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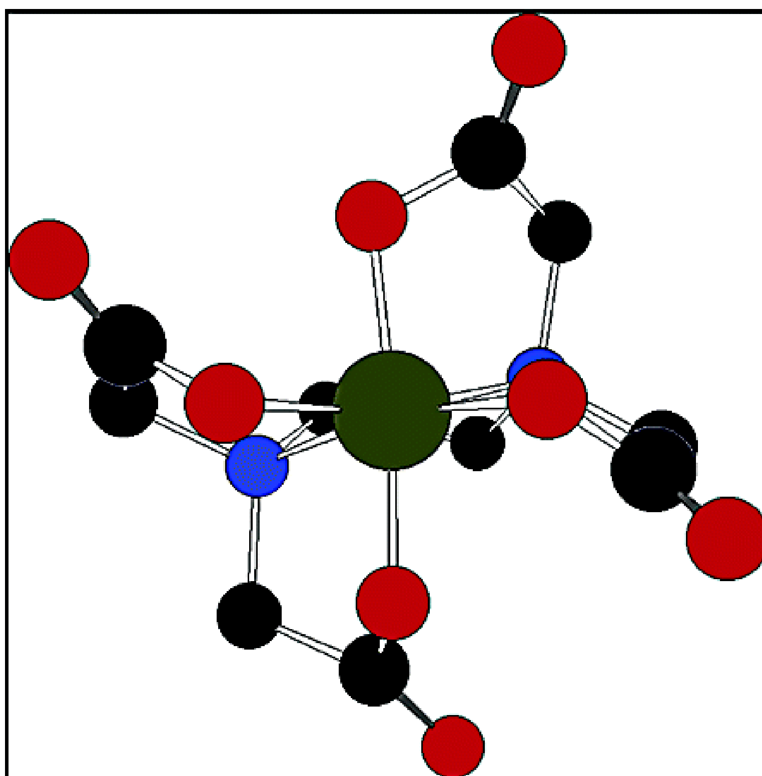

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Additivity and the Physical Basis of Multivalency Effects: A Thermodynamic Investigation of the Calcium EDTA Interaction

Trine Christensen, David M. Gooden, Joyce E. Kung, and Eric J. Toone*

Contribution from the Department of Chemistry, Duke University, B120 LSRC, Durham, North Carolina 27708

Received October 3, 2002; E-mail: eric.toone@duke.edu

Abstract: To better understand the origin of multivalency effects in ligand binding, the binding of a series of mono-, bi-, tri- and tetravalent carboxylate ligands to Ca(II) was examined by isothermal titration calorimetry (ITC). The data are inconsistent with an entropic origin of enhanced affinity, but rather show that at least in this instance the multivalency effect is enthalpic in origin. Analysis of binding data using the Jencks model shows the addition of incremental carboxylate "ligands" produces an unfavorable interaction entropy that is more than offset by a strongly favorable interaction enthalpy. The most likely source of this interaction enthalpy is the relief of repulsive Coulombic interactions in the unbound state. The conformational entropy penalty arising from the restriction of flexible dihedrals is negligible, within experimental error. On the other hand, an enthalpic contribution from linker restriction contributes strongly to the overall thermodynamics of ligand binding. Together, these data suggest that enthalpic effects dominate ligand binding, and design strategies should seek to optimize these interactions. The incorporation of unfavorable interactions in the unbound ligand that are relieved during binding provides an important mechanism by which to enhance ligand affinities.

Introduction

The ability to synthesize small molecule ligands with predetermined affinities for binding sites of known structure is perhaps the most fundamental goal of medicinal chemistry. Molecular-resolution structures are now available for over 16 000 proteins, with new structures deposited at the rate of more than 3000 per year.[1] Despite this wealth of data, the ability to create high affinity small molecule ligands for binding sites with well-defined dimensions and character remains highly limited; these failures highlight the complexity of structure–function relationships in aqueous solution.

Many proteins contain more than one binding site. These sites can be homologous, for example a quaternary assembly having multiple copies of the same receptor, or heterologous, a mono- or multimeric assembly possessing affinity for more than one type of ligand. In the latter case, these conceptual binding sites might reside on a single contiguous surface or at distinct loci separated by an intervening noninteracting surface. From this basis, a variety of multivalent ligands have been developed in the search for high affinity.[2] A recent well-studied instance of multivalent ligand binding involves the lectins, the myriad carbohydrate binding proteins that virtually always exist as polymeric aggregates.[3] The so-called "cluster glycoside" effect, or enhancement in affinity on a concentration corrected basis, ranges to nearly 10^9.[4]

The molecular basis of these enhancements is unclear. Any thermodynamic parameter characterizing the binding of a multivalent ligand to a multivalent receptor is related to that for the corresponding monovalent ligand by the expression

$$\Delta J°_{obs} = n\Delta J°_{mono} + \Delta J°_{int} \quad (1)$$

where $J°$ represents any thermodynamic parameter, $\Delta J°_{mono}$ represents the thermodynamic quantity for the monovalent ligand, and n is the valency of the ligand. In this formalism the interaction term, $\Delta J°_{int}$, describes the energetic consequences of physically tethering monovalent ligands. Both entropic and enthalpic terms contribute to interaction free energies, and the magnitude and sign of each term is unpredictable. Interaction energies are typically considered from an entropic perspective, largely as a balance between a favorable translational/rotational interaction entropy and an unfavorable conformational interaction entropy.[5−7] In the former instance, the molecularity of the interaction is reduced: the binding of a bivalent ligand to a bivalent receptor involves the conversion of two particles to

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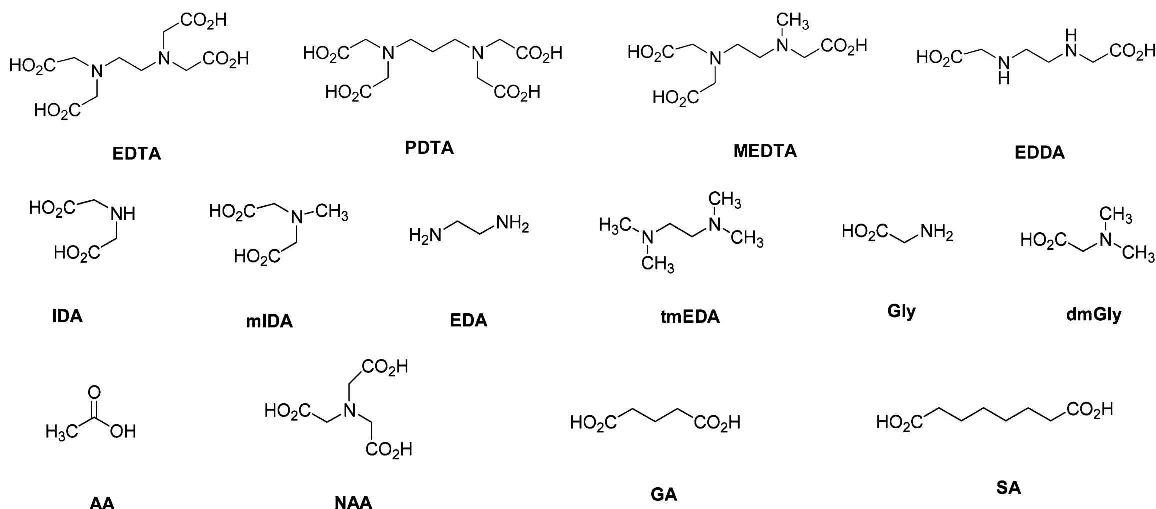

Figure 1. EDTA and the conceptual EDTA fragments used in this study.

one rather than of three particles to one. Because translational and rotational entropies scale as the logarithm of the molecular weight, this effect provides an increment to the interaction entropy equivalent to the translational and rotational entropy of one of the monovalent ligands.[5-7] On the other hand, conformational degrees of freedom are lost as a linker domain is anchored at both ends, restricting rotation about flexible torsion angles. The magnitude of each term is poorly understood. Estimates of the translational/rotational barrier to bimolecular complex formation in aqueous solution range from 2 to 15 kcal mol^{-1} near room temperature,[8,9] whereas the loss of conformational entropy accompanying the restriction of a single rotor has been estimated at between 0.1 and 1.5 kcal mol^{-1}.[8,10,11]

The binding of a multivalent ligand to a multivalent receptor can proceed in either an intra- or an intermolecular fashion. During intramolecular binding a single discrete complex is formed, whereas in intermolecular binding a complex of indeterminate size is produced. Assuming both binding motifs are feasible, the distribution between the two can be either thermodynamically or kinetically controlled. In the former case, reversible binding ultimately yields a distribution controlled by the thermodynamic stabilities of the various interconverting species. In the latter instance bound complexes are trapped—perhaps by a diminished solubility—and the various structures ultimately cease interconverting.

