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Published on Web 11/20/2002

Ion-Pairing Molecular Recognition in Water: Aggregation at Low Concentrations That Is Entropy-Driven

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Received April 29, 2002

Abstract: Investigations into the thermodynamic parameters that characterize the binding of citrate to tris-guanidinium host **1** in water are reported. The parameters K_a, $\Delta H°$, $\Delta S°$, and $\Delta G°$ for the binding event were quantified using isothermal titration calorimetry (ITC) techniques. The 1:1 binding stoichiometry was verified by a Job plot derived from NMR data, and the microcalorimetry data was collected for solutions of **1** and citrate ranging from 1 to 100 mM using phosphate buffer concentrations of 5 and 103 mM. At low buffer concentrations (low ionic strength) complexes with greater than 1:1 stoichiometries were observed by ITC, and K_1 was determined to range from 2.0×10^3 to 3.0×10^3 M^{-1}. At higher buffer concentrations (high ionic strength) the higher-order complexes were not detected, and K_1 was determined to be 409 M^{-1}. The 1:1 association of host **1** and citrate is characterized by a large favorable entropy component and negative enthalpy. However, the complexes with higher-order stoichiometry arise from desolvation processes that result from the association of polyions in aqueous media and is entirely entropy driven. This leads to an unusual observation: the dilution of one component of the host/guest complex leads to the formation of the higher-order complexes. The reason for this observation is discussed.

Introduction

Entropy is a thermodynamic measure of a system's disorder, or a function that directly correlates to probability.[1] 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 interactions that are favorable enthalpically. Yet, interactions between biological molecules rely predominately on a series of weak noncovalent interactions, with low activation energies for formation. Hence, a large series of these weak interactions is required to counteract the unfavorable entropy. This is a general phenomena. Yet, 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. For example, it is well know that the interfaces of interacting proteins are more likely to incorporate nonpolar amino acids than the remainder of the protein exterior,[2a] and thus desolvation of these nonpolar amino acids (the hydrophobic effect)[2] has a significant impact on the overall thermodynamics of association.[3] However, 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.

As implied in the discussion above, the relatively small enthalpy changes associated with weak noncovalent interactions, by themselves, are often not sufficient to render many supramolecular systems thermodynamically stable.[4] Further, as the number of components in a supramolecular system increases, the enthalpy contribution or desolvation must increase to counteract the inherent negative entropy that derives from association. Therefore, complexes having greater than 1:1 stoichiometry should be driven by increasing enthalpy or desolvation.

Solvent release can occur upon complex formation, thereby giving an overall positive entropy.[5,6] This was shown experimentally in one case by comparing the stability of 1:1 and 1:2 γ-cyclodextrin complexes with various guests at different reagent concentrations,[7] and many molecular recognition events

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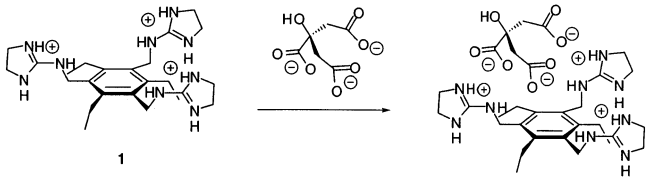
have been found to be favored entropically.[8] Recently, Schmidtchen[9] and Inoue[7a] have explored the control of solvation/desolvation processes as a possible strategy to overcome the loss of entropy upon binding, even in systems that do not exploit the hydrophobic effect, such as ion-pairing. Although ion-pairing may be expected to be associated with negative enthalpy, it has been found that it only sometimes behaves this way and that commonly entropy in part drives the interaction.[5,6,8,10] Therefore, controlling complexation via focusing upon entropy effects may be a more general strategy than the focus commonly given to enthalpy considerations, especially with regard to ion-pairing-driven molecular recognition.[11]

Our initial intention in this study was simple. We wanted to characterize the thermodynamic parameters associated with the complexation of host **1** with citrate, a previously reported host/guest interaction.[12] We anticipated that the binding would be favorable, both enthalpically and entropically, as may be expected on the basis of the discussion given above. However, we also found that entropy-driven association results in highly complex assemblies at low reagent concentrations. Aggregation of ions at low concentrations has been observed in other ion-pairing molecular recognition systems,[13] although the aggregation was not explored in depth. Therefore, the effect we report here is not an isolated phenomenon associated with our system. Our discovery was surprising and seemed to us at first to be contradictory. As reported herein, we observed entropy-driven complexation with increased complexity upon reduction of the concentration of one component while the concentration of the other is held constant. The use of electrostatic interactions of oppositely charged organic polyions as the driving force for the association of organic molecules gave this result. The reason for this unusual behavior is the vantage point taken in discussing the ITC experiments presented.



