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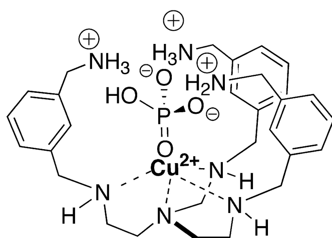

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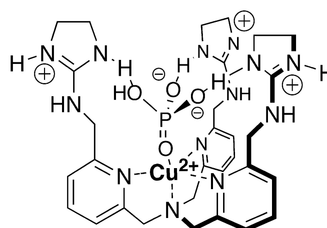

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Energetics of Phosphate Binding to Ammonium and Guanidinium Containing Metallo-Receptors in Water

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Abstract: The design and synthesis of receptors containing a Cu(II) binding site with appended ammonium groups (**1**) and guanidinium groups (**2**), along with thermodynamics analyses of anion binding, are reported. Both receptors **1** and **2** show high affinities (10^4 M^{-1}) and selectivities for phosphate over other anions in 98:2 water:methanol at biological pH. The binding of the host−guest pairs is proposed to proceed through ion-pairing interactions between the charged functional groups on both the host and the guest. The affinities and selectivities for oxyanions were determined using UV/vis titration techniques. Additionally, thermodynamic investigations indicate that the **1**:phosphate complex is primarily entropy driven, while the **2**:phosphate complex displays both favorable enthalpy and entropy changes. The thermodynamic data for binding provide a picture of the roles of the host, guest, counterions, and solvent. The difference in the entropy and enthalpy driving forces for the ammonium and guanidinium containing hosts are postulated to derive primarily from differences in the solvation shell of these two groups.

Introduction

One goal of molecular recognition is the creation of synthetic receptors demonstrating both a high affinity and a high selectivity for guest binding in water.[1] The high affinity and high selectivity found within natural receptors such as enzymes and antibodies derives from multiple weak nonbonded interactions between the functional groups on the binding partners. These natural systems provide the inspiration for the rational design of synthetic receptors that can be used to garner an understanding of the binding forces that contribute to complex formation.

A significant portion of this understanding can be gleaned by probing the thermodynamics of binding interactions. The formation of a host−guest complex through noncovalent interactions has an associated enthalpy and entropy change. Additionally, changes in the solvent structure and the solvation spheres of the host and the guest contribute to the overall entropy and enthalpy changes of the system. The interplay of the differing thermodynamic contributions arising from complex formation and concomitant solvation/desolvation processes is not immediately discernible from the determination of a binding affinity alone. Therefore, the dissection of the Gibbs free energy of binding ($\Delta G°$) into its component parts ($\Delta H°$ and $\Delta S°$) through van't Hoff analyses or isothermal titration calorimetry (ITC) provides useful insight into the nature of the binding interactions, especially in cases where the differences in the $\Delta G°$ values of binding were too subtle to draw any conclusions.[2−4]

Extensive studies by Diederich,[3] using both van't Hoff methods and ITC, have showed that host−guest binding via hydrophobic interactions in water can proceed with favorable enthalpy changes and unfavorable entropy changes. Alternatively, Schmidtchen[2] and Hamilton[3,6] have focused their efforts on electrostatic interactions. Their work shows binding via electrostatic interactions in organic media proceeds with favorable entropy changes. Classically, one might expect hydrophobic binding in water to have an entropic driving force[7] and electrostatic binding in organic media to have a favorable enthalpy change. These experimental data suggest otherwise, indicating that thermodynamic investigations have the potential to provide a understanding of a single binding event, sometimes with surprising results. To further highlight the power of a thermodynamic analysis, Diederich, Schmitdchen, and Hamilton were able to use the component thermodynamic values, $\Delta H°$ and $\Delta S°$, to differentiate the roles of the solvent and the counterions in host−guest complex formation.

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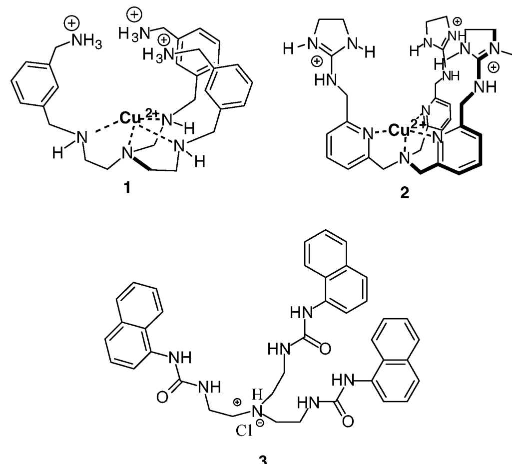
Recent work from our group and Inoue describes the thermodynamic investigations of host:guest binding that proceeds through ion-pairing interactions in aqueous media.[8] ITC studies on a citrate-tris-guanidinium host:guest complex in water revealed that the 1:1 complexation was entropy driven, with an accompanying exothermic enthalpy change. This result was observed at high ionic strength, whereas at low ionic strength higher ordered complexes were observed, having an endothermic enthalpy change and a strongly favorable entropy change. The switch between different complexes at different ionic strengths was revealed through the changes in the thermodynamic profile of the associations. Again, ITC played a key role in revealing the components of the free energy, thereby leading to a method of controlling the binding behavior.