We have previously demonstrated in at least some instances the cluster glycoside effect arises from an aggregative model of binding.[2,3] We thus turned our attention to other examples of multivalent interaction in an attempt to identify molecular structures that predispose an interacting system to one or the other model of multivalent binding. The metal chelate ethylenediaminetetraacetic acid (EDTA) is perhaps the simplest multivalent ligand, with four carboxylates and two amino groups available to participate in binding (Figure 1). EDTA−metal complexation is the subject of myriad reports during the past several years.[12-14] The mechanism by which EDTA binds metal ions with such facility remains the subject of some debate. In 1952, Schwarzenbach introduced the concept of the chelate effect, and attributed the exceptionally high affinity of various multivalent amine and carboxylate ligands to a reduction in the loss of translational entropy during binding, compared to the corresponding intermolecular case.[15] Similar conclusions were reached by other researchers and an entropic model of chelation persists, albeit with the addition of the related concept of rotational entropy.[16-20] On the other hand, Williams stressed the importance of enthalpic effects in chelate binding, invoking both a diminished repulsive force between neighboring ligands in a chelate as compared to those in a complex with monodentate ligands and solvation.[21] A number of researchers have considered the effect of varying concentration scales, parsing entropies into unitary and cratic terms. Adamson noted that the anomalous entropy effect virtually vanishes if entropy changes during the binding of a variety of multivalent amine ligands are calculated using mole fraction concentration scales.[22] Because adoption of mole fraction concentration scales in many ways explicitly accounts for differences in translational and rotational entropy, the fact that the unitary chelation entropies are near zero is causal.[12] Today the chelate, or multivalency, effect is viewed largely from an entropic perspective.[5]

Many of the conceptual fragments of EDTA are known, providing a particularly attractive system with which to consider additivity in ligand binding. A variety of simultaneous events contribute to the net measured thermodynamic properties characterizing metal ion binding, including release of metal ions bound to buffer and proton-transfer resulting from shifting pK_a values. For these and other reasons, little can be deduced from consideration of the thermodynamic parameters characterizing a single binding event. On the other hand, a comparison of

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values for the binding of a closely related series of ligands is highly instructive, and can provide considerable detail regarding the molecular origin of affinity. The thermodynamics of association of EDTA and some of its constitutent ligands have previously been reported.[13,14,23−32] Unfortunately, no single comprehensive study that fairly compares binding under an equivalent set of conditions and that properly accounts for the role of buffer binding and proton transfer has, to the best of our knowledge, been reported. Here, we report a thermodynamic study of the binding of EDTA and its lower valent fragments and use these data to consider the molecular origin of the chelate effect.

Results and Discussion

To consider the effect of multivalency in metal chelate binding, we considered the EDTA−Ca(II) system as a tetravalent ligand, consisting of four individual acetate ligands, binding a tetravalent "receptor". From this formalism, binding of the corresponding mono-, bi-, and trivalent ligands were also examined (Figure 1). Our data are interpreted assuming no cooperativity in the metal, i.e., binding of each acetate ligand has no effect on the electronic character of the calcium ion. The consequences and validity of this assumption are considered further below. In the event, reduction of raw data using a model that invokes multiple classes of sites or of interaction between sites fails to improve the fit beyond that expected statistically from the incorporation of additional variables. An Occam's razor approach thus requires adoption of the single site model of binding.

ITC evaluation of metal ion−chelate binding presents a variety of experimental challenges. Data from titration calorimetry can be deconvoluted to yield both a binding constant and an enthalpy of binding where c, a unitless constant numerically equivalent to the product of the binding constant and the concentration of binding sites, ranges from 1 to 1000. At the low affinity limit, the calorimetric experiment fails over solubility concerns, as the minimum concentration of binding sites in the cell becomes prohibitively large. At the high affinity limit, instrument sensitivity becomes limiting, as the maximum allowable concentration of binding sites becomes prohibitively small. Most of the ligands used here, including all of the mono- and bivalent compounds and EDTA, have affinities for Ca(II) such that direct titration is not possible. Accordingly, thermodynamic parameters were evaluated by displacement titration.[33,34] In this experiment, a high affinity ligand displaces a low affinity competitor as high affinity ligand is titrated into a solution of receptor saturated with low affinity ligand. If the thermodynamic parameters characterizing the binding of one of the two ligands are known, then those characterizing binding of the other are revealed by subtraction. For the high affinity ligand EDTA, displacement of the weakly bound Ba(II) by Ca(II) provided the Ca(II)−EDTA parameters. Thermodynamic parameters for the binding of low affinity ligands (mono- and bivalent ligands ethylenediaminediacetic acid, iminodiacetic acid, methyliminodiacetic acid, ethylenediamine, glycine, dimethylglycine, acetic acid, glutaric acid, and suberic acid) were derived by displacement of each ligand by nitrilotriacetic acid. The binding of Ca(II) by buffer salts was determined in the same way. Representative data for displacement titration of Ba(II)−EDTA by Ca(II) are shown in Figure 2. Details of data reduction protocols are supplied in Supporting Information.

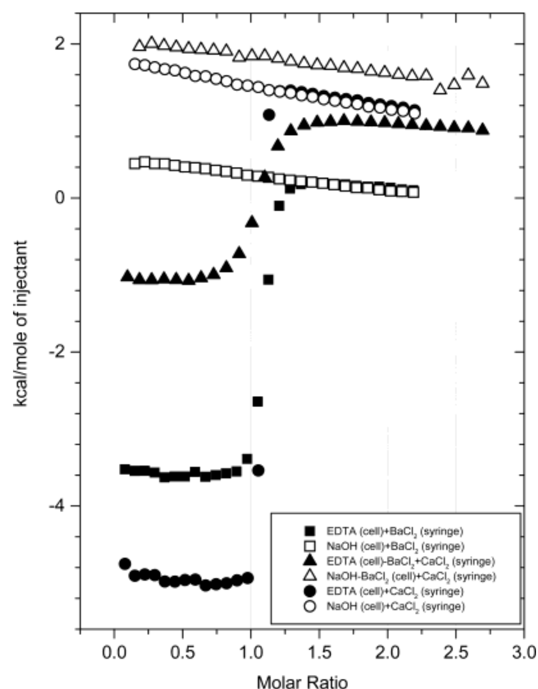

Figure 2. Displacement experiment in NaOH at pH 13. The circles show the EDTA-Ca(II) titration (filled circles) and heat of dilution, Ca(II) titrated into NaOH pH 13 (open circles). The squares show the EDTA-Ba(II) titration (filled squares) and the corresponding heat of dilution (Ba(II) into NaOH pH 13; open squares). The triangles show the displacement titration, in which $CaCl_2$ is titrated into an EDTA-Ba(II) solution (filled triangles), the heat of dilution experiment (open triangles) is the addition of $CaCl_2$ into a solution of EDTA-Ba(II). In the first titration, the concentrations of EDTA and $BaCl_2$ are 0.49 and 5.03 mM, respectively. In the displacement titration, the concentrations of EDTA and $BaCl_2$ in the cell are 0.40 and 0.92 mM, respectively, and the concentration of $CaCl_2$ in the syringe is 5.05 mM.

Thermodynamic parameters are state functions, and calorimetry measures the sum of all processes that occur during a titration. Prior to the construction of a molecular model with which to rationalize the thermodynamic behavior of a series of ligands, all of the contributing events must be evaluated separately. Here, two important contributors to measured thermodynamic parameters were considered, namely metal ion binding by buffers and proton transfer.

Table 1 shows thermodynamic parameters for Ca(II) binding to various buffers at pH 6, 8, and 9 and for Ba(II) binding to HEPES at pH 8. Ca(II) binds to both TRICINE and citric acid with an affinity that significantly alters apparent thermodynamic

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Table 1. Thermodynamic Parameters of Metal Binding to Various Buffers at 25 °C

buffer	pH	metal Ion	K (M^{-1})	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$T\Delta S°$ (kcal mol^{-1})
TRICINE	9	Ca(II)	215.8 ± 80.3	−3.18 ± 1.18	−4.33 ± 0.20	−1.15 ± 0.40
HEPES	8	Ba(II)	6.7 ± 3.6	−1.13 ± 0.61	+1.49 ± 0.38	+2.62 ± 0.78
HEPES	8	Ca(II)	6.7 ± 0.3	−1.13 ± 0.05	+0.67 ± 0.03	+1.80 ± 0.11
MOPS	8	Ca(II)	3.9 ± 0.1	−0.81 ± 0.02	+0.90 ± 0.03	+1.71 ± 0.07
TRICINE	8	Ca(II)	99.6 ± 8.5	−2.73 ± 0.23	−2.41 ± 0.11	+0.32 ± 0.03
TRIS	8	Ca(II)	3.4 ± 0.3	−0.72 ± 0.06	−1.17 ± 0.01	−0.45 ± 0.05
citric acid	6	Ca(II)	170.8 ± 11.6	−3.05 ± 0.21	−0.13 ± 0.47	+2.92 ± 10.55
PIPES	6	Ca(II)	negl [a]	negl	negl	negl
MES	6	Ca(II)	3.7 ± 0.1	−0.78 ± 0.02	−0.095 ± 0.016	+0.69 ± 0.12