1

Experimental Section

Materials. Compound **1** was synthesized as described previously.[12] The purity of **1** was verified by elemental analysis and ^{1}H and ^{13}C NMR. All other chemicals used in the microcalorimetric, 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.[16] The thermodynamic parameters for the complexation reaction of cyclohexanol with β-CD were also in good agreement with previous results.[17]

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,[17] 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 1) 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.[7a]

Each microcalorimetric titration experiment consisted of 20−40 successive injections as described previously.[7a] Initial concentrations of citrate and **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,[18] and thus at least 90% of the citrate is trianionic at pH

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Table 1. Complex Stability Constant (K), Standard Free Energy ($\Delta G°$), Enthalpy ($\Delta H°$), and Entropy Changes ($T\Delta S°$) for 1:1 and 2:1 Complexation of the Macrocycle with Citrate in Aqueous Buffer Solutions at 298.15K.

reaction	[citrate] (mM)	[**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 + **1** = (C·**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·**1**)+**1** = (C·**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 + **1** = (C·**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·**1**)+ **1** = (C·**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 + **1** = (C·**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·**1**)+ **1** = (C·**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 + **1** = (C·**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·**1**)+ **1** = (C·**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 + **1** = (C·**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·**1**)+**1** = (C·**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 + **1** = (C·**1**)	100.3	6.26	N = 40 (5 mM)	3180 (K_1)	−19.99	−4.1	15.9
(C·**1**)+ **1** = (C·**1**$_2$)	100.3	6.26	N = 40 (5 mM)	330 (K_2)	−14.38	5.6	20.0
C + **1** = (C·**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·**1**)+ **1** = (C·**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)

[a] Number of data points (or number of injections) in microcalorimetric titration experiment and concentration of phosphate buffer (pH 7.4) in mM.

7.4. At this pH, host **1** is tricationic, since the pK_a's are all above 11.0.[19] 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 nonideality corrections are not necessary under the experimental conditions employed.[17]

NMR Experiments. The ^{1}H NMR experiments used to determine Job plots[20] at a total citrate/**1** concentration of 10 mM were performed on a Varian UNITY 300 MHz instrument. The ^{1}H NMR experiment at a total citrate/**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 were collected on a 100 mM sample of **1** in water (pH = 7.4) using the electrospray ionization mode. The standard conditions employed were 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**/isocitrate mixtures were obtained in a conventional quartz cell (10 mm × 10 mm × 45 mm) at room temperature by using a JASCO J-720 instrument.

Results

Aggregation State of Interacting Molecules in the Solution. Before studying the molecular recognition of **1** with citrate we needed to delineate the aggregation state of these structures under the experimental conditions to be employed. Microcalorimetric dilution experiments of citrate in aqueous buffer solutions demonstrate heat patterns typical of dilution of simple electrolytes[21] (Supporting Information). This supports the assumption that citrate (−3) does not self-associate under the experimental conditions employed.

Host **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 or through micelle-like interactions of six ethyl groups from two molecules of **1**. Dilution microcalorimetric experiments were performed by injection of a 75 mM solution of **1** in 5 mM phosphate buffer (pH 7.4) into the reaction microcalorimetric cell charged with the same buffer solution. This exhibited relatively small endothermic heat effects (Supporting Information) which are consistent with the range of heat effects observed for the dilution of a simple electrolyte.[21] Opposite signs of the heat effects (endothermic for **1** and exothermic for citrate) do not support aggregation of **1** in the solution. It is well-known that the sign of heat effects upon dilution of conventional inorganic or organic electrolytes varies depending on the particular electrolyte employed and on its specific analyte concentrations in solution.[21] The dilution experiments also do not support significant complexation of **1** with the phosphate buffer.

High concentrations of **1** and added salt would be expected to enhance the hydrophobically driven aggregation. Therefore, further dilution experiments were pursued to confirm the absence of aggregation of **1**. Dilution microcalorimetric experiments were repeated in the presence of a higher salt concentration (103 mM phosphate buffer pH 7.4). The heat of dilution of 77.3 mM **1** into 103 mM phosphate buffer, pH 7.4 (Supporting Information) 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.[21] 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 vs 5 mM phosphate buffer pH 7.4). The curves also show that even at the higher phosphate buffer concentrations, no significant complexation of **1** with phosphate is evident.

Thus, the results of our dilution microcalorimetric experiments give no indication of significant aggregation of **1** in the solution. Nevertheless, a small degree of the aggregation of **1** in the range of several percent cannot be ruled out on the basis of the microcalorimetric experiments, therefore ESI mass spectra were obtained to identify all species present in a solution of **1**. The

(19) Titration of **1** reveals pK_a values all above 11. Perreault, D.; Anslyn, E. V. Unpublished results.

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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 **1** likely would not be detected. This is consistent with a previous report of little to no binding between phosphate and **1** in water.[12]

ESI measurements performed on a 100 mM solution of **1** using standard conditions indicate that the predominant species is monomeric, with some dimer, in a 97:3 ratio (Supporting Information). 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 (Supporting Information) correspond to the transfer of chloride ions from the solution phase into the gas phase together with **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 did not support higher levels of dimerization.