The binding selectivities and thermodynamic profiles of electrostatic interactions in water at biological pH are the focus of this report. A specific design approach for phosphate[9] using metalloreceptors (**1** and **2**) is described.[10] The design of each of the host employs a platform that is preorganized[11] upon binding Cu(II), along with appended functional groups as binding sites: ammonium or guanidinium groups. The use of several binding sites endows the receptor with the ability to form multiple nonbonded interactions with the guest. We find that the vast majority of the free energy of binding derives from the Cu(II) binding, while the appended groups tune the affinity and selectivity of binding. The binding is found to be entropy driven at the metal, whereas the ammoniums or guanidiniums offer primarily entropy[12] and enthalpy assistance to the binding. Speculation as to why the ammonium and guanidinium groups act differently is given, focusing primarily upon the differences in hydration of these two functional groups.

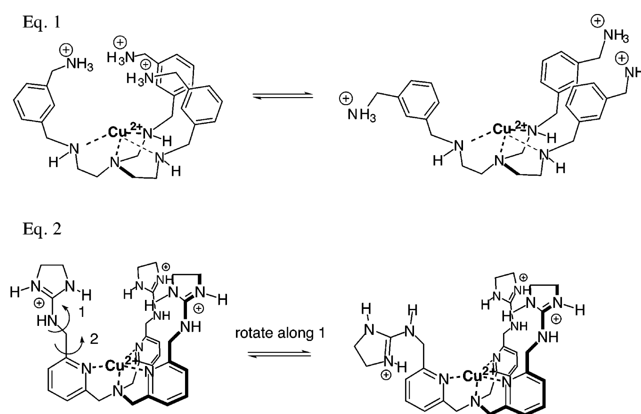
Results and Discussion

Design Criteria. The design of receptors **1** and **2** features a C_{3v} symmetric cavity derived from the coordination of a tripodal ligand to a Cu(II) center. The Cu(II) center is intended to provide one binding site for a guest, while three additional functional groups bearing positive charges are positioned to provide binding interactions with oxygens on phosphate. The shapes of the cavities are complementary to the three faces of a tetrahedron.[10] One recent example (**3**) of this design principle is found in work by Xie,[13] but studies in water are not reported.



Receptor **1** is derived from a tris(2-ethylamino)amine (TREN) unit with appended benzylamine groups, similar to the design exploited by Fabbrizzi[14] and others.[15] Similarly, receptor **2** is derived from a tris[(2-pyridyl)methyl]amine (TPA) subunit functionalized with appended guanidinium groups (embedded in aminoimidazoline groups). This is analogous to compounds used by Canary,[16] Karlin,[17] and others.[18]

Though the design of both **1** and **2** are intended to be complimentary to a tetrahedral anion as drawn, they are not completely rigid. Receptor **1** is more flexible than receptor **2**, albeit both have alternative conformations than those shown. For example, one expects that the preferred conformation of **1** in solution is completely open with the ammonium groups diverging away from the metal center (eq 1). Likewise, conformations with the amines both divergent and convergent likely exist in water.



The manner that we draw compound **2** is more representative of the conformation in solution, but others exist. In our picture,

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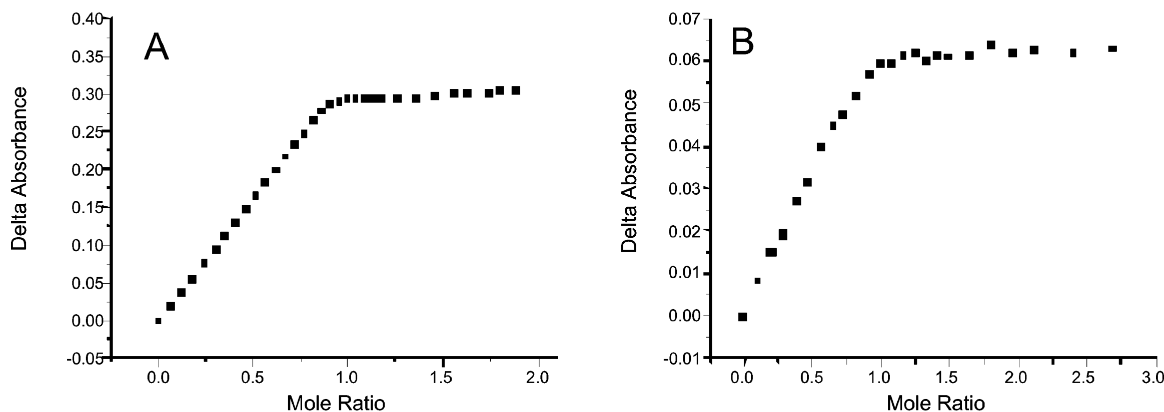
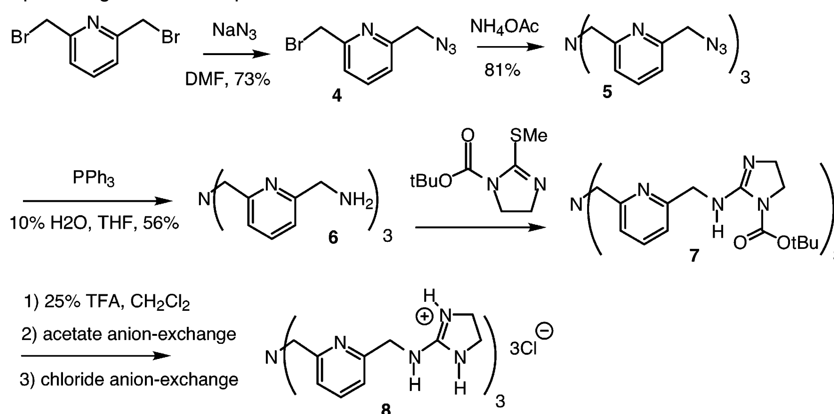


Figure 1. Mole ratio plots for the tripodal ligands binding to Cu(II). (A) Aliquots of a millimolar solution of $CuCl_2$ were added to a solution of ligand (0.98 mM), and the absorbance was monitored. (B) Aliquots of a solution of $CuCl_2$ were added to a solution of ligand (1.16 mM), and the absorbance was monitored.