[a] Neglectable[55]

Table 2. Thermodynamic Parameters of Ca(II) Binding to EDTA and EDTA Fragments Determined by Direct Titrations in Different Buffers at 25 °C[a]

EDTA derivative	buffer	pH	K(M^{-1})	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$T\Delta S°$ (kcal mol^{-1})	stoichiometry
EDTA	citric acid	6	$(1.66 \pm 0.06) \times 10^5$	−7.12 ± 0.26	+1.58 ± 0.12	+8.70 ± 0.37	1.04 ± 0.02
EDTA	MES	6	$(2.01 \pm 0.05) \times 10^6$	−8.60 ± 0.21	−4.08 ± 0.23	+4.52 ± 0.14	0.97 ± 0.02
EDTA	PIPES	6	$(7.96 \pm 0.34) \times 10^5$	−8.05 ± 0.34	−2.50 ± 0.05	+5.55 ± 0.13	1.02 ± 0.02
EDTA	HEPES	8	$(5.91 \pm 0.26) \times 10^8$	−11.97 ± 0.53	−5.25 ± 0.12	+6.72 ± 0.17	0.95 ± 0.02
EDTA	MOPS	8	$> 2 \times 10^6$	< -8.6	−5.64 ± 0.08	$> +3.0$	0.96 ± 0.02
EDTA	TRICINE	8	$(2.97 \pm 0.42) \times 10^8$	−11.56 ± 1.63	−8.58 ± 0.05	2.98 ± 0.21	0.94 ± 0.03
EDTA	TRIS	8	$> 2 \times 10^6$	< -8.6	−11.67 ± 0.09	> -3.1	1.01 ± 0.01
MEDTA[b]	HEPES	8	$(7.57 \pm 1.03) \times 10^4$	−6.66 ± 1.14	−1.22 ± 0.04	+5.44 ± 0.47	1.00 ± 0.02
MEDTA	MOPS	8	$(8.22 \pm 0.30) \times 10^4$	−6.70 ± 0.24	−1.41 ± 0.08	+5.29 ± 0.18	0.95 ± 0.02
MEDTA	TRICINE	8	$(1.35 \pm 0.06) \times 10^5$	−7.00 ± 0.31	−4.47 ± 0.15	+2.53 ± 0.07	0.93 ± 0.02
MEDTA	TRIS	8	$(1.21 \pm 0.05) \times 10^5$	−6.93 ± 0.29	−7.54 ± 0.31	−0.61 ± 0.02	0.98 ± 0.02
PDTA[c]	HEPES	8	$(5.49 \pm 0.29) \times 10^4$	−6.47 ± 0.34	−1.01 ± 0.05	+5.46 ± 0.20	1.00 ± 0.04
PDTA	MOPS	8	$(1.71 \pm 0.13) \times 10^4$	−5.77 ± 0.44	−1.21 ± 0.06	+4.56 ± 0.21	1.02 ± 0.02
PDTA	TRICINE	8	$(4.51 \pm 0.46) \times 10^4$	−6.35 ± 0.32	−6.11 ± 0.05	+0.24 ± 0.01	1.03 ± 0.02
PDTA	TRIS	8	$(3.32 \pm 0.19) \times 10^4$	−6.17 ± 0.35	−10.99 ± 0.39	−4.82 ± 0.16	0.96 ± 0.02
PDTA	TRICINE	9	$(1.35 \pm 0.18) \times 10^6$	−8.36 ± 1.11	−5.98 ± 0.15	+2.38 ± 0.16	1.05 ± 0.02
NAA[d]	HEPES	8	$(3.48 \pm 0.17) \times 10^4$	−6.20 ± 0.30	−1.40 ± 0.03	+4.80 ± 0.13	1.00 ± 0.02
NAA	MOPS	8	$(6.21 \pm 0.29) \times 10^4$	−6.54 ± 0.31	−1.45 ± 0.08	+5.09 ± 0.19	1.05 ± 0.02
NAA	TRICINE	8	$(1.02 \pm 0.05) \times 10^5$	−6.83 ± 0.33	−4.51 ± 0.15	+2.32 ± 0.07	1.01 ± 0.02
NAA	TRIS	8	$(8.22 \pm 0.37) \times 10^4$	−6.70 ± 0.30	−7.56 ± 0.31	−0.86 ± 0.03	1.03 ± 0.02

[a] All parameters are corrected for metal-buffer binding. [b] MEDTA, *N*-methylethylenediaminetriacetic acid. [c] PDTA, 1,3-diaminopropanetetracetic acid. [d] NAA, nitrilotriacetic acid.

parameters for Ca(II) binding. Ligand binding data in Tables 2−6 are corrected for buffer metal ion binding.

Calorimetric enthalpies include contributions from buffer protonation/deprotonation according to the equation[35]

$$\Delta H°_{obs} = \Delta H°_{b} + N\Delta H°_{ion} \quad (2)$$

where $\Delta H°_{obs}$ is the observed enthalpy of binding, $\Delta H°_{b}$ is the enthalpy arising from solute−solute interactions, and $\Delta H°_{ion}$ is the buffer ionization enthalpy arising from proton transfer to or from buffer. A plot of $\Delta H°_{obs}$ versus $\Delta H°_{ion}$ yields a straight line with a slope equivalent to the numbers of protons transferred during binding. Figure 3 shows the observed calorimetric enthalpy of EDTA-Ca(II) binding as a function of buffer ionization enthalpy. A total of 1.73 ± 0.29 and 0.87 ± 0.04 protons are released from EDTA to buffer during Ca(II) binding at pH 6 and 8, respectively, suggesting EDTA binds in the fully deprotonated form. Table 2 shows thermodynamic parameters for Ca(II) binding to EDTA, *N*-methylethylenediaminetriacetic acid, 1,3-diaminopropanetetraacetic acid, and nitrilotriacetic acid in different buffers. Table 3 shows the expected and measured number of protons released during Ca(II) binding and $\Delta H°_{b}$.

The experimentally measured proton release during the binding of Ca(II) to EDTA differs slightly from that expected on the basis of pK_a values (Table 3). The amino groups of EDTA have pK_a values of 6.19 and 10.39,[31] and EDTA should bind 1.6 protons at pH 6 and 1 proton at pH 8. To resolve this discrepancy, EDTA was titrated against HCl in the presence and absence of $CaCl_2$ (Figure 4). Clearly, Ca(II) complexation alters EDTA pK_a values, and this shift likely explains the discrepancy in expected and observed proton transfers.

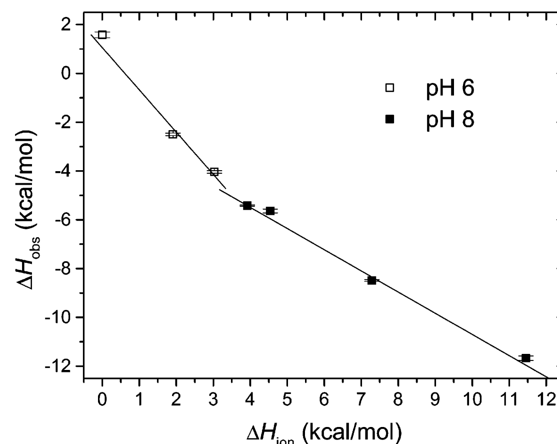

Figure 3. Graph of the calorimetric enthalpy obtained by ITC as a function of the buffer ionization enthalpy at pH 6 (open squares) and at pH 8 (filled squares). At pH 6, 1.73 ± 0.29 protons are released during binding and at pH 8, 0.87 ± 0.04 protons are released to the buffer. The used buffers are: Citric acid, PIPES, and MES at pH 6, and HEPES, MOPS, TRICINE, and TRIS at pH 8 (Table 2).