In addition to the above experimental evidence, a Beer's law plot of UV absorbance (270 nm) versus concentration of **1** (Supporting Information) shows no curvature, which is consistent with the existence of **1** in the solution in a monomeric form.[22] Therefore, on the basis of microcalorimetric, ESI, and spectrophotometric data, both **1** and citrate are considered to exist as monomers in aqueous buffer solutions.

Microcalorimetric Experiments at Low Concentrations of 1 (1−2 mM) and Low Ionic Strength (5 mM Phosphate Buffer). Titration curves for three microcalorimetric experiments performed at various citrate/**1** concentrations are presented in Figure 1. In each of the three cases the shapes of the curves indicate that there are at least two different complexation events occurring as citrate and **1** interact. The complexation reaction which 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. The inflections in the curves signal binding events other than simple 1:1 complexation. 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 equiv, a (Citrate·$\mathbf{1}_2$) complex is likely.

As described in an earlier article,[7a] the simplest and the most reasonable theoretical model to fit titration curves presented in Figure 1a−c is a stepwise 2:1 complexation (eqs 1 and 2). In our previous study, we used the most common fitting procedure of a stepwise 2:1 complexation model with "host" in the reaction microcalorimetric cell and "ligand" in the syringe. In the present study the "deconvolution" method[23] was successful in fitting the experimental data. It should be noted that the ORIGIN software used for all calculations in this study defines "host" as the species that possess more than one interacting site ($n \geq 1$) and the "ligand" as the species with only one interacting site ($n = 1$). By definition, in the "deconvolution" method it is assumed that citrate is in the syringe and **1** is in the reaction microcalorimetric cell. Consequently in light of the experimental data presented in Figure 1a−c, one should consider the following equilibria to occur in the reaction mixture of citrate and **1**:

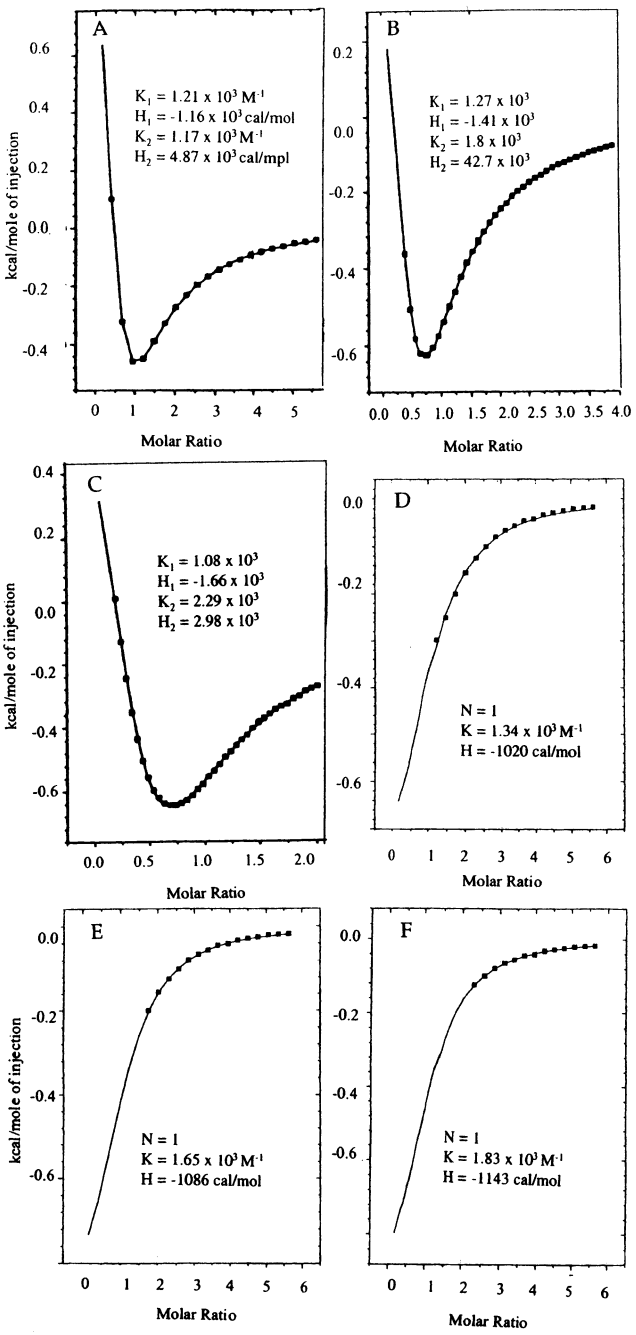


Figure 1. Microcalorimetric titration curve of addition of citrate solution (5 mM phosphate buffer pH 7.4) into reaction cell charged with **1** (5 mM phosphate buffer pH 7.4): (a**)** 99.9 mM citrate and 1.41 mM **1**; (b**)** 100.3 mM citrate and 1.98 mM **1**; (c**)** 51.7 mM citrate and 1.98 mM **1**. The curve fits as they appear are a result of applying an identical interacting sites model with n=2. (d−f) The effect of the gradual deletion of data points from the initial. Part of the titration curve shown in (a). The curve fit was derived from application of a 1:1 model.

