, the strong dependence of equilibrium constants upon changes in ionic strength is anticipated. Thus, the results of ^{1}H NMR and ITC titrations are comparable. Indeed, the equilibrium constants determined by ITC titrations using a 5 mM phosphate buffer fall within the range of values determined by ^{1}H NMR titrations in 100 mM phosphate buffer and pure D_2O.[12] Furthermore, the ITC data are more consistent with the values determined in pure D_2O than those in 100 mM phosphate buffer. This is reasonable if the magnitude of the ionic strength of the solutions is considered. It should be mentioned that differences in affinities of corresponding complexation reactions in H_2O vs D_2O does not exceed 10-20%, and is thus insignificant in the context of the discussion given.[22]

4.4.5 Determination of the Driving Force for Aggregation.

We sought to uncover whether the higher order aggregation arises from ion-pairing or the hydrophobic effect. If hydrophobic interactions are the dominant forces for the formation of (citrate•**4.1**$_2$) species, the addition of methanol to the reaction mixture should destroy this complex. Addition of 10% methanol to the 5 mM phosphate buffer at pH 7.4 has a significant impact on the heat of dilution of a 100 mM citrate solution. However, the pattern of the heat effects during consecutive injections of 100 mM citrate into a solution of **4.1** are qualitatively very similar (pure 5 mM phosphate buffer at pH 7.4 versus 5 mM phosphate buffer pH 7.4 at with 10% methanol added). Furthermore, after the appropriate corrections to the heat of dilution of the initial citrate solutions, (with and without methanol) the data can be fit using an identical interacting sites model with six parameters (K_1, ΔH^o_1; K_2, ΔH^o_2; K_3, ΔH^o_3). As discussed above K_3 and ΔH^o_3 values are not thermodynamic parameters that characterize the

formation of a (Citrate•**4.1**$_3$) species, but rather are an indication of high order aggregation. In both scenarios, the K_3 and ΔH^o_3 values are similar enough to suggest that aggregation does occur, and the physical nature of the solutions are the same in both cases. Therefore the higher order aggregation is not affected considerably by the presence of methanol. These results indicate that hydrophobic interactions do not play a significant role in the complexation of **4.1** and citrate. Therefore, association through electrostatic interactions with attendant solvation/desolvation processes appears to be the most likely driving force for both 1:1 and higher order aggregates.

Complex formation of oppositely charged poly-electrolyte ions driven predominantly by electrostatic interactions can be easily perturbed by the presence of another poly-electrolyte at high concentration, e.g. HPO_4^{2-}. To address this possibility, a microcalorimetric titration of 75 mM **4.1** into the reaction cell charged with 2.27 mM citrate solution in 103 mM phosphate buffer (pH 7.4) was performed. The pattern of heat effects during this experiment (Figure 4.5) differ from what was observed previously at the low buffer concentration (Figures 4.1a,b). It should be emphasized that all injections (Figure 4.6) up to **4.1**/citrate molar ratio ≈2.5 result in heat production (exothermic heat effects). In contrast, endothermic heat effects were observed at the lower buffer concentration (5 mM), and were attributed to a 2:1 complex. This indicates that the high ionic strength suppresses 2:1 complexation as well as higher order **4.1**/citrate aggregation. Secondly, by applying the same computer simulation model of a stepwise 2:1 complexation described above, values were 5-6 times