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Table 3. Expected and Measured Proton Release and Intrinsic Enthalpies of Ca(II) Binding to EDTA and EDTA Fragments[a] Measured at pH 8 and 25 °C

compd	(amino group free molecule) pK_{a1}[f]	pK_{a2}[f]	proton release expected	measured	ΔH_b (kcal mol^{-1})
EDTA[b]	6.19	10.39	1.61	1.73 ± 0.29	+1.06 ± 0.70
EDTA[c]	6.19	10.39	1.00	0.87 ± 0.04	−2.02 ± 0.22
PDTA	7.88	10.30	1.43	1.37 ± 0.10	+4.51 ± 0.74
MEDTA	5.42	10.31	1.00	0.88 ± 0.10	+2.29 ± 0.46
NAA [d)]	n.a.	9.69	0.98	0.84 ± 0.13	+1.94 ± 0.55
NAA [e)]	n.a.	9.69	0.98	0.89 ± 0.05	+2.18 ± 0.37
AA	n.a.	n.a.	0	−0.04 ± 0.01	+0.94 ± 0.10
Gly	n.a.	9.56	0.97	1.50 ± 0.49	+14.02 ± 3.64
dmGly	n.a.	9.94	0.99	1.61 ± 0.32	+11.60 ± 2.39
IDA	n.a.	9.17	0.94	0.74 ± 0.18	+5.76 ± 1.34
mIDA	n.a.	9.59	0.98	1.07 ± 0.05	+4.76 ± 0.34
EDA	7.13	9.91	1.11	1.09 ± 0.42	+11.16 ± 3.10
tmEDA	6.13	9.28	0.95	1.35 ± 0.25	+9.36 ± 1.85
EDDA	6.55	9.60	1.01	0.62 ± 0.23	+4.72 ± 1.70
GA	n.a.	n.a.	0	−0.02 ± 0.01	+1.03 ± 0.05
SA	n.a.	n.a.	0	0.03 ± 0.01	+1.13 ± 0.03

[a] PDTA, 1,3-diaminopropanetetracetic acid: MEDTA, *N*-methylethylenediaminetriacetic acid; NAA, nitrilotriacetid acid; AA, acetic acid; Gly, glycine; dmGly, *N*,*N*-dimethylglycine; IDA, iminodiacetic acid; mIDA, methylimimodiacetic acid; EDA, ethylenediamine; tmEDA, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; EDDA, ethylenediamine-*N*,*N*′-diacetic acid; GA, glutaric acid; SA, suberic acid. [b] pH 6. [c] pH 8. [d] Nitrilotriacetic acid in the cell, $CaCl_2$ in the syringe. [e] $CaCl_2$ in the cell, nitrilotriacetic acid in the syringe. [f] EDTA;[31] PDTA, NAA, dmGly, EDA, tmEDA;[52] MEDTA;[24] Gly, mIDA, EDDA;[56] IDA.[25]

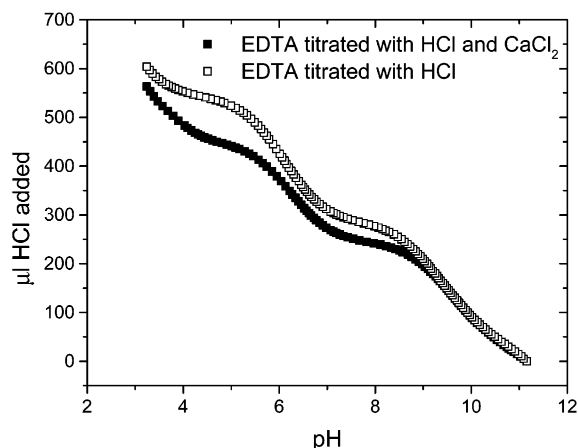

Figure 4. EDTA pK_a values in the presence and absence of Ca(II).

Thermodynamic parameters characterizing the EDTA−Ca(II) interaction were determined by displacement of Ba(II) at pH 8 in HEPES and TRICINE buffers and at pH 13, where water buffers effectively (Table 4).

To determine the contributions of individual functional groups to the overall binding of Ca(II) by EDTA, the binding of various conceptual EDTA fragments was considered. Figure 5 shows a representative thermogram and binding isotherm for *N*-methylethylenediaminetriacetic acid binding to Ca(II). Two additional ligands that are not formally EDTA fragments, nitrilotriacetic acid and 1,3-diaminopropanetetracetic acid, were included in this study (Table 2). To consider the role of amino moieties in the association, the thermodynamics of association of glutaric and suberic acid were considered (Table 5).

We next considered the energetic contribution of each interacting segment of EDTA to the overall thermodynamic parameters for Ca(II) binding. We assume at the outset that the thermodynamic parameters describing complexation arises from favorable interactions between the metal ion and electronegative elements of the ligands (O, N) and desolvation. We also assume that the calcium ion possesses four identical binding sites. In this model, iminodiacetic acid is considered as a combination of two acetates, and nitrilotriacetic acid as a combination of three acetates.[1] Similarly, ethylenediamine-*N*,*N*′-diacetic acid can be considered as a combination of two acetates, *N*-methylethylenediaminetriacetic acid as a combination of three acetates, and EDTA and 1,3-diaminopropanetetracetic acid as a combination of four acetates. On the basis of this analysis, Table 6 shows interaction energies—the discrepancy between predicted and observed thermodynamic behavior—for each ligand. From these data, we reach three primary conclusions: the basis of affinity in EDTA−metal ion binding is enthalpic, not entropic; conformational entropy contributes negligibly to the overall thermodynamics of metal ion binding; the loss of linker conformational freedom during binding yields a much greater impact on binding enthalpies than on binding entropies.

An Enthalpic Origin of EDTA Affinity. Multivalency effects are typically considered from an entropic perspective; that is, following an initial conceptual interaction, subsequent interactions proceed without any loss in translational and rotational entropy. This entropic "savings" is partially or wholly offset by losses in conformational entropy, as rotors in the linker domain are restricted. This model predicts an favorable enthalpy of binding of the first ligand, offset by an unfavorable entropy arising from losses of translational and rotational entropy during bimolecular association. Subsequent binding events provide a similar increment to the binding enthalpy but with a less unfavorable entropic term.

The observed thermodynamics of ligand binding are inconsistent with this model of association. Binding of the first carboxylate ligand is strongly opposed by the enthalpic component of the free energy, at least near room temperature; a strongly favorable entropic contribution nearly offsets this unfavorable enthalpy (Table 6). The enthalpic contribution from subsequent binding events is remarkably constant, and the addition of each carboxylate moiety provides an increment of roughly 5 kcal mol^{-1} to the overall enthalpy of binding. Because our interaction enthalpies are based on the enthalpy of the monovalent ligand, we take these values to represent the enthalpic "cost" of the initial association and, less this value, additional ionic interactions are more exothermic than predicted from the association of the monovalent ligand.

In each and every case, the interaction entropy, or the entropy arising from physical linkage of binding epitopes, is strongly *unfavorable*. We are left, however, with the clear observation that metal ion affinities scale as ligand valency. Absent favorable entropic effects, a self-consistent model of this behavior is not straightforward. First, solvation could contribute significantly to the overall thermodynamics of binding. The most sensitive probe of changes in solvation during ligand binding is the change in molar heat capacity that accompanies binding; we and others have previously demonstrated that this term is a measure of solvent reorganization during binding.[36−39] ΔC_p values were

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Table 4. Thermodynamic Parameters for Ca(II) Binding to EDTA at Different pH Values[a]

cell content	syringe content	buffer	K (M^{-1})	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$T\Delta S°$ (kcal mol^{-1})	stoichiometry
EDTA	Ba(II)	HEPES	$(8.22 \pm 0.34) \times 10^5$	-8.07 ± 0.33	-3.75 ± 0.05	$+4.32 \pm 0.09$	1.05 ± 0.02
EDTA·Ba(II)[b]	Ca(II)	HEPES	$(9.04 \pm 0.33) \times 10^5$	-8.12 ± 0.30	-1.32 ± 0.04	$+6.80 \pm 0.16$	0.95 ± 0.02
EDTA[c]	Ca(II)	HEPES	$(5.91 \pm 0.26) \times 10^8$	-11.97 ± 0.53	-5.25 ± 0.12	$+6.72 \pm 0.17$	0.95 ± 0.02
EDTA[d]	Ca(II)	HEPES	$> 2 \times 10^6$	< -8.6	-5.42 ± 0.02	$> +3.2$	1.01 ± 0.01
EDTA	Ba(II)	TRICINE	$(8.62 \pm 0.27) \times 10^5$	-8.10 ± 0.25	-7.17 ± 0.05	$+0.93 \pm 0.01$	1.00 ± 0.01
EDTA·Ba(II)[b]	Ca(II)	TRICINE	$(1.36 \pm 1.06) \times 10^5$	-7.00 ± 5.46	$+0.17 \pm 0.01$	$+7.17 \pm 2.80$	0.94 ± 0.03
EDTA[c]	Ca(II)	TRICINE	$(2.97 \pm 0.42) \times 10^8$	-11.56 ± 1.63	-8.58 ± 0.05	$+2.98 \pm 0.21$	0.94 ± 0.03
EDTA[d]	Ca(II)	TRICINE	$> 2 \times 10^6$	< -8.6	-8.49 ± 0.04	$> +0.1$	0.99 ± 0.05
EDTA[b]	Ba(II)	NaOH	$(2.68 \pm 0.15) \times 10^6$	-8.77 ± 0.49	-4.05 ± 0.05	$+4.72 \pm 0.14$	1.03 ± 0.02
EDTA·Ba(II)[b]	Ca(II)	NaOH	$(6.92 \pm 0.27) \times 10^5$	-7.97 ± 0.31	-2.21 ± 0.05	$+5.76 \pm 0.13$	1.05 ± 0.02
EDTA[b,c]	Ca(II)	NaOH	$(1.03 \pm 0.10) \times 10^9$	-12.29 ± 1.19	-6.15 ± 0.05	$+6.14 \pm 0.30$	1.05 ± 0.02

[a] Experiments are performed by the displacement method at 25 °C in 20 mM HEPES, 20 mM TRICINE, pH 8; or in 0.1 M NaOH, pH 13. [b] These values have not been corrected for metal-buffer binding (see Materials and Methods). [c] Calculated from the displacement titrations. [d] Parameters determined directly.