$$\text{citrate}^{3-} + \mathbf{1}^{3+} = (\text{Citrate}\cdot\mathbf{1})^{0} \quad (1)$$

$$(\text{Citrate}\cdot\mathbf{1})^{0} + \mathbf{1}^{3+} = (\text{Citrate}\cdot\mathbf{1}_2)^{3+} \quad (2)$$

(22) Jencks, W. P. *Catalysis in Chemistry and Enzymology*, 2nd ed.; Dover Publications: New York, 1987.
(23) Yang, C. P. ITC Data Analysis in Origin v.2.9, MicroCal Inc.: Northampton, MA, 1993.

The equilibrium constants reported for all fitting procedures (Figure 1a−c) are intrinsic equilibrium constants[23,24] ($K_1°$, $K_2°$) 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 3 and 4) using the relationship shown in eq 5. These K values are representative of the binding constants with the degeneracies removed, that is, affinities assuming no interactions between binding groups. These K values were then used to determine $\Delta G°$ values (Table 1).

$$K_1 = [(\text{Citrate}\cdot\mathbf{1})^0]/[\text{Citrate}^{3-}]\,[\mathbf{1}^{3+}] \quad (3)$$

$$K_2 = [(\text{Citrate}\cdot\mathbf{1}_2)^{3+}]/[(\text{Citrate}\cdot\mathbf{1})^0]\,[\mathbf{1}^{3+}] \quad (4)$$

$$K_i = [(n - i + 1)/i]\cdot K_i° \quad (5)$$

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.[7a] Several considerably different sets of parameters (K_1, $\Delta H_1°$; K_2, $\Delta H_2°$) 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.[23,25]

The majority of the experimental data points (Figure 1a−c) are located on the portion of the curve that exponentially approaches 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 $(\text{Citrate}\cdot\mathbf{1})^0$ complex from monomeric citrate and **1** are well defined. Indeed, the values of K_1 (Table 1) range from 2200 to 2580 M^{-1} (less than 10% from the average) and values of $\Delta H_1°$ are in the range from −4.85 to −6.95 kJ mol^{-1} (they deviate about 20% from the average). The quantitative estimations of the K_1 and $\Delta H_1°$ values were verified by applying a simple 1:1 model to the final part of the titration curves (Figure 1d−f). It is reasonable to assume that deletion of data points from the initial part of titration curve, endothermic formation of $(\text{Citrate}\cdot\mathbf{1}_2)^{3+}$, 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, $\Delta H_1°$, $\Delta H_2°$) can fit the curve, one finds that there is only one set of parameters (K_1 and $\Delta H_1°$) that fits the later points in the titration. Also, because citrate is in excess at the final portion of the titration curve, formation of $(\text{Citrate}\cdot\mathbf{1}_2)^{3+}$ 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 $\Delta H_1°$ values become more closely matched to the range of the values obtained by the stepwise 2:1 complexation model (Figure 1a−c). Deletion of about one-third of the data points (Figure 1f) followed by curve fitting with the 1:1 model results in K_1 equal to 1830 M^{-1} and $\Delta H_1°$ equal to −4.8 kJ mol^{-1}. This indicates that at large enough citrate/**1** ratios, the values obtained by both fitting procedures (simple 1:1 model and stepwise 2:1 complexation model) are in quantitative agreement with each other.