Scheme 1. Synthesis of Tripodal Ligand for Receptor **2**



the guanidinium groups are aligned to create a cavity. This places them the furthest from each other relative to other possible conformations along bond rotation 2 shown in eq 2. Further, large rotations along the bonds indicated as 1 in eq 2 create steric interactions with the pyridine rings. However, rotations about the bonds indicated in eq 2 are feasible. The conclusion from our analysis is that **1** and **2** are both flexible, yet the flexibility is expected to be lower with **2** than with **1**. The largest difference between the designs is the ammonium and guanidinium groups as the peripheral binding elements.

Synthesis. The synthesis of **1** has been reported previously.[19] The synthesis of **2** commences with the reaction of 2,6-bis-(bromomethyl)pyridine with sodium azide in dimethylformamide. The resulting monoazidomethyl product **4** is stirred with ammonium acetate and potassium carbonate in dry acetonitrile, while recovered starting material was recycled. Trisazido tripod adduct **5** is reduced to the tris-amine under Staudinger conditions. The resulting amine (**6**) is combined with 3 equiv of boc imidazoline to yield **7**. Boc protected imidazoline[20] was deprotected using trifluoroacetic acid, and the salt was isolated. The TFA salt was eluted from an acetate anion exchange column to verify the presence of three acetate counterions. The acetate salt was subsequently placed on a chloride anion exchange column to afford the trischloride salt (**8**) (Scheme 1).

The introduction of a stoichiometric amount of copper(II) chloride preorganizes the tripodal tren derived ligand to yield the desired receptor **1**. 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} M^{-1} by Anderegg.[21] The coordination[22] 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 **1**. The change in absorbance was used to generate a mole ratio plot and verified a 1:1 binding stoichiometry for ligand-to-metal binding (Figure 1A).

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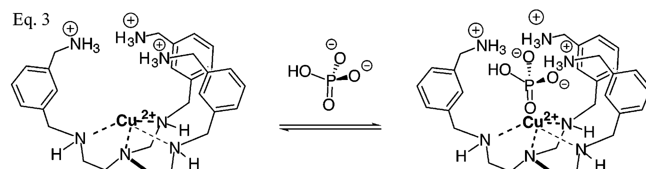
A similar study was used to verify the coordination of the Cu(II) to ligand **8** by following the absorbance at 669 nm. This was carried out in an aqueous solution buffered at pH 7.4 with tris(hydroxymethyl)aminomethane (TRIS) buffer. The chelating ability of TRIS to metals prompted investigations into 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 (Figure 1B). The binding isotherm was not curve fit because the binding affinity was too high to generate a binding affinity with reasonable error. However, a binding constant of Cu(II) to a TPA ligand is reported in the literature as 10^{17} M^{-1}, which further substantiates the Cu(II) binding primarily to the TPA derived ligand. The TRIS Cu(II) interaction was negligible for our purposes.

Affinities, Selectivities, and Protonation States Summary of Binding. The binding affinities and selectivities of each of the receptors, **1** and **2**, were determined using UV/vis titrations with several anionic guests. This work was previously reported[10] and is briefly summarized below as background for the present thermodynamic studies. We choose to use UV/vis spectroscopy and ITC to characterize binding constants rather than potentiometric titrations because there are seven amines that undergo protonation state changes in the potentiometric titrations of apo-**1** and apo-**2**. Further, the metal complexes can undergo hydrolysis giving bound hydroxides, and phosphate has three pK_a values of its own that shift upon binding to the receptors (see eq 3 to visualize an idealized binding mode). The number of variables



to be fit by a potentiometric titration modeling program available to us, such as HYPERQUAD,[24] makes such a method unreliable. With UV/vis spectroscopy or ITC, the anion binding event itself is all that needs to be modeled with a curve fitting program, thereby making us more confident of the results.

The change in the absorbance of **1** at 800 nm was monitored by UV/vis spectroscopy as aliquots of a NaH_2PO_4 solution were introduced (98:2 water:methanol buffered to pH 7.4 with 10mM Tris). For consistency, in all the UV/vis and ITC studies described herein, a 98:2 water:methanol solution is used for solubility purposes, even though this minimum amount of methanol was only needed in certain cases to maintain homogeneous solutions. The resulting binding isotherm was fit with a curve derived from a 1:1 binding algorithm to yield a binding affinity of 2.5×10^4 M^{-1}. Similar titration methods were used to determine the binding affinities of several other anions to **1** in aqueous media (Table 1). The lower affinities observed for other tetrahedral anions as well as anions of various charges and sizes demonstrate the selectivity of **1** for phosphate.