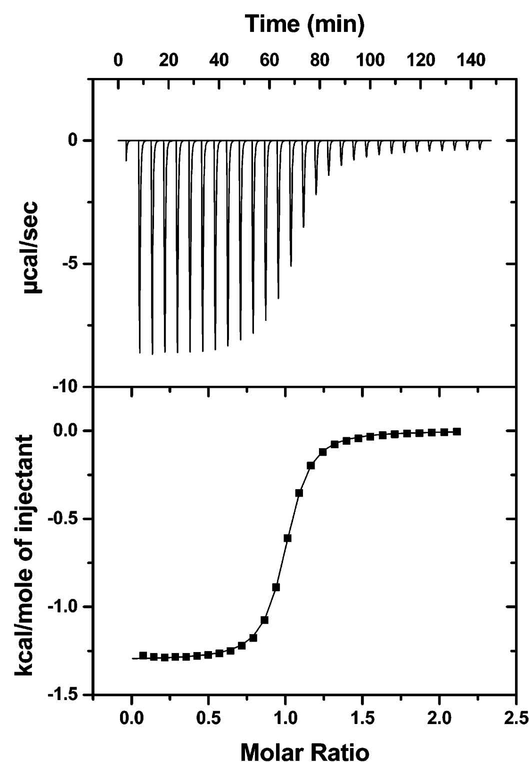


Figure 5. Thermogram (top) and binding isotherm (bottom) showing the addition of 24.19 mM $CaCl_2$ (syringe) into 2.46 mM *N*-methylethylenediaminetriacetic acid (cell) in 20 mM HEPES at pH 8, 25 °C. From the curve fit (using the Origin software), the following parameters have been determined; the association constant, $K = (7.57 \pm 1.03) \times 10^4$ M^{-1}, the enthalpy, $\Delta H° = -1.22 \pm 0.04$ kcal/mol, and the stoichiometry is 1.00 ± 0.02 (Table 2). The thermodynamic parameters have been corrected for Ca(II)-buffer binding.

measured for Ca(II) binding by nitrilotriacetic acid, *N*-methylethylenediaminetriacetic acid, 1,3-diaminopropanetetraacetic acid, and EDTA. In all instances, ΔC_p values are small and positive, between +22 to +40 eu (data not shown). Although a direct correlation between ΔC_p and a solvation-associated enthalpy of binding is unclear, the consistency of the values demonstrates that solvation-associated contributions to binding thermodynamics across the range of ligands are similar and almost certainly insufficient to explain the patterns observed here.

A second plausible explanation for the observed effect involves 'cooperativity' in binding at the level of the metal ion. This explanation questions our assumption, used during data

Table 5. Thermodynamic Parameters of Ca(II) Binding to Weak Binding EDTA Fragments[a] Determined by the Displacement Method in 20 mM HEPES pH 8, 25 °C

EDTA fragment	K (M^{-1})	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$T\Delta S°$ (kcal mol^{-1})
AA	16.3 ± 2.3	-1.7 ± 0.2	$+1.3 \pm 0.1$	$+3.0 \pm 0.2$
Gly	1.6 ± 0.2	-0.3 ± 0.03	$+8.8 \pm 0.6$	$+9.1 \pm 1.1$
dmGly	1.4 ± 0.1	-0.2 ± 0.05	$+5.0 \pm 0.5$	$+5.2 \pm 0.7$
IDA	43.5 ± 16.2	-2.2 ± 0.8	$+2.9 \pm 0.4$	$+5.1 \pm 1.0$
mIDA	239.2 ± 40.5	-3.2 ± 0.5	$+0.25 \pm 0.04$	$+3.45 \pm 0.4$
EDA	13.1 ± 5.0	-1.5 ± 0.6	$+7.3 \pm 0.5$	$+8.8 \pm 1.8$
tmEDA	2.4 ± 0.3	-0.6 ± 0.06	$+4.2 \pm 0.2$	$+4.8 \pm 0.3$
EDDA	58.0 ± 3.1	-2.4 ± 0.1	$+2.4 \pm 0.05$	$+4.8 \pm 0.2$
GA	65.0 ± 1.5	-2.5 ± 0.06	$+1.3 \pm 0.1$	$+3.8 \pm 0.2$
SA	75.1 ± 14.5	-2.6 ± 0.5	$+1.3 \pm 0.5$	$+3.9 \pm 0.8$

[a] Abbreviations as in Table 3.

reduction, that the metal ion contains four equivalent, noninteracting binding sites. Failure of this assumption would imply that binding of a first carboxylate ligand alters the binding activity of the second, and so on; such an impact of incremental carboxylate binding hardly seems unexpected. To the extent that this effect is operative, however, it must be unfavorable; that is, each sequential carboxylate binding makes subsequent binding *less* favorable. Were this not true—if carboxylate binding increased the affinity of a calcium ion for subsequent carboxylate binding—then the stoichiometry of the binding of calcium to a monovalent ligand would not be unity. Furthermore a *negative* cooperativity seems intuitively more reasonable; binding of a carboxylate ligand presumably diminishes the electropositive character of the metal ion and ameliorates Coulombic interactions during subsequent associations. To the extent that negative cooperativity exists, then, it causes us to *underestimate* the interaction enthalpies and reinforces our assertion of an enthalpic origin of affinity. The interaction energies of Table 6 are thus lower limits and cooperative effects, if they exist, magnify and reinforce our conclusions.

We next considered repulsive interactions in the unbound state as a source of affinity in multivalent ligands. Repulsive ion—ion interactions in the unbound tetracarboxylate ligand are presumably relieved during ligand binding. This effect would be roughly additive, enthalpic, and extensively diminished by solvation. As a simple test of this hypothesis, we compared the predicted and measured standard enthalpies of formation of ethylenediaminediacetic acid, nitrilotriacetic acid, and EDTA. The standard heat of formation of any of these compounds can be related to those of their constituent fragments additively, and

Table 6. Predicted, Observed, and Interaction Thermodynamic Parameters of Calcium Binding to EDTA and Selected EDTA Derivatives[a] Using Acetic Acid as the Monovalent Ligand According to eq 1[b]

ligand	$\Delta G°_{obs}$ (kcal mol^{-1})	$n\Delta G°_{momo}$ (kcal mol^{-1})	$\Delta G°_{int}$ (kcal mol^{-1})	$\Delta H°_{obs}$ (kcal mol^{-1})	$n\Delta H°_{momo}$ (kcal mol^{-1})	$\Delta H°_{int}$ (kcal mol^{-1})	$T\Delta S°_{obs}$ (kcal mol^{-1})	$T\Delta S°_{int}$ (kcal mol^{-1})	$-T\Delta S°$/rotor (kcal mol^{-1} bond^{-1})
AA	−1.7 ± 0.2	n.a.	n.a.	+1.3 ± 0.1	n.a.	n.a.	+3.0 ± 0.2	n.a.	n.a.
IDA	−2.2 ± 0.8	−3.4 ± 0.6	+1.2 ± 0.5	+2.9 ± 0.4	+2.6 ± 0.3 (+1.9 ± 0.3)	+0.3 ± 0.05 (+1.0 ± 0.2)	+5.1 ± 1.0	−0.9 ± 0.4 (−0.2 ± 0.09)	0.2 (0.05)
EDDA	−2.4 ± 0.1	−3.4 ± 0.6	+1.0 ± 0.2	+2.4 ± 0.1	+2.6 ± 0.3 (+1.9 ± 0.3)	−0.2 ± 0.02 (+0.5 ± 0.1)	+4.8 ± 0.2	−1.2 ± 0.3 (−0.5 ± 0.1)	0.2 (0.07)
NAA	−6.2 ± 0.3	−5.1 ± 1.0	−1.1 ± 0.2	−1.4 ± 0.03	+3.9 ± 0.5 (+2.8 ± 0.5)	−5.3 ± 0.7 (−4.2 ± 0.8)	+4.8±0.1	−4.2 ± 0.9 (−3.1 ± 0.8)	0.7 (0.5)
MEDTA	−6.7 ± 1.1	−5.1 ± 1.0	−1.6 ± 0.4	−1.2 ± 0.04	+3.9 ± 0.5 (+2.8 ± 0.5)	−5.1 ± 0.7 (−4.0 ± 0.7)	+5.4 ± 0.5	−3.5 ± 1.0 (−2.4 ± 0.7)	0.4 (0.3)
EDTA	−12.0 ± 0.5	−6.8 ± 1.6	−5.2 ± 1.2	−5.3 ± 0.1	+5.2 ± 0.8 (+3.8 ± 0.8)	−10.5 ± 1.6 (−9.1 ± 1.9)	+6.7 ± 0.2	−5.3 ± 1.5 (−3.9 ± 1.2)	0.5 (0.4)
PDTA	−6.5 ± 0.3	−6.8 ± 1.6	+0.3 ± 0.07	−1.0 ± 0.1	+5.2 ± 0.8 (+3.8 ± 0.8)	−6.2 ± 0.3 (−4.8 ± 1.1)	+5.5 ± 0.2	−6.5 ± 1.5 (−5.1 ± 1.7)	0.5 (0.4)
GA	−2.5 ± 0.1	−3.4 ±0.6	+0.9 ± 0.2	+1.3 ± 0.1	+2.6 ± 0.3 (+1.9±0.3)	−1.3 ± 0.2 (−0.6±0.1)	+3.8 ± 0.2	−2.2 ± 0.6 (−1.5±0.4)	0.6 (0.4)
SA	−2.6±0.5	−3.4±0.6	+0.8±0.2	+1.3±0.5	+2.6±0.3 (+1.9±0.3)	−1.3±0.5 (−0.6±0.2)	+3.9±0.8	−2.1±0.7 (−1.4±0.6)	0.3 (0.05)