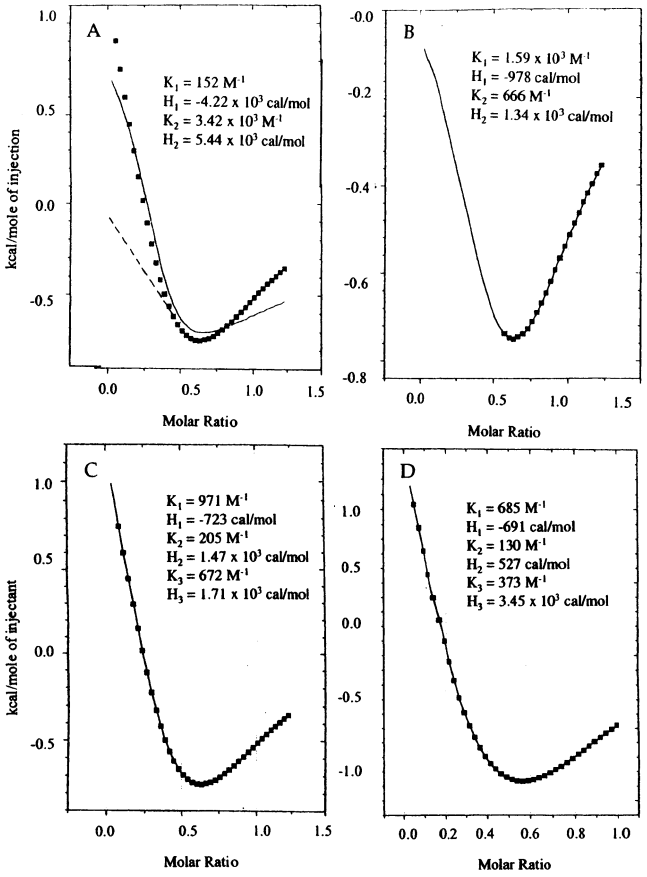


Figure 2. Microcalorimetric titration curve of the addition of 100.3 mM citrate solution (5 mM phosphate buffer pH 7.4) into the reaction cell charged with 6.26 mM **1**: (a**)** The solid line represents the curve fitting analysis using a four parametric fit. The dotted line represents the four parametric curve fit obtained from Figure 2B. (b**)** Four parametric curve fit to the data at citrate/**1** molar ratios larger than 0.5. (c) Six parametric fit of all the data points obtained during titration of 100.3 mM citrate solution into reaction cell charged with 6.26 mM **1**. (d) Six parametric fit of all data points obtained during titration of 145 mM citrate solution into reaction cell charged with 11.2 mM **1**.

Given the above discussion, the estimation for K_1 and $\Delta H_1°$ values are reliable. However, the complexity of a stepwise 2:1 binding algorithm does not allow the same conclusion for K_2 and $\Delta H_2°$ values. Further, the magnitude of these values vary depending on the experimental conditions, as discussed below.

Microcalorimetric Experiments at Higher Concentrations of 1 (6−11 mM) and Low Ionic Strength (5 mM Phosphate Buffer). These titrations were repeated using higher initial concentrations of **1** in the reaction cell. There were two reasons for doing this. First, the higher concentration of **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 microcalorimetric titration of 100 mM citrate solution (in the syringe) into the reaction cell charged with 6.3 mM **1** in 5 mM phosphate buffer (pH = 7.4) are presented in Figure 2a−c. In contrast to the experimental data obtained at the lower concentrations of **1** (1.4−2.0 mM) (Figure 1a−c), the experimental data obtained at higher concentrations of **1** (Figure 2a) 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 $(\text{Citrate}\cdot\mathbf{1})^0$ and $(\text{Citrate}\cdot\mathbf{1}_2)^{3+}$ in solution, when **1** is at higher concentra-

(24) van Holde, K. E. *Physical Biochemsitry*, 2nd ed.; Prentice-Hall: New Jersey, 1985.
(25) Rekharsky, M. V. Application of Microcalorimetry in Biochemistry. Thesis Dr. of Science, Institute of Biological and Medical Chemistry, Russian Academy of Medical Sciences, Moscow, 1997.

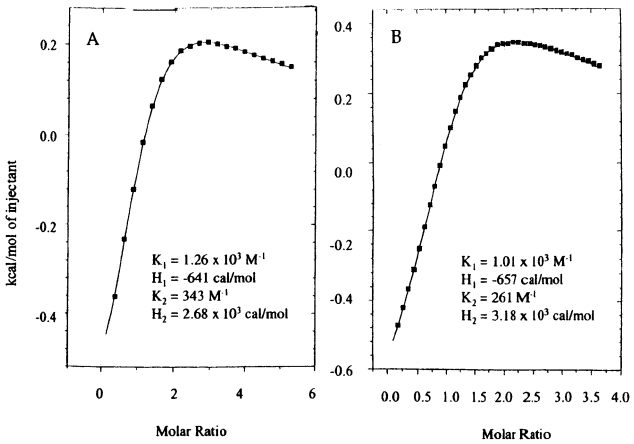


Figure 3. (a,b) Microcalorimetric titration curve of addition of **1** (5 mM phosphate buffer pH 7.4) into reaction cell charged with citrate solution (5 mM phosphate buffer pH 7.4): (a) 1.16 mM citrate and 77.3 mM **1**; (b) 2.27 mM and 107.7 mM **1**.

tion. Large deviations from the stepwise 2:1 complexation model are observed only at very large excess of **1** in the solution (Figure 2a), but all the data points at the molar ratios larger than 0.5 are well characterized by a stepwise 2:1 complexation model (Figure 2b). 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, $\Delta H_1°$; K_2, $\Delta H_2°$; K_3, $\Delta H_3°$) (Figure 2c). Furthermore, the results of titration experiments using a higher concentration of **1** (11.2 mM) (Figure 2d) can also be described by stepwise 3:1 complexation model with a comparable set of parameters.