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Table 1. Binding Affinities of Several Different Anions to Receptor **1** Were Investigated Using UV/vis Titrations[a]

receptor	anion	stoichiometry anion:**1**	binding constants (M^{-1})
1	HPO_4^{2-}	1:1	2.5×10^4 ($\pm$ 6×10^2)
1	$HAsO_4^{2-}$	1:1	2.5×10^4 ($\pm$ 6×10^2)
1	ReO_4^-	1:1	2.0×10^3 ($\pm$ 7×10^2)
1	HSO_4^-		nd
1	AcO^-	1:1	<900
1	NO_3^-	1:1	<20
1	HCO_3^-	2:1[b]	nd
1	Cl^-	2:1[b]	nd
9	HPO_4^{2-}	1:1	9.0×10^2 ($\pm$ 3×10^2)

[a] The absorbance of the Cu(II) center of the receptor was observed as aliquots of the guest (20 mM) were added to a solution of the receptor (0.7 mM). All solutions were buffered at pH 7.4 with HEPES buffer (5 mM), and the counterions to the receptor were chlorides. The counterions to the guests were sodium. [b] Stoichiometry was estimated from the shape of the isotherms, although even higher order complexes cannot be ruled out.

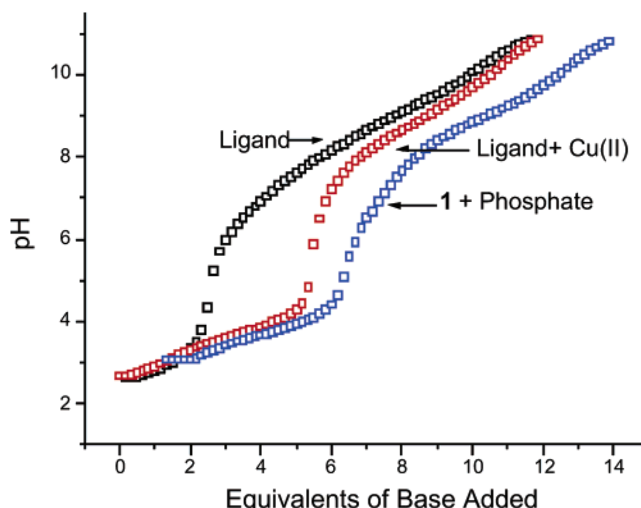


Figure 2. pH titration data for the ligand, the ligand and copper, and the 1-phospahte complex. For each titration, aliquots (10 μL) of a standardized solution of NaOH (83.5 mM) were added to a solution containing 0.005 mmol of the compounds of interest. The ligand-Cu and **1**-phosphate were all present in 1:1 mole ratios.

The species present in solution at this pH become important for interpreting the binding data. The pH titrations of the ligand, the ligand in the presence of Cu(II), and the **1**/phosphate complex are shown (Figure 2). As Cu(II) begins to coordinate to the ligand, we would expect a shift in the ammonium pK_a values. Indeed, this expectation is observed with a concomitant appearance of a blue color as the Cu(II) coordinates at approximately pH 3. The pK_a values of the secondary amines of the ligand decrease by approximately 4−5 pK_a units in the presence of the Cu(II), and the primary amines have pK_a values above 8. In the presence of phosphate, the titration curve is shifted to the right by one proton equivalent, which is reasonable because the first pK_a value for phosphate is 2.15. The Cu(II) coordinates as discussed before, and the remaining pK_a values are above 8.

Further, based on curve fitting, the host−guest species has an additional pK_a around 6−7. We cannot distinguish whether the proton resides on an amine or on phosphate. However, this value would be appropriate for a second pK_a of phosphate that is depressed due to the presence of the host. Although the host/guest complex is likely to be a mixture of species with several different protonations states, we are currently postulating that, at pH 7.4, the phosphate is predominately in the form of HPO_4^{-2}

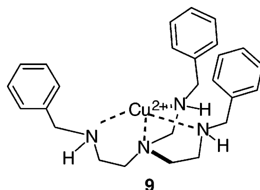
Table 2. Binding Affinities of Several Different Anions to Receptor **2** Were Investigated Using UV/vis Titrations[a]

receptor	anion	stoichiometry anion:**2**	binding constants (M^{-1})
2	HPO_4^{2-}	1:1	1.5×10^4 ($\pm 6 \times 10^2$)
2	$HAsO_4^{2-}$	1:1	1.7×10^4 ($\pm 6 \times 10^2$)
2	ReO_4^-		<100
2	HSO_4^-		
2	AcO^-		<100
2	NO_3^-		<100
2	HCO_3^-		<100
2	Cl^-		<100
10	HPO_4^{2-}	1:1	3.0×10^2 ($\pm 6 \times 10^s$)[a]

[a] The absorbance of the Cu(II) center of the receptor was observed as aliquots of the guest (20 mM) were added to a solution of the receptor (1.1 mM). All Solutions were buffered at pH 7.4 with TRIS buffer (10 mM), and the counterions to the receptor were chlorides. The counterions to the guests were sodium.

when bound. The purpose of the pH titrations was to establish with certainty that the ammonium groups on the periphery of the cavity were protonated at the working pH of the host/guest titrations and to verify that the phosphate is bound primarily as a dianion.