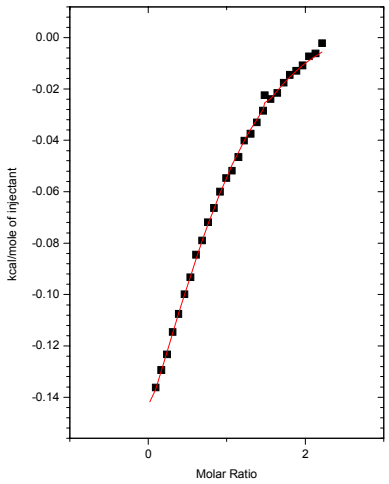


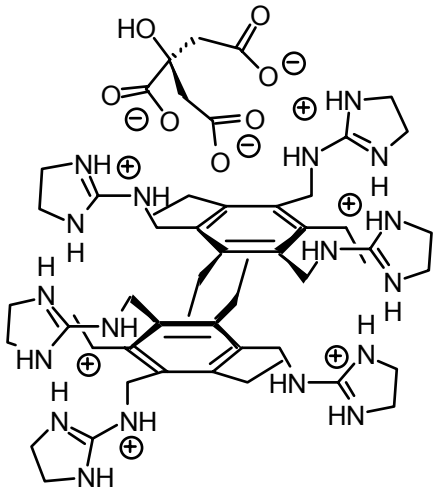
Figure 4.5 Binding Isotherm. This represent the addition of aliquots of a solution of 4.1 (107 mM) into a solution of citrate (2.27 mM) buffered with a phosphate buffer (103 mM) at pH 7.4.

lower for K_2 and about 3 times lower for K_1 (Figure 4.5) were obtained. The suppression of 2:1 complex formation and higher order **4.1**/citrate aggregation, combined with the reduced affinity constant for 1:1 complexation, shows that electrostatic interactions associated with profound solvation/desolvation processes are solely responsible for the stability of all the complex species formed by the interaction of citrate and **4.1**.

4.5 DRIVING FORCE FOR COMPLEX FORMATION

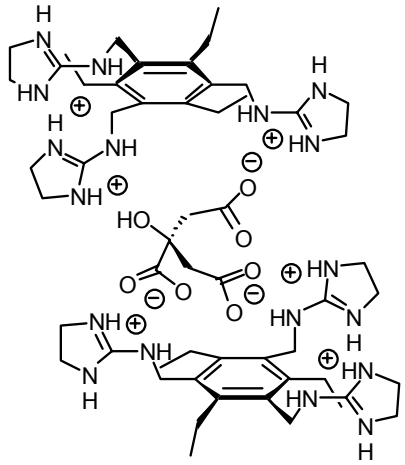
The formation of a (Citrate•**4.1**) complex is certainly driven by electrostatic interactions between oppositely charged reactants, and indeed the enthalpy change is negative. However, the driving force of (Citrate•**4.1**$_2$)

complex formation is not so obvious. One possibility lies in the hydrophobic interactions between the aliphatic and aromatic groups of two host molecules upon formation of a (Citrate•**4.1**$_2$) complex. Such hydrophobic interactions would be facilitated by the presence of citrate. The (Citrate•**4.1**) complex is electrically neutral and thus electrostatic repulsion between two hosts should occur to a lesser extent when compared with the dimerization of hosts themselves. Furthermore, small amounts of **4.1**$_2$ in a concentrated solution of **4.1** are observed (3%). Dimerization (stacking or micelle like) would become more thermodynamically favorable in the presence of oppositely charged citrate anions. Schematically the structure of such a complex could be presented as follows: citrate interacts from one side of **4.1** and a second molecule of **4.1** interacts from the other side of the same plane. In this case the dominant driving force for the formation of a (Citrate•**4.1**$_2$) complex is hydrophobic interactions. The data do not support such a structure.



Another possibility involves the sharing of three negative charges of a single citrate anion between two molecules of **4.1** that do not interact with each other. Some support for the existence of such a structure can be found by consideration of the structure of citrate/**4.1** complex reported previously.[6] Two

different unit cells were found. In one crystal cell three carboxylate groups of a citrate anion interact with three guanidinium cations of single molecule of **4.1**. However, in another crystal cell only two carboxylate groups of the citrate anion interact with one molecule of **4.1**, and the third carboxylate group of the same citrate anion interacts with another molecule of **4.1**. Formation of ion-pairs and release of a considerable amount of water molecules from the originally separated hydration shells of citrate and **4.1** into the bulk water would result in significant entropy gain and thus entropy could serve as a driving force of higher order complex formation. Our data supports this kind of complex.



Here we begin to examine the question of why the higher stoichiometry complexes are entropy driven? The formation of a simple 1:1 complex between citrate and **4.1** is driven by both favorable (negative) reaction enthalpy changes and favorable (positive) reaction entropy changes. This is reasonable when electrostatic interactions associated with profound desolvation processes are responsible for complex stability. Indeed, strong electrostatic interactions manifest themselves by exothermic heat effect of complexation. At the same time, the close proximity of oppositely charged groups on citrate and on the host

imply the overlapping of hydration shells, thereby resulting in the release of water molecules to bulk solution, giving a positive entropy gain.