An excellent curve fit using a stepwise 3:1 complexation does not verify the existence of (Citrate·$\mathbf{1}_3$)$^{6+}$ in the solution, and indeed such an aggregate seems implausible. Intuitively, the existence of such a species is rather unlikely. A (Citrate·$\mathbf{1}_3$)$^{6+}$ species having a +6 charge should possess a high affinity toward a second negatively charged (−3) citrate anion. Thus, it would be more logical to assume a higher-order aggregation species with empirical stoichiometries near 1:1 such as (Citrate$_2$·$\mathbf{1}_3$)$^{3+}$, (Citrate$_3$·$\mathbf{1}_4$)$^{3+}$, and so forth. 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.

Microcalorimetric Titration of Citrate by a Solution of 1. To confirm the aggregation state and endothermic peaks we performed reverse titrations. The results of the reverse microcalorimetric titrations, 77−107 mM **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 Figure 3a,b. As expected, excess **1** in the reaction cell (molar ratio > 1) during the latter part of titration experiment increases the probability of the formation of a (Citrate·$\mathbf{1}_2$)$^{3+}$ species, and endothermic heat effects again dominate. In principle, the predominant formation of (Citrate·$\mathbf{1}_2$)$^{3+}$ species in the reaction mixture should facilitate reliable determination of complexation thermodynamic parameters for these species.

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 **1** is in large excess. In contrast, the K_2 values presented in Figure 1a−c and Figure 3a,b are vastly different. This experimental observation indicates that the reaction mixture is more complicated than merely having the coexistence of two

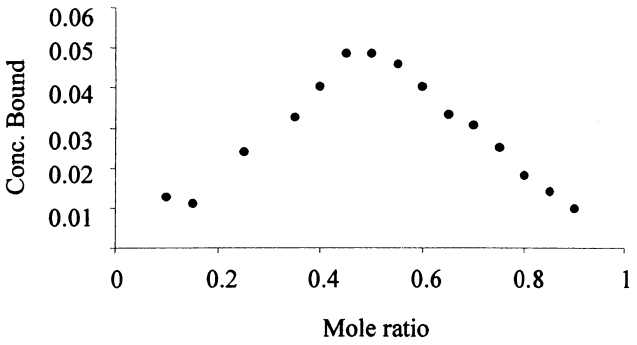


Figure 4. Job plot of of NMR titration data for a solution of total concentration 4.01 mM. Solutions were buffered with at pH 7.4. Note the maximum at 0.5 mole ratio.

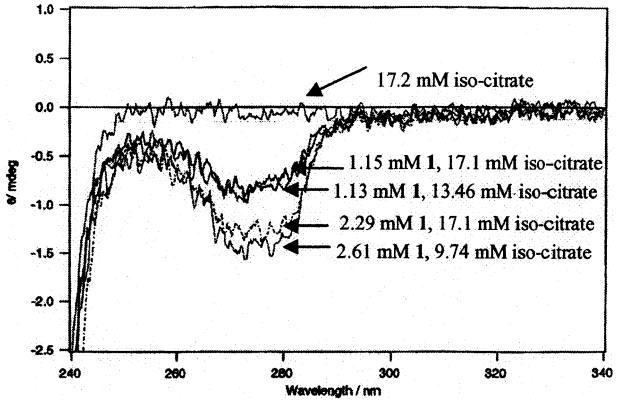


Figure 5. Circular dichroism (CD) spectra of a solution of **1** at two concentrations (1.1 mM and 2.2−2.6 mM) with a large excess of chiral isocitrate (10−17 mM).

complex species, that is, (Citrate·**1**)^0 and (Citrate·$\mathbf{1}_2$)$^{3+}$. Again, the results indicate the existence of more complex aggregates with excess **1** in solution.

Stoichiometry of 1/Citrate Association As Derived from NMR, ESI, and CD Measurements. To better understand the stoichiometry of the complexes in solution at the high concentrations of **1** Job plots using ^{1}H NMR were obtained. Job plots obtained at two different total **1**/citrate concentrations, namely 10 mM and 4.01 mM (Figure 4), reveal the existence of only one maximum at molar ratio 0.5. These results are counter to the suggestion that multiple complexes such as (Citrate·$\mathbf{1}_2$)$^{3+}$ exist. There are two possible reasons for the disagreement between the stepwise 2:1 complexation model curve fit (Figures 1a,b and 3a,b) and the Job plot. One is aggregation in which the species maintain an empirical stoichiometry close to 1:1, such as (Citrate$_2$·$\mathbf{1}_3$)$^{3+}$, (Citrate$_3$·$\mathbf{1}_4$)$^{3+}$, etc. Another is that the chemical shifts of the aggregates and the 1:1 complex are all very similar.

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·**1**)$_2$, CD spectra of the solutions containing chiral isocitrate and **1** at various concentrations were obtained (Figure 5). A 2-fold increase of the isocitrate concentration (from 9.74 to 17.1 mM) does not lead to an enhancement of the CD signal. This shows that a 4-fold excess of isocitrate (9.74 mM) over **1** is enough for the complete saturation of **1**. A 2-fold reduction in concentration of **1** decreases the CD signal by only 2-fold. This result was obtained under conditions where **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 if a 2:2 complex dissociated upon dilution. This supports the existence of a Citrate·**1** complex rather than a complex dimer (Citrate·**1**)$_2$.