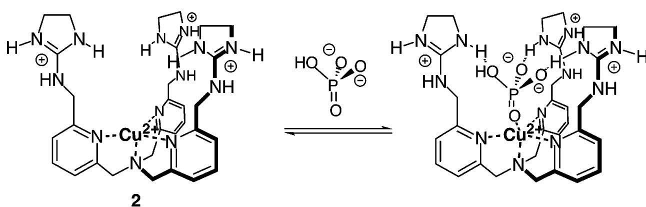
To decipher the roles of various binding sites on **1**, we examined host **9**. Phosphate binds to **9** with an affinity of 900 M^{-1}, thereby indicating that the ammonium groups offer a 27-fold enhancement of phosphate complexation in compound **1**.



Below we show van't Hoff plots that indicate **1** is better than **9** by about 100-fold (Table 3). A factor of a 27−100 increase over a metal baseline affinity of 900 M^{-1} supports the notion that the largest portion of the binding of phosphate to **1** is due to the metal center, a key feature in the host design. Further, it indicates that each ammonium of **1** likely does not adopt a conformation making a contact to the bound phosphate. Because solvent exposed ion pair $\Delta G°$ values are estimated to be worth near 1 kcal/mol in water,[25] the binding of **1** to phosphate probably only involves a little over one ion pair on average between the phosphate and the ammoniums resulting from the flexibity of this host, although this is clearly speculation.

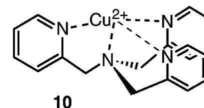
Titrations of solutions containing receptor **2** with anions were also performed (Table 2). Modulations in the UV/vis absorbance at 790 nm were monitored as aliquots of a phosphate solution buffered at pH 7.4 were added. The resulting binding isotherm was fit with a 1:1 algorithm to yield a binding affinity of 1.5×10^4 M^{-1} (see eq 4 for an idealized binding mode). Titrations

Eq. 4



with various other inorganic analytes were performed using identical experimental conditions, and no binding was observed. Host **2**, therefore, has both a high affinity and excellent selectivity in water at neutral pH for phosphate and arsenate.

Our first studies indicated that the guanidinium groups added to the selectivity and affinity of **2** relative to a control where the guanidinium was replaced by azides.[10] We now report additional titration data using control host **10**. Titration of a



phosphate solution (29.3 mM) to a solution of receptor **10**[26] (1.5 mM), which lacks the guanidinium groups, demonstrates a binding affinity of 300 M^{-1}. Therefore, **2** is a better phosphate binder than **10** by a factor of 50 (ITC data gives a factor of only 10; see below). The lower affinity compared to **2** verifies the cooperative effect of the guanidinium groups and the Cu(II) center. However, as with the comparison between **1** and **9**, the comparison between **2** and **10** indicates that the largest binding free energy derives from the phosphate−metal interaction. Speculation is again needed to state how many guanidinium-phosphate binding interactions occur in the complex. All that can really be stated with confidence is that the free energy gain with **2** relative to **10** is similar to that between **1** and **9**. It is difficult to judge whether more or fewer ion pairs are formed with the appended functional groups with **1** or **2**. It is simply the increased preorganization of **2** that implies more contacts than with **1**.

Irrespective of the fact that the metal center dominates the affinity, the selectivity of both **1** and **2** for phosphate derives from the appended groups, which provide shape, size, and charge complimentarity to tetrahedral oxyanions. In our original interpretation of these data,[10] we concluded that the inherent flexibility of **1** relative to that of **2** decreased its selectivity for phosphate. In contrast, the increased rigidity of **2** led to a decrease in affinity while increasing its selectivity for phosphate and arsenate. To further investigate this postulate, we attempted to determine the enthalpy and entropy origin of these differences in selectivity and affinity. Our original conclusion is still supported, but it is now clear that solvation differences between ammonium and guanidinium groups are also major contributors to the differences in the binding behavior of **1** and **2**.

Entropy and Enthalpy Changes. A. Binding to Host 1. Isothermal titration calorimetry (ITC)[27] was used to probe the driving force for the high affinity of phosphate with **1** by quantifying the thermodynamic parameters ($\Delta G^{m°}$, $\Delta H°$, and $\Delta S°$) of binding. The titration proceeded with the addition of 5 μL aliquots of a 5.1 mM solution of **1** to the titration cell containing a 0.32 mM solution of NaH_2PO_4 at 25 °C (buffered to pH 7.4 with 5 mM HEPES). Less than 1 kcal/mol of heat was generated per injection for the duration of the titration. The lack of a significant amount of evolved heat was independent of the concentration of the host and guest we used. The

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Table 3

receptor	guest	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$T\Delta S°$ (kcal/mol)
1[a]	HPO_4^{2-}	−6.5	+0.6	+5.9
1[b]	ReO_4^{1-}	−3.7	−2.2	+1.5
1[b]	AcO^-	−3.4	+0.7	+4.1
2[a]	HPO_4^{2-}	−5.3	−3.8	+1.5
9[b]	HPO_4^{2-}	−3.8	−0.9	+2.9
10[b]	HPO_4^{2-}	−4.1	−0.8	+3.3

[a] These thermodynamic values were determined using a van't Hoff analysis for the UV/vis data at different temperatures. [b] These thermodynamic values were determined using isothermal titration calorimetry techniques

experiment was repeated using arsenate as the guest, only to yield a similar result. This result, 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 at each temperature were comparable within experimental error of the data, resulting in a van't Hoff plot with a slightly positive slope (no correction was needed in this plot as described below for host **2**). The values determined from the plot were $T\Delta S° = +5.9$ (±1.0) kcal/mol and $\Delta H° = +0.6$ (±0.5) kcal/mol, thus confirming the major contribution of the entropy changes to the overall strength of the binding.