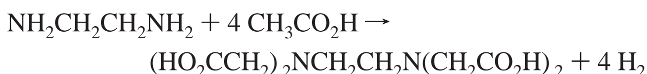
[a] Abbreviations as in Table 3. [b] Numbers in parentheses are corrected for buffer ionization.

Table 7. Measured and Calculated Enthalpies of Formation of Multivalent Ligands

compound	$\Delta H_{f,calc}$ (kcal mol^{-1})	$\Delta H_{f,meas}$[a] (kcal mol^{-1})	$\Delta\Delta H$ (kcal mol^{-1})
iminodiacetic acid	−242.1	−222.9	+19.2
nitrilotriacetic acid	−357.7	−313.6	+44.1
EDTA	−478.1	−420.5	+57.6

[a] Ref 57.

EDTA can be considered as a synthesis of ethylenediamine and acetic acid



$$NH_2CH_2CH_2NH_2 + 4\ CH_3CO_2H \rightarrow (HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2 + 4\ H_2$$

Variations between measured and calculated enthalpies of formation arise from the molecular structure of the complete molecule: higher-order stabilizing events increase the favorable enthalpy of formation of the complete molecule while destabilizing structures diminish the enthalpy of formation. In the event, iminodiacetic acid, nitrilotriacetic acid and EDTA all show condensed-phase enthalpies of formation considerably less exothermic than predicted (Table 7).[56−57]

The cumulative placement of carboxylate recognition epitopes in a constrained space is an energetically disfavored process. We submit that this destabilization is, at least in part, the origin of the extraordinary affinity of EDTA for metal ions: to the extent that charge−charge repulsion is mitigated by metal ion binding the change in free energy for binding is enhanced. The effect is predicted to be enthalpic, in keeping with the observed behavior. The values included in Table 7 are calculated and measured for the pure, condensed state; presumably solvation of the charges diminishes this unfavorable interaction. This solvation in turn ameliorates the energetic advantage derived from metal ion binding and the observed enthalpic effect is less than that available in the absence of solvation. The favorable enthalpic contribution is offset by an unfavorable entropic term; this term presumably arises from the displacement of highly disordered water found near ionic species.[40] This supposition is further supported by a positive change in constant pressure heat capacity.[40]

Enthalpic destabilization of the unbound state has previously been suggested as a contributor to binding energies in chelate complexes. For example, Hancock and Marsicano modified an empirical predictor of affinity of polyamine chelates for applicability to polycarboxylates by adding a term that explicitly accounts for carboxylate-carboxylate repulsive interactions.[41] Although the term "preorganization" has traditionally referred to conformational restriction in the unbound state, Cram pointed out that the geometric restrictions associated with such organization in crowns resulted in an energetically unfavorable desolvation of oxygen lone pairs.[42] This energetic "price", enthalpic in nature and "paid" during ligand synthesis, is recouped during ligand binding. In the sense that this energy contributes favorably to the overall free energy of ligand binding and arises from unfavorable interactions in the unbound state, the concepts put forth by Cram anticipate the suggestions made here. Finally, we note that enthalpies of chelate−metal complexation are strongly dependent on the nature of the metal ion,[43] and the generality of the model proposed here awaits further study.

Conformational Entropy Contributes Negligibly to Metal Ion Binding. The importance of losses in conformational degrees of freedom from the restriction of flexible torsions to the overall thermodynamics of binding remains controversial. On initial inspection, entropies associated with the binding of a series of ligands of increasing valence—iminodiacetic acid, nitrilotriacetic acid, ethylenediaminediacetic acid, *N*-methylethylenediaminetriacetic acid and EDTA—seem in good accord with the notion of an increasingly unfavorable interaction entropy arising from the loss of translational degrees of freedom. Thus, the unfavorable interaction energy per freely rotating bond through the series of 0.2, 0.7, 0.2, 0.4, and 0.5 kcal mol^{-1} bond^{-1}, respectively (Table 6). These values are close to the value recently suggested by Whitesides and co-workers.[10] In this model, then, the favorable translational and rotational contribution to the interaction entropy is small and the unfavorable conformational contribution to the interaction entropy is

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large. Only when the relatively constant favorable enthalpic term grows faster than the entropic penalty does the construct move forward in free energy.

On the other hand, this model is discounted by the behavior of homologous ligands of equivalent valency. For example, iminodiacetic acid and ethylenediamine-*N*,*N*′-diacetic acid and glutaric and suberic acids show equivalent free energies and enthalpies of binding despite a factor of 2 difference in the number of freely rotating rotors (Table 5). The addition of nitrogen to the ligand has a greater effect on the enthalpy of binding than on the free energy, but the equivalent binding enthalpies of glutaric and suberic acid are certainly consistent with the notion that each carboxylate ligand makes a roughly equivalent contribution to the binding enthalpy. Likewise nitrilotriacetic acid and *N*-methylethylenediaminetriacetic acid, the two trivalent ligands for which data are available, show virtually identical free energies of binding (−6.20 versus −6.66 kcal mol^{-1}) and enthalpies (−1.40 versus −1.22 kcal mol^{-1}), despite a strikingly different loss in conformational degrees of freedom. The remarkably similar thermodynamics of the two pairs of homologous ligands strongly suggests that losses in conformational entropy do not contribute significantly to the overall thermodynamics of binding. In these extremely simple ligands, it appears as though contributions to overall binding entropies from losses of conformational degrees of freedom are small.

The apparently negligible contribution of conformational entropy to the overall binding energy is remarkable, and somewhat difficult to rationalize. Still, the similarity of thermodynamic parameters for pairs of homologous ligands differing only in the number of freely rotating dihedrals is striking. There are two possible explanations for this observation: either the bound complex retains considerable conformational flexibility or the unbound state is considerably restrained. We favor for the latter explanation. It is difficult to visualize a bound geometry that retains considerable conformation flexibility, given the size of the ligands. We note parenthetically that a "preorganization" explanation of the phenomonology does not require organization of the ligand in the correct orientation for binding, but rather only that the ligand lack flexibility. Presumably some combination of solvophobic and hydrogen bonding effects contribute to this diminished flexibility.

This remarkably small conformational entropy is also consistent with various recent reports in other unrelated systems. Bundle and Boons have independently prepared a variety of conformationally restricted glycoside ligands.[44,45] Despite localizing various carbohydrate structures near their bound conformation, none showed affinities greater than their flexible counterparts. Calorimetric evaluation of the binding of one series of ligands showed that flexible and tethered ligands showed identical thermodynamic parameters, ruling out offsetting effects in at least that case. Various researchers have prepared a wide range of β- and γ-peptides.[46−49] Despite a significant increase in the number of flexible dihedrals restricted during folding, many of these polymers adopt three-dimensional shapes similar to those found in presumably less flexible α-peptides.