Mass Spectroscopy To Probe Complex Stoichiometry. To further identify the species present in the reaction mixtures during the ITC analyses, various mass-spectrometric techniques, for example, ESI, MALDI, and CI were applied.[26] The most logical set of results comes from the ESI data in which (Citrate·**1**$_2$) dominates at low citrate concentrations and high host concentration. As the citrate concentration is increased (Citrate·**1**) (MW 646) begins to dominate the spectra. This observation rules out the possibility of (Citrate·**1**) dimerization. If dimerization occurs to form (Citrate·**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 $+$**1** $=$ (Citrate·**1**), and consequently the concentration of the dimers should also increase on the basis of the equilibrium (Citrate·**1**) $+$ (Citrate·**1**) $=$ (Citrate·**1**)$_2$. Yet, (Citrate·**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 **1** (1−2 mM) is a stepwise 2:1 complexation. The coexistence of only two complex species, namely, (Citrate·**1**) and (Citrate·**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 **1**, higher-order aggregates exist.

Comparison of Equilibrium Constants Obtained by Microcalorimetric and NMR Titrations. ^{1}H NMR titrations in previous work[10] 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 **1**, or vice versa, shows little to no complex formation in water.[12]

The affinity constant of a 1:1 complexation for **1** with citrate obtained using microcalorimetric data in this study is in the range of 2×10^3 to 3×10^3 M^{-1} using a 5 mM phosphate buffer. In dealing with the association of oppositely charged polyelectrolytes, the strong dependence of equilibrium constants upon changes in ionic strength is anticipated. Thus, the results of ^{1}H NMR and microcalorimetric titrations are in complete agreement. Indeed, the equilibrium constants determined by microcalorimetry 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 microcalorimetry 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 versus D_2O do not exceed 10−20% and are thus insignificant in the context of the discussion given.[27]

(26) (a) Vincenti, M. *J. Mass. Spectrom.* **1995**, *30*, 925; Brodbelt, J. S.; Dearden, D. V. *Comprehensive Supramolecular Chemistry*; Davies, J. E. D., Ripmeester, J. A., Eds.; Elsevier Science Ltd.: Oxford, 1996; Vol. 8, pp 567−589.

(27) Rekharsky, M.; Inoue, Y. Unpublished results

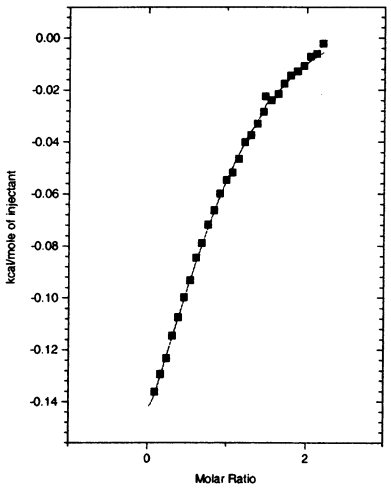


Figure 6. Microcalorimetric titration of **1** (107 mM) into a solution of citrate (2.27 mM) with 103 mM buffer at pH 7.4.

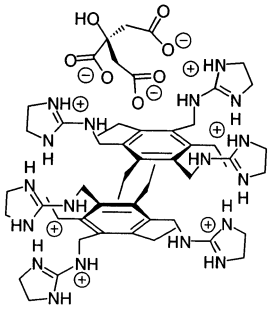
Determination of the Driving Force for Aggregation. We sought to uncover whether the higher-order aggregation arises from ion-pairing or the hydrophobic effect. If hydrophobic interactions are the dominant forces for the formation of (Citrate·**1**$_2$) species, the addition of methanol to the reaction 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 (Supporting Information). However, the pattern of the heat effects during consecutive injections of 100 mM citrate into a solution of **1** are qualitatively very similar (pure 5 mM phosphate buffer at pH 7.4 vs 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, $\Delta H_1°$; K_2, $\Delta H_2°$; K_3, $\Delta H_3°$) (Supporting Information). As discussed above K_3 and $\Delta H_3°$ values are not thermodynamic parameters that characterize the formation of a (Citrate·**1**$_3$) species, but rather are an indication of higher-order aggregation. In both scenarios, the K_3 and $\Delta H_3°$ 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 **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 polyelectrolyte ions driven predominantly (or solely) by electrostatic interactions can be easily perturbed by the presence of another polyelectrolyte at high concentration, for example, HPO_4^{2-}. To address this possibility, a microcalorimetric titration of 75 mM **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 6) differ from what was observed previously at the low buffer concentration (Figure 1a,b). It should be emphasized that all injections (Figure 6) up to **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

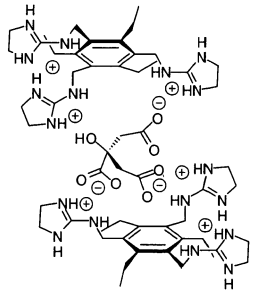
attributed to a 2:1 complex. This indicates that high ionic strength suppresses 2:1 complexation as well as higher-order **1**/citrate aggregation. Second, by applying the same computer simulation model of a stepwise 2:1 complexation described above, values were 5−6 times lower for K_2 and about 3 times lower for K_1 (Figure 6) were obtained. The suppression of 2:1 complex formation and higher-order **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 **1**.