Association of a second host molecule with the simple 1:1 complex is not expected to be accompanied by stronger electrostatic interactions as compared to the 1:1 complex. The host was originally designed to complex citrate through complimentary electrostatic interactions and thus the location and position of positively charged guanidinium groups of **4.1** were preorganized to compliment the negatively charged carboxylate groups of citrate. However, the addition of a second host molecule would lead to further desolvation processes during (Citrate•$\mathbf{4.1}_2$) complex formations. It is therefore reasonable to anticipate that the formation of this complex could be primarily entropy driven. This simple rationale agrees with the microcalorimetric data (Table 4.0). To further develop this idea one could suggest that further aggregation, e.g. (Citrate$_2$•$\mathbf{4.1}_3$), (Citrate$_3$•$\mathbf{4.1}_4$), and etc., would also be driven by entropy. Indeed, large deviations from the stepwise 2:1 complex model observed at high excess of macrocycle in the solution (Figures 4.1a-b) indicate that higher order aggregation is accompanied by endothermic (unfavorable) enthalpy, leaving entropy as the only possible thermodynamic driving force for aggregation.

The fundamental difference between the ion-pairing system analyzed here and aggregation of organic compounds due to the hydrophobic effect in water should be emphasized.[23-26] First, we have shown that the aggregation is not due to the hydrophobic effect. Second, many organic compounds possessing hydrophobic and hydrophilic (often charged) moieties spontaneously aggregate at some critical concentration to form micelles. Dilution of the aggregate inevitably leads to their spontaneous degradation into single separate molecules. The most distinguishing feature of the **4.1**/citrate system is the increasing complexity with reduction of citrate concentration while holding the concentration of **4.1** constant. We would like to discuss this issue in more detail.

reaction	[Citrate] (mM)	[**4.1**] (mM)	Injections (*N*), [buffer].(mM)[a]	*K* (M^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})
C + **4.1** = (C•**4.1**)	99.9	1.41	*N*=20 (5 mM)	2420 (K_1) (± 194)	-19.31 (± 0.21)	-4.8 (± 1.02)	14.5 (± 1.21)
(C•**4.1**)+**4.1** = (C•**4.1**$_2$)	99.9	1.41	*N*=20 (5 mM)	585 (K_2) (± 283)	-15.79 (± 0.39)	20.4 (± 0.50)	36.2 (± 4.10)
C + **4.1** = (C•**4.1**)	100.3	1.98	*N*=40 (5 mM)	2540 (K_1) (± 194)	-19.43 (± 0.21)	-5.9 (± 1.02)	13.5 (± 1.21)
(C•**4.1**)+ **4.1** = (C•**4.1**$_2$)	100.3	1.98	*N*=40 (5 mM)	900 (K_2) (± 283)	-16.86 (± 0.39)	11.3 (± 0.50)	28.2 (± 4.10)
C + **4.1** = (C•**4.1**)	51.7	1.98	*N*=40 (5 mM)	2160 (K_1) (± 194)	-19.03 (± 0.21)	-6.9 (± 1.02)	12.1 (± 1.21)
(C•**4.1**)+ **4.1** = (C•**4.1**$_2$)	51.7	1.98	*N*=40 (5 mM)	1150 (K_2) (± 283)	-17.47 (± 0.39)	12.5 (± 0.50)	30.0 (± 4.10)
C + **4.1** = (C•**4.1**)	1.16	77.3	*N*=20 (5 mM)	2520 (K_1) (± 353)	-19.42 (± 0.39)	-2.7 (± 0.50)	16.7 (± 0.35)
(C•**4.1**)+ **4.1** = (C•**4.1**$_2$)	1.16	77.3	*N*=20 (5 mM)	170 (K_2) (± 28)	-12.73 (± 0.46)	11.2 (± 1.48)	23.9 (± 1.06)
C + **4.1** = (C•**4.1**)	2.27	107.7	*N*=40 (5 mM)	2020 (K_1) (± 353)	-18.87 (± 0.39)	-2.7 (± 0.50)	16.2 (± 0.35)
(C•**4.1**)+**4.1** = (C•**4.1**$_2$)	2.27	107.3	*N*=40 (5 mM)	130 (K_2) (± 28)	-12.07 (± 0.46)	13.3 (± 1.48)	25.4 (± 1.06)
C + **4.1** = (C•**4.1**)	100.3	6.26	*N*=40 (5 mM)	3180 (K_1)	-19.99	-4.1	15.9
(C•**4.1**)+ **4.1** = (C•**4.1**$_2$)	100.3	6.26	*N*=40 (5 mM)	330 (K_2)	-14.38	5.6	20.0
C + **4.1** = (C•**4.1**)	2.27	107.7	*N*=30 (103 mM)	410 (K_1) (± 28)	-14.91 (± 0.21)	-0.9 (± 0.21)	14.0 (± 0.35)
(C•**4.1**)+ **4.1** = (C•**4.1**$_2$)	2.27	107.3	*N*=30 (103 mM)	48 (K_2) (± 5.0)	-9.60 (± 0.46)	0.9 (± 0.50)	10.5 (± 1.06)