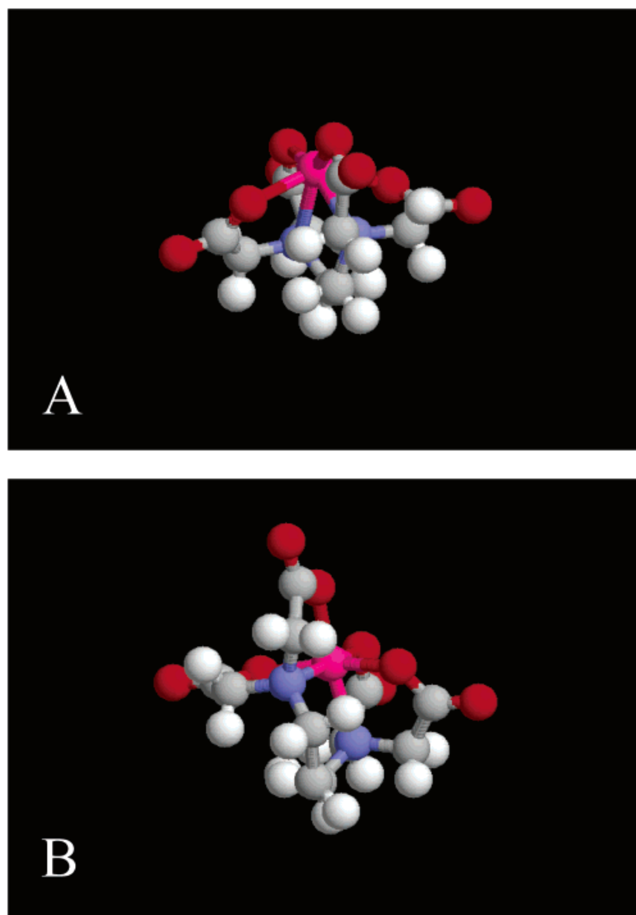

Figure 6. Crystal structure image of (A) EDTA bound to antimony.[50] Antimony is shown in pink, carbon atoms in gray, hydrogen atoms in white, nitrogen atoms in blue and oxygen atoms in red. The four acid groups and the two amino groups participate in binding of the metal ion. It is obvious that the two methylene groups of the EDTA 'backbone' are staggered and therefore in a low energy conformation. B) 1,3-diaminopropanetetracetic acid bound to vanadium.[51] The same color code as above, except vanadium is shown in pink. Two of the methylene groups in the 1,3-diaminopropanetetracetic acid 'backbone' are in an eclipsed conformation.

Enthalpic Effects of Linker Restriction Greatly Exceed Entropic Effects. We next considered the binding of homologated EDTA, propylenediaminetetracetic acid. The addition of a single methylene unit to the backbone of EDTA diminishes the binding free energy by some six kcal mol^{-1}, the greatest portion of which is enthalpic (Tables 2 and 6). There exist two possible origins for this diminution. First, destabilization of the unbound form of the ligand arising from Coulombic repulsion should be minimized by increasing the distance between carboxylates. Unfortunately, the standard heat of formation of 1,3-diaminopropanetetracetic acid is, to the best of our knowledge, unknown. Second, unfavorable steric interactions from eclipsing interactions along the propylenediamine backbone likely contribute unfavorably to the overall free energy and enthalpy. This suggestion is strongly supported by the known structure of other EDTA−metal complexes. For example, while

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the crystal structure of the ethylenediamine tetraacetate-antimony complex shows perfectly staggered dihedrals down the ethylenediamine backbone, the analogous complex with 1,3-diaminopropanetetracetic acid shows an almost perfectly eclipsed interaction down the C_1-C_2 bond (Figure 6).[50,51] Such effects are predicted to be large and unfavorable, and could easily account for the entire loss of binding energy compared to EDTA

In summary, the binding of calcium by various metal chelates has been studied by isothermal titration microcalorimetry. Thermodynamic data for several of these bindings are inconsistent with a view of multivalent ligand binding driven by favorable interaction entropies. Rather, the enhancement of binding with increasing valency is enthalpic in origin. Although we cannot unambiguously attribute this increasingly favorable binding to a specific intermolecular interaction, the phenomenology is at least consistent with an increased change in free energy arising from a destabilization of the unbound state, rather than a stabilization of the bound form. Additionally, the enthalpic effect of unfavorably oriented dihedrals in the linker moiety contribute strongly to the overall interaction free energy, and are almost certainly more important than apparently minor contributions to interaction entropies. We continue our studies of multivalency effects in ligand binding and will report our results in due course.

Materials and Methods

Ethylenediaminetetraacetic acid, glycine, acetic acid, HEPES, TRIS, and citric acid are from EM Science; *N*,*N*-dimethylglycine, iminodiacetic acid, methylimimodiacetic acid, *N*,*N*,*N'*,*N'*-tetramethylethylenediamine, ethylenediamine, ethylenediamine-*N*,*N'*-diacetic acid, nitrilotriacetic acic, glutaric acid, suberic acid, MOPS, PIPES, MES, and $BaCl_2$ were purchased from Aldrich Chemical Corp.: 1,3-diaminopropanetetracetic acid were from Fluka, TRICINE were from ICN Biomedicals Inc.: $CaCl_2$ were from Fisher Scientific and NaOH were from Mallinckrodt. These compounds were used without further purification. *N*-methylethylenediaminetriacetic acid was synthesized as follows:

***N*-Methyl-*N*,*N'*,*N'*-tris(*tert*-butyloxycarbonylmethyl)-ethylenediamine.** To a suspension of potassium carbonate powder (1.86 g, 13.6 mmol) in dry CH_3CN (15 mL) was added *N*-methylethylenediamine (0.336 g, 4.5 mmol) in one portion followed by *tert*-butyl bromoacetate (2.64 g, 13.5 mmol) also in one portion. The reaction mixture was stirred at room temperature for 24 h. The reaction mixture was concentrated under reduced pressure and the residue was extracted with CH_2Cl_2 (2 × 40 mL). Organic phases were combined, washed with saturated aqueous NaCl (2 × 20 mL), then dried over anhydrous $MgSO_4$. Drying agent was removed by filtration and the filtrate was concentrated under reduced pressure to give a yellow oil. Flash column chromatography (silica gel, 100% EtOAc) gave the desired ester as an amber oil (1.25 g, 66%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.48 (s, 4H), 3.21 (s, 2H), 2.86 (t, 2H), 2.69 (t, 2H), 2.40 (s, 3H), 1.46 (s, 27H). The unresolved AA′XX′ spin system prohibited coupling constant determination. ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.42, 170.01, 80.70, 59.34, 56.17, 55.00, 51.53, 42.23, 28.12. IR (neat, cm^{-1}): 1735. MS (*m*/*z*, CI): 417 [M$^+$+1], 361, 305. Anal. Calcd for $C_{21}H_{40}N_2O_6$: C, 60.55; H, 9.68; N, 6.73. Found: C, 60.00; H, 9.55; N, 6.20.

***N*-Methylethylenediamine-*N'*,*N*,*N*-triacetic acid dihydrochloride (MEDTAA2HCl).** *N*-Methyl-*N*,*N'*,*N'*-tris(*tert*-butyloxycarbonylmethyl)-ethylenediamine (1.05 g, 2.5 mmol) was stirred overnight at room temperature with 12 *M* HCl (3.5 mL). The precipitate was filtered off by vacuum filtration, washed with CH_2Cl_2 (2 × 3 mL), and dried in vacuo to give a white powder (0.77 g, 95%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.23 (s, 2H), 3.68 (s, 4H), 3.37 (br s, 2H), 3.21 (br s, 2H), 2.92 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.77, 167.02, 55.15, 54.53, 52.33, 49.10, 41.34. IR (KBr, cm^{-1}): 3500−2400, 1743. MS (*m*/*z*, FAB): 249 [M$^+$+ 1]. Anal. Calcd for $C_9H_{16}N_2O_6$A2HCl: C, 33.66; H, 5.65; N, 8.72. Found: C, 32.02; H, 5.99; N, 8.40.

Isothermal Titration Calorimetry. An isothermal VP−ITC titration calorimeter from MicroCal, Inc. (Northampton, MA) was used for the titration experiments, the volume of the sample cell is 1.4346 mL. The calorimeter was calibrated using electrical pulses. All samples were degassed before loading the calorimeter cells and syringe, during the titration the stir rate was 310 rpm. The reference cell was loaded with the buffer used in the experiment. All experiments were carried out at 25 °C.

Metal Binding to Buffers. Buffer concentrations ranging from 10 to 500 mM were prepared and $CaCl_2$ was added to each buffer in a final concentration of 0.5−1.0 mM and placed in the sample cell. At pH 8 and 9, nitrilotriacetic acid (10.08−10.23 mM) was titrated from an H_2O solution into the cell and at pH 6 EDTA (5.0−10.08 mM) was titrated from an H_2O solution into the cell. To determine $BaCl_2$ binding to HEPES at pH 8, 1.0 mM $BaCl_2$ was dissolved in 353−611 mM HEPES and placed in the sample cell, $BaCl_2$ was displaced with 10.6 mM EDTA. The association constant for metal binding was measured for each buffer and for the displacing molecule (either nitrilotriacetic acid or EDTA) using eq 5. Second the enthalpies were determined using eq 6. If the association constant for the metal-buffer interaction is very low and $[L]_0 \geq 1/K1$ is not fulfilled, only the thermodynamic parameters for the displacing molecule can be determined. To measure the weak binding to between the buffer and the metal ion a very high buffer concentration was used and the thermodynamic parameters can be calculated according to eqs 5 and 6.