The binding of phosphate to control compound **9** was also analyzed using a van't Hoff approach, yielding $\Delta H° = -0.9$ (±0.5) kcal/mol and $T\Delta S° = +2.9$ (±1.0) kcal/mol. These values indicate that, in the absence of ammonium groups for binding, the primary mode of phosphate binding to **9** is through a slightly exothermic ligation to the Cu(II) center with a dominate entropic driving force. The favorable entropy change is thought to arise from the release of solvent and/or counterions from the host and the guest upon complex formation. The ammonium groups in **1** enhance phosphate binding by 2.7 kcal/mol relative to **9**. The dominant entropy change for **1** derives from interaction of the phosphate with not only the Cu(II) center, but from the additional interactions with the ammoniums, leading to more solvent and/or counterion release.

Thermodynamic studies were used to examine the binding energetics of anions with differing affinities to **1** (Table 3). By UV/vis analysis, arsenate showed almost identical thermodynamic parameters to those of phosphate. The binding of perrhenate to **1** was quantified (500 M^{-1}) using ITC techniques. The addition of 5 μL aliquots of a solution of **1** to a solution of the guest displayed exothermic heats of binding. The raw data, when fit with a binding isotherm, yielded a $\Delta H°$ value of −2.2 (±0.5) kcal/mol and a $T\Delta S°$ value of +1.5 (±1.0) kcal/mol. Similar methods for acetate as the guest resulted in $\Delta H° = +0.71$ (±0.5) kcal/mol and $T\Delta S° = +4.12$ (±1.0) kcal/mol.

In all cases, the binding of the guest to **1** 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 serves to add yet more examples to the literature which involve ion-pairing interactions in water with a significant contribution to the binding energetics arising 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[28] and molecular recognition events using synthetic receptors.[29]

It is interesting to note that the host design renders **1** 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 **1**, as well as smaller associated entropy changes. The perrhenate anion occupies a larger molar volume and has a more loosely held solvation shell due to its smaller charge density relative to phosphate. Therefore, it is reasonable that the diminished $\Delta S°$ arises from the release of solvent from a less organized solvent sphere around perrhenate relative to phosphate. Overall, the entropy changes for complex formation with perrhenate are less favorable than phosphate binding, and the data show a more favorable enthalpy change relative to phosphate compensates for this decreased entropy.

When looking at acetate, less solvent is expected to be displaced from the binding cavity than with phosphate due to its smaller size. Yet, because it is smaller, acetate has a higher charge density and, thus, a more organized solvent shell than dihydrogen phosphate.[30] However, at the working pH of these titrations, hydrogen phosphate is present, having a higher charge density than dihydrogenphosphate, thereby leading to a species having a more ordered solvation shell.[31] The data show that the entropy change is indeed significant for acetate binding. However, phosphate has a larger entropy change than in the case of acetate. For phosphate, this may arise from the larger more ordered solvent shell which is dispersed upon binding to the host.

B. Binding to Host 2. Thermodynamic investigations on the binding of phosphate to **2** were pursued using ITC techniques. Aliquots (4.5 μL) 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 $\Delta H°$ 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.

The possible presence of multiple equilibria lead to a Job plot analysis to verify the binding stoichiometry of the **2**:phos-

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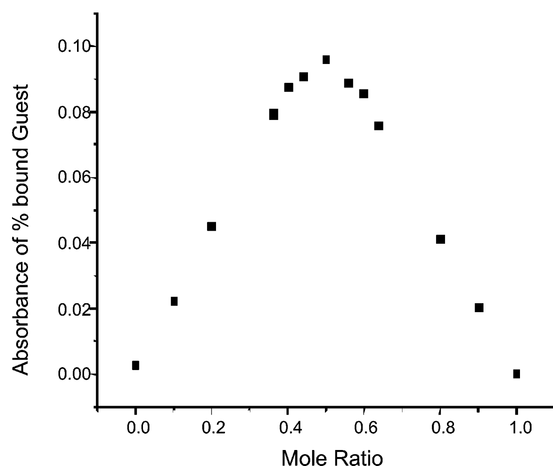

Figure 3. Job plot of receptor **2** binding phosphate. A total concentration of 2.1 mM was used buffered with TRIS at pH 7.4. A maximum at 0.5 mole ratio indicates a 1:1 host−guest stoichiometry for binding.

phate 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. 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 3). This result is a clear indication that the host−guest stoichiometry is 1:1 at the concentrations used for the UV/vis analysis.

The verification of a 1:1 binding stoichiometry allowed investigations to proceed with a van't Hoff analysis using the same concentration range as in the Job analysis. UV/vis spectroscopy was used to determine the binding affinities of phosphate to **2** over a range of temperatures from 16 °C to 55 °C. The general trend observed was a decrease in binding affinity with temperature increase. The resulting data (Figure 4A) were fit with linear regression from which a $\Delta H°$ value of −6.3 (±0.5) kcal/mol and a $T\Delta S°$ value of −0.7 (±1.0) kcal/mol were determined. These values indicate that there was a strong favorable enthalpy change associated with phosphate binding, with a slightly negative entropy change, suggesting that the guanidinium groups offer an enthalpic advantage in binding phosphate to **2**.