Table 4.0 Tabulated ITC Data. The thermodynamic parameters for host guest binding in 1:1 and 2:1 stoichiometries are reported. The values were determined from ITC titrations using a curve fitting algorithm in Origin.

As stated in the introduction, entropy changes serve as a destructive factor upon dilution of any and all host/guest complexes formed by non-covalent intermolecular interactions. However, we have found that it is possible to increase aggregation upon lowering the concentration of one reactant involved in the complexation event if the other component is kept at constant concentration. If one compares the species involved in the final part of titration curves to those involved in the initial part of the curves, we find what appears to be the more complex species (C_n**4.1**$_m$, where C = citrate) in the more dilute solutions. We conclude that this may arise whenever aggregation is accompanied by solvation/desolvation processes.

The vantage point taken during the experiment explains this finding. We are following complex formation, which leads to the release of water. All equilibria will shift toward the reactants or the products that possess the most number of free entities upon dilution due to entropy. Examine Eqs. 4.6 and 4.7, which show the 1:1 and 1:2 complexation events studied herein (C = citrate). When diluting only citrate, we find larger and larger amounts of free **4.1**, which

can release water upon complexation with (citrate•**4.1**). Thus, upon dilution of citrate, one creates more free entities due to water release by addition of another host, but since we are following the aggregates, the system appears to become more complex. Only when we approach an equal amount of citrate and **4.1** do we find the simpler (citrate•**4.1**) complexes, because now both enthalpy changes and entropy changes drive their formation, which combined are stronger driving forces than solvent release alone.

$$C(H_2O)_{n+a} + \mathbf{4.1}(H_2O)_{m+b} \Leftrightarrow C\bullet\mathbf{4.1}(H_2O)_{a+b} + (n+m)H_2O \quad (4.6)$$

$$C\bullet\mathbf{4.1}(H_2O)_{a+b} + \mathbf{4.1}(H_2O)_{m+b} \Leftrightarrow C\bullet\mathbf{4.1}_2(H_2O)_{a+b} + (b+m)H_2O \quad (4.7)$$

4.6 CONCLUSION

Our study shows that higher order complexes can be achieved using reduced concentrations of one reactant. Increases in the apparent complexity of the system can be driven exclusively by entropy. Of course, the apparent increase in the complexity results from a decrease in overall order due to solvent release. The finding that a host/guest system can increase aggregation state exclusively due to favorable entropy at reduced concentrations may have some practical implications. Potentially, this serves as a way in which to design supramolecular systems at lower concentrations with self-controlled affinity toward a particular substrate. There is the possibility of gradual and controllable interconversion of supramolecular architectures by variations in the component concentrations and/or supplementary electrolyte concentrations. Most importantly, the complexity of the supramolecular architectures would be higher at the lower concentrations when one component is in excess.

4.7 EXPERIMENTAL

Materials.