Ligand Displacement Titration, Tight Binding. Displacement experiments to determine the binding parameters for the EDTA−Ca(II) interaction were carried out at pH 8 (20 mM HEPES and 20 mM TRICINE) and pH 13 (0.1 M NaOH). Initial concentrations of EDTA, $BaCl_2$, and $CaCl_2$ were 0.43−0.52 mM, 5.23 mM, and 5.03 mM in HEPES, initial concentrations of EDTA, $BaCl_2$, and $CaCl_2$ were 0.43−0.97 mM, 5.21−10 mM, and 4.91−8.5 mM in TRICINE, and initial concentrations of EDTA, $BaCl_2$, and $CaCl_2$ were 0.26−0.49 mM, 2.72−5.03 mM, and 2.61−5.05 mM at pH 13. At pH 13, $BaCl_2$ and $CaCl_2$ were dissolved in H_2O. First, $BaCl_2$ was titrated into the cell containing EDTA and the thermodynamic parameters for that reaction was obtained using the Origin software. In the following titration, $CaCl_2$ was titrated into the cell containing EDTA and $BaCl_2$ (Figure 2). The EDTA and $BaCl_2$ concentrations were corrected for dilution before starting the second titration. After the second titration, apparent binding constants and apparent enthalpies were obtained and the corresponding values for the EDTA-Ca(II) interaction was determined using the software for displacement titrations provided by Dr. B. W. Sigurskjold[33] according to eqs 5 and 6. When calculating the binding parameters for the EDTA-Ca(II) interaction, the Ba(II)-buffer interaction is not corrected. Ba(II) binding to TRICINE has not been measured.

Direct Titrations. Direct titrations were done in 20 mM citric acid pH 6.0 (0.49 mM EDTA and 5.05 mM $CaCl_2$), 10 mM MES pH 6.0 (0.50 mM EDTA and 5.00 mM $CaCl_2$), 20 mM PIPES pH 6.0 (0.49 mM EDTA and 5.05 mM $CaCl_2$), 20 mM MOPS pH 8.0 (0.49 mM EDTA and 5.02 mM $CaCl_2$), 20 mM HEPES pH 8.0 (0.51−1.0 mM EDTA and 5.03−10.0 mM $CaCl_2$), 20 mM TRIS pH 8.0 (0.50 mM EDTA and 5.00 mM $CaCl_2$), 20 mM HEPES pH 8.0 (0.49−4.92 mM *N*-methylethylenediaminetriacetic acid and 4.84−48.37 mM $CaCl_2$), 20 mM MOPS pH 8.0 (1.97 mM *N*-methylethylenediaminetriacetic acid and 19.95 mM $CaCl_2$), 20 mM TRICINE pH 8.0 (1.54 mM *N*-methylethylenediaminetriacetic acid and 14.63 mM $CaCl_2$), 20 mM TRIS pH 8.0 (1.27 mM *N*-methylethylenediaminetriacetic acid and 13.09 mM $CaCl_2$), 20 mM HEPES pH 8.0 (0.50 mM 1,3-diamino-

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propanetetracetic acid and 5.23 mM $CaCl_2$), 20 mM MOPS pH 8.0 (1.97 mM 1,3-diaminopropanetetracetic acid and 19.95 mM $CaCl_2$), 20 mM TRICINE pH 8.0 (0.58−1.48 mM 1,3-diaminopropanetetracetic acid and 5.81−14.63 mM $CaCl_2$), 20 mM TRIS pH 8.0 (0.36 mM 1,3-diaminopropanetetracetic acid and 3.93 mM $CaCl_2$), 20 mM TRICINE pH 9.0 (0.58 mM 1,3-diaminopropanetetracetic acid and 5.83 mM $CaCl_2$), 20 mM HEPES pH 8.0 (5.01 mM nitrilotriacetic acid and 47.46 mM $CaCl_2$), 20 mM MOPS pH 8.0 (1.01 mM nitrilotriacetic acid and 10.47 mM $CaCl_2$), 20 mM TRICINE pH 8.0 (1.01 mM nitrilotriacetic acid and 9.98 mM $CaCl_2$), 20 mM TRIS pH 8.0 (1.01 mM nitrilotriacetic acid and 10.71 mM $CaCl_2$).

Displacement Titrations, Weak Binding. The thermodynamic parameters of calcium binding to the weak binding ligands acetic acid (10.3−153.9 mM), glycine (34.0−495.8 mM), dimethylglycine (31.0−516.5 mM), iminodiacetic acid (11.0−153.9 mM), methyliminodiacetic acid (9.6−24.7 mM), ethylenediamine (93.4−200.4 mM), tetramethylethylenediamine (49.2−524.8 mM), ethylenediaminediacetic acid (25.8 mM), suberic acid (20.0−173.1 mM), and glutaric acid (24.8−53.1 mM) were determined by displacement titrations in 20 mM HEPES pH 8: 0.99−1.04 mM $CaCl_2$ and the weak binding ligand were in the cell and nitrilotriacetic acid was titrated into that solution, displacing the weak binding ligand. The thermodynamic parameters for the weak binding ligand were obtained as described above.

Heat of Ionization Measurements. At pH 6, binding of $CaCl_2$ to EDTA was measured in 20 mM citric acid, 10 mM MES, and 20 mM PIPES, the $CaCl_2$ concentrations were 5.00−5.05 mM and the EDTA concentrations were 0.49−0.50 mM. At pH 8, the experiments were done in 20 mM HEPES, 20 mM MOPS, 20 mM TRICINE, and 20 mM TRIS. The experimental details for the HEPES buffer are listed above. The EDTA concentrations were 0.49−0.50 mM and $CaCl_2$ concentrations were 5.0−5.02, *N*-methylethylenediaminetriacetic acid concentrations were 1.27−1.97 mM and $CaCl_2$ concentrations were 13.09−19.95 mM, 1,3-diaminopropanetetracetic acid concentrations were 0.36−1.97 mM and $CaCl_2$ concentrations were 5.81−19.95 mM, nitrilotriacetic acid concentrations were 1.01 mM and $CaCl_2$ concentrations were 9.98−10.71 mM, for the opposite titrations in 20 mM HEPES nitrilotriacetic acid (10.02−10.20 mM) were placed in the syringe and $CaCl_2$ (0.99−1.05 mM) were placed in the cell. For the weak binding ligands, displacement titrations were done where the weak binding ligand and $CaCl_2$ (0.99−1.05 mM) were placed in the cell and Ca(II) was displaced with nitrilotriacetic acid (10.02−10.20 mM. The concentrations of the weak binding ligands were 102.60 mM acetic acid, 495.84 mM glycine, 356.99 mM dimethylglycine, 52.64 mM iminodiacetic acid, 24.66 mM methyliminodiacetic acid, 148.08 mM ethylenediamine, 524.77 mM tetramethylethylenediamine, 25.75 mM ethylenediaminediacetic acid, 24.84 mM glutaric acid, and 24.74 mM suberic acid. The binding values for nitrilotriacetic acid without any weak binding ligands present were calculated from the opposite titration. From the displacement titrations in HEPES buffer, the association constants were determined for each of the weak binding ligands. Here, one concentration of weak binding ligand was chosen so the conditions for displacement titrations are fulfilled and the thermodynamic parameters were calculated according to eqs 5 and 6. The buffer ionization constants are 0 kcal mol^{-1} for citric acid,[52] 3.03 kcal mol^{-1} for MES, 2.08 kcal mol^{-1} for PIPES, 3.92 kcal mol^{-1} for HEPES, 4.54 kcal mol^{-1} for MOPS, 7.29 kcal mol^{-1} for TRICINE,[53] and 11.45 kcal mol^{-1} for TRIS.[52]

Data Analysis. All data analyses were done using Origin (MicroCal Software, Inc.). From an isothermal titration experiment the observed, calorimetric enthalpy (ΔH_{obs}), the association constant (K), and the stoichiometry (n), can be obtained directly using eq 4. The Gibbs free energy and the entropy were calculated according to known thermodynamic relations. Details of the curve fitting have been described by Wiseman et al.[54]

Metal Ion Binding to Buffers. The obtained binding isotherms are fitted to a one site model from the Origin software. The experiments are done at different buffer concentrations and the association constant and the enthalpy for the metal-buffer binding process is calculated according to eqs 5 and 6.

For analyzing displacement titration experiments software designed to calculate displacement values was provided by Dr. B. W. Sigurskjold.[33] In tight binding experiments (EDTA + Ca(II)), the initial values are not corrected for Ba(II) binding to the buffer since the Ba(II) will be released to the buffer upon Ca(II) binding and will be canceled out in the final calculation of the EDTA−Ca(II) interaction.

Ca(II) binding to weak binding ligands were measured by displacing the Ca(II) ion from the ligand, at different ligand concentrations, by a tighter binding ligand. First the binding isotherms were analyzed by the displacement software.

Equations 5 and 6 are used to correct the obtained binding data for buffer-metal binding.

Abbreviations Used: EDTA, ethylenediaminetetraacetic acid; PDTA, 1,3-diaminopropanetetracetic acid; NAA, nitrilotriacetid acid; MEDTA, *N*-methylethylenediaminetriacetic acid; AA, acetic acid; Gly