The enthalpic advantage offered by the guanidinium groups is even more apparent when comparing the ITC data for the **10**:phosphate complex to that for **2**. Compound **10** is slightly exothermic ($\Delta H° = -0.8$ (±0.5) kcal/mol), but the binding is primarily driven by a favorable entropy change ($T\Delta S° = +3.3$ (±1.0) kcal/mol). Using this ITC data, the advantage of appending the guanidinium groups to **10** creating **2** is only a factor of near 10 for binding phosphate, but the driving force has switched from primarily entropy with **10** to primarily enthalpy with **2**.

Although a linear fit to the data in the above van't Hoff analysis for **2** 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 found it instructive to fit the data with a modified van't Hoff equation (eq 5) which incorporates the heat capacity change.

$$R \ln K = -\Delta H_0(1/T) + \Delta C_p^\circ \ln T + (\Delta S_0 - \Delta C_p^\circ) \quad (5)$$

A plot of $R \ln K$ against T can be fit with eq 5, 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 (eqs 6 and 7) that account for the temperature dependence of the enthalpy and entropy changes of the system to yield $\Delta H°$ and $\Delta S°$ values.

$$\Delta H° = \Delta H_0 + T\Delta C_p$$

$$\Delta S° = \Delta S_0 + \Delta C_p° \ln T$$

Treatment of the binding data in this fashion (Figure 4B) yielded a $\Delta H°$ value of −3.8 (±0.5) kcal/mol and a $T\Delta S°$ value of +1.5 (±1.0) kcal/mol for the binding of phosphate to **2**. Because binding data were collected over a 40° temperature range, we are inclined to be more confident in the values obtained from the corrected van't Hoff plot. More importantly, the data from either analysis show the same trend. The binding is characterized by a favorable entropy change, but the complex formation is primarily driven by a favorable enthalpy change.

Heat Capacity Change. The van't Hoff analysis yielded a $\Delta C_p°$ value of −174 (±44) cal/molK. The heat capacity 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 ($\Delta C_p^\circ = -12$ to -190 cal/mol K) for hydrophobic interactions have been reported for cyclophane and cyclodextrin systems in water.[32] The change in heat capacity for electrostatic binding interactions in abiotic host−guest complexes (porphyrin−mannoside, quinone−porphyrin, and diacid−aminopyrimidine) in polar solvents have been reported to range from −30 to −560 cal/mol K.[33]

We attribute the ΔC_p° value found for **2** to the solvation differences between the host and the guest versus the host−guest complex. Receptor **2** is highly charged with solvent exposed functional groups; therefore, it is reasonable to expect the host and guest to be well solvated in aqueous media. Upon binding, the solvent spheres are disrupted and water excluded. The increase in entropy observed and the negative heat capacity change fit well with this scenario, which is very similar to the classical hydrophobic effect.[15]

Our abiotic host−guest systems differ from natural systems because 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.[34] We find a very significant heat capacity change with **2**.

Structure and Energetics. The results described above clearly indicate that the binding of phosphate to **1** and **2** have comparable Gibbs free energies of binding. Yet, the component enthalpy and entropy changes are different. Receptor **2** complexes phosphate with a dominant enthalpy driving force, whereas the binding of phosphate to receptor **1** is entirely entropically driven. Similar results were recently reported by

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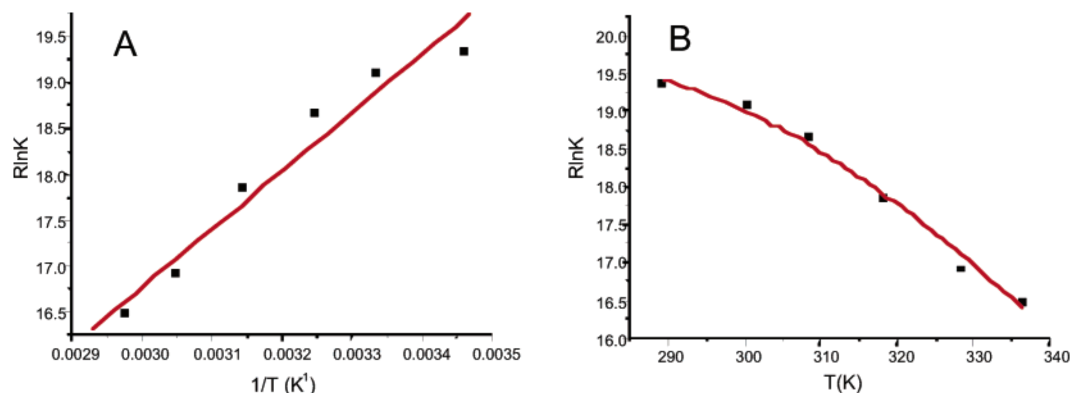

Figure 4. van't Hoff plots for phosphate binding to receptor **2**. (A) A plot of RlnK vs 1/*T*. The data were fit with a straight line from which the enthalpy and entropy changes were extrapolated. The linear fit has an R^2 value of 0.95. (B) The data in Figure 3 was plotted as RlnK versus T and fit with a van't Hoff equation that incorporated the temperature dependence of the enthalpy and entropy changes.

Smith and co-workers.[35] 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. A discussion of the differences in our hosts, which may also be relevant to their hosts, offers insight into the differences in the thermodynamic profiles.