Compound **4.1** was synthesized as described previously.[6] The purity of **4.1** was verified by elemental analysis, ^{1}H NMR, and ^{13}C NMR. All other chemicals used in the ITC, NMR, and other spectroscopic experiments were commercially available from Aldrich, and they were used without further purification.

Microcalorimetric Measurements.

An isothermal titration calorimeter (ITC), purchased from Microcal Inc., MA, was used in all microcalorimetric experiments. Titration microcalorimetry[14] allows one to determine simultaneously the enthalpy and equilibrium constant from a single titration curve. The ITC instrument was periodically calibrated using an internal electric heater.[15] The instrument was also calibrated chemically by using the neutralization enthalpy of the reaction of HCl with NaOH, and the ionization enthalpy of TRIS buffer. These standard reactions were in excellent agreement (±1-2%) with the literature data.[27; 28] The thermodynamic parameters for the complexation reaction of cyclohexanol with β-CD were also in good agreement with previous results.[29-31]

ORIGIN software (Microcal Inc.) was used to calculate the equilibrium constant and standard molar enthalpy of reaction from the titration curves in the cases of simple 1:1 and stepwise 2:1 complexation. The standard deviation based on the scatter of the data points in a single titration curve was also calculated. As reported previously[29-31], the accuracy of the calculated thermodynamic quantities for 1:1 complexations was checked by performing several independent titrations. The uncertainties in the observed thermodynamic quantities for 1:1 complexation (Table 4.0) are two standard deviations of the mean value unless otherwise stated.

The basis for the estimation of uncertainties for stepwise 2:1 complexation reactions (Table 1) is discussed in a previous paper.[11]

Each microcalorimetric titration experiment consisted of 20-40 successive injections as described previously.[6a] Initial concentrations of citrate and **4.1** in each run are indicated in Table 1. Phosphate buffer (pH=7.4) [NaH_2PO_4 + $NaHPO_4$], either 5 or 103 mM was used for all microcalorimetric and spectroscopic experiments. The third pK_a of citrate is 6.4,[32; 33] and thus at least 90% of the citrate is trianionic at pH 7.4. At this pH, host 1 is tricationic, since the pK_a's are all above 11.0.[34] The pH of the solutions before and after ITC and NMR titrations were always within 0.1 pH units, and therefore the complexity in the binding curves presented below are not due to pH effects. Further, we have previously shown that non-ideality corrections are not necessary under the experimental conditions employed.[17]

NMR experiments.

The ^{1}H NMR experiments used to determine Job Plots[14-17] at a total citrate/**4.1** concentration of 10 mM were performed on a Varian UNITY 300 MHz instrument. The ^{1}H NMR experiment at a total citrate/**4.1** concentration of 4.01 mM were run on a Varian INOVA 500 MHz instrument.

ESI mass-spectrometric measurements.

Mass-spectroscopy experiments were performed using a LCQ Finnigan-MAT(San Jose, CA) instrument. The data was collected on a 100 mM sample of **4.1** in water (pH = 7.4) using electrospray ionization mode. The standard conditions employed are as follows: vaporization temperature of 240° C, capillary voltage of 30 V, and spray voltage of 5 kV. Samples run at milder conditions were run at a

vaporization temperature of 50° C, a capillary voltage of 0 V, and a spray voltage of 4 kV.

Circular dichroism measurements.

Circular dichroism spectra of aqueous solutions of 1/iso-citrate mixtures were obtained in a conventional quartz cell (10 x 10 x 45 mm) at room temperature by using a JASCO J-720 instrument.

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Vita

Suzanne Lai Tobey was born in Providence, Rhode Island on May 26, 1976, the daughter of Alrica Beverly Tobey and Robert Sheppard Tobey. After completing her work at Hillel High School, Kingston, Jamaica in 1993, she entered Kent State University in Kent, Ohio. In the spring of 1995 she transferred to the University of Florida in Gainesville, Florida. During the summer of 1997 she participated in a research program at Duke University that was sponsored by the National Science Foundation. She received a Bachelor of Science from the University of Florida in May 1998. In August 1998 she entered the Graduate School of the University of Texas.

Permanent address:12148 Jollyville #316, Austin Texas, 78759

This dissertation was typed by the author.