Discussion

Driving Force of Complex Formation: Hydrophobic or Electrostatic Interactions? The formation of a (Citrate·**1**) complex is certainly driven by electrostatic interactions between oppositely charged reactants, and indeed the enthalpy is negative. However, the driving force of (Citrate·$\mathbf{1}_2$) 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·$\mathbf{1}_2$) complex. Such hydrophobic interactions would be considerably facilitated by the presence of citrate. The (Citrate·**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 $\mathbf{1}_2$ in a concentrated solution of **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 **1** and a second molecule of **1** interacts from the other side. In this case the dominant driving force for the formation of a (Citrate·$\mathbf{1}_2$) 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 **1** that do not interact with each other. Some support for the existence of such a structure can be found by considering the structure of citrate/**1** complex reported previously.[12] 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 **1**. However, in another crystal cell only two carboxylate groups of the citrate anion interact with one molecule of **1**, and the third carboxylate group of the same citrate anion interacts with another molecule of **1**. Formation of ion-pairs and release of a considerable amount of water molecules from the originally separated hydration shells of citrate and **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.



Why Are the Higher Stoichiometry Complexes Entropy-Driven? The formation of a simple 1:1 complex between citrate and **1** is driven by both favorable (negative) reaction enthalpy and favorable (positive) reaction entropy. This is reasonable when electrostatic interactions associated with profound desolvation processes are responsible for complex stability. Simple consideration of Coulomb's law predicts enthalpy-driven ion-pairing. 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 **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·$\mathbf{1}_2$) 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 1). To further develop this idea one could suggest that increasing aggregation, for example (Citrate$_2$·$\mathbf{1}_3$), (Citrate$_3$·$\mathbf{1}_4$), and so forth, 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 (Figure 2a,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.[28] 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

(28) (a) Nintrode, P. L.; Privalov, P. L. *J. Mol. Biol.* **1997**, *5*, 1050. (b) Park, S. J.; Hong, J.-I. *Tetrahedron Lett.* **2000**, *41*, 8311. (c) Iszatt, P. M.; Oscarson, J. L.; Gillepsie, S. E.; Chen, X.; Wang, P.; Watt, G. D. *Pure Appl. Chem.* **1995**, *4*, 543. (d) Teulade-Fichou, M.-P.; Vinneron, J.-P.; Lehn, J.-M. *J. Chem. Soc., Perkin Trans. 2* **1996**, *2*, 2169.

micelles. Dilution of the aggregate inevitably leads to their spontaneous degradation into single separate molecules. The most distinguishing feature of the **1**/citrate system is the increasing complexity with reduction of citrate concentration while holding the concentration of **1** constant. We would like to discuss this issue in more detail.

As stated in the Introduction, entropy serves as a destructive factor upon dilution of any and all host/guest complexes formed by noncovalent 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 microcalorimetric curves to those involved in the initial part of the curves, we find what appears to be the more complex species ($C_n\mathbf{1}_m$, 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 the surprising 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 6 and 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 **1**, which can release water upon complexation with (Citrate·**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 **1** do we find the simpler (Citrate·**1**) complexes, because now both enthalpy and entropy drive their formation, which combined are stronger driving forces than solvent release alone.

$$C(H_2O)_{n+a} + \mathbf{1}(H_2O)_{m+b} \rightleftarrows C\cdot\mathbf{1}(H_2O)_{a+b} + (n+m)H_2O \quad (6)$$

$$C\cdot\mathbf{1}(H_2O)_{a+b} + \mathbf{1}(H_2O)_{m+b} \rightleftarrows C\cdot\mathbf{1}_2(H_2O)_{\alpha(a+2b+m)} + (1-\alpha)(a+2b+m)H_2O \quad (7)$$

where $1 > \alpha \gg 0$.

Conclusions

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 or supplementary electrolyte concentrations or both. Most importantly, the complexity of the supramolecular architectures would be higher at the lower concentrations when one component is in excess.

Acknowledgment. We gratefully acknowledge the support for this research from NIH (GM65515) and JST.

Supporting Information Available: Isothermal data for dilution of **1** and citrate, ESI-MS data, Beer's Law plot for **1**, ITC data for titrations with solutions containing methanol (PDF).

JA020612E