We designed both **1** and **2** to possess a binding cavity to match the size and tetrahedral shape of oxyanions using a lock-and-key analogy. 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 enhance the favorable enthalpy change upon binding. Receptor **1** employs ammonium groups for this purpose, as they are known to be effective binding epitopes.[36] However, the ammonium groups in **1** did not contribute to an overall enthalpy change, but instead their presence led to enhanced binding when compared to **9** due to an increase in favorable entropy.

This observation may be explained by solvation considerations. We start with the assumption that the inherent flexibility in **1** renders the ammonium groups exposed and well solvated. If ion pairs between the phosphate and ammonium groups offer any electrostatic enthalpic advantage, it must be countered by endothermic changes in the solvent structure. In other words, the heats of solvation of the phosphate and ammonium groups separately, with their respective organized solvation spheres, must be nearly identical to the heats of interaction between ammoniums and phosphate in the complex and in the released water. Upon binding phosphate, however, solvent molecules and/or counterions would be released, leading to the favorable entropy change observed. This favorable entropy change must overcome any restriction of degrees of freedom upon organizing the structure of **1** to phosphate binding.

In contrast, the guanidinium groups of receptor **2** are likely less well solvated enthalpically than the ammonium groups of receptor **1** for several reasons. First, receptor **2** is predicted to be more preorganized (see the earlier discussion) and, hence, has a smaller cavity containing less waters of solvation. Second, guanidinium groups in general are predicted to be less hydrated than ammonium groups. Smaller more localized ionic charges, such as an ammonium relative to a guanidinium group, are better solvated enthalpically and have a larger cytobactic region. This means that the water around an ammonium is more organized, and the extent of organized solvation sphere being replaced by interaction of phosphate would be smaller with a guanidinium group but larger with an ammonium group. Thus, upon losing their solvation shell, the ammonium groups are expected to release more solvent molecules into bulk solution, but importantly, they release solvent that is more organized than would guanidinium groups.

The solvation differences of an ammonium versus a guanidinium group leads to the reasoning given above for why there is an entropy difference between receptors **1** and **2**, but differences in solvation also predict the associated enthalpy difference. We note that the free energy of transfer from an organic solvent to water is actually larger for a guanidinium group than an ammonium group.[37] However, this does not mean that the heat of hydration of a guanidinium group is larger than that of an ammonium group. In fact, recent studies show that water is not well organized around guanidinium groups,[38] and therefore, it is postulated that the water does not sit in a deep enthalpic well when solvating guanidinium groups. This finding means there is likely a much smaller enthalpic attraction between water and a guanidinium group relative to an ammonium group, and that the higher hydrophilicity of a guanidinium group relative to an ammonium group almost completely results from entropy. This favorable entropy of hydration arises from the ability of a guanidinium group to break the structure of bulk water, a well-known chaotropic property.[39] Therefore, we postulate that the more exothermic enthalpy of phosphate binding to **2** relative to **1** arises from a weaker enthalpic solvation of guanidinium groups in **2**, allowing the enthalpic interactions with phosphate to manifest themselves to a greater extent than with **1**. In other words, the heats of solvation of separate guanidinium groups and phosphate are less favorable than the electrostatic attraction between the guanidinium-phosphate and the released water primarily because guanidiniums are not as well solvated enthalpically initially.

This enthalpy argument further supports the discussion given

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above related to the increased favorable entropy for binding with ammonium groups relative to guanidinium groups. Based again upon the recent studies indicating an unorganized solvation sphere around a guanidinium group,[38] and upon the postulate that guanidinium groups act as chaotropic agents by breaking the structure of bulk water,[39] we again postulate that the release of water from a guanidinium group is not expected to impart as large an entropy advantage relative to the release of water involved in organized solvation spheres around ammonium groups.

The comparison of binding groups given above assumes that differences in solvation of the bound and unbound form of each receptor are nearly identical in all other respects. This assumption seems reasonable based on the thermodynamic data for the binding of phosphate to both control hosts **9** and **10**. They both interact with phosphate through the Cu(II) center with a small enthalpy change and almost identical entropy changes. Therefore, the differences in the thermodynamics of phosphate binding to **1** and **2** arise from the functional groups on the periphery of the respective cavities.

Conclusion

In summary, a specific design approach yielded two metalloreceptors having high selectivity and high affinity for phosphate in water at neutral pH. The binding energies of the host−guest complexes were very similar; however, the subtle differences in the binding were identified by further quantifying the enthalpic and entropic contributions to the complex formation. The Cu(II) center was shown to be an effective binding site for the anion. Yet, the cavity design appears to be more significant in creating a high affinity and high selectivity complex. In addition to the specific receptor design, the dominant entropy change in phosphate:**1** binding further supports recent proposals that solvation/desolvation processes can be an important design criteria. The thermodynamic profile of **1** is quite similar to the accepted thermodynamic character of the "classical" hydrophobic effect, having a favorable entropy change. In contrast, the phosphate:**2** complex formation was predominantly driven by a favorable enthalpy change, reflecting the lower extent of solvent organization around guanidinium groups relative to the ammonium groups, and lower enthalpy of solvation of guanidinium groups. It is clear that thermodynamic investigations are a powerful means by which complex formations in solution can be characterized and thereby further our understanding of fundamental binding forces.

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Supporting Information Available: The Experimental Section. This material is available free of charge via the Internet at http://pubs.acs.org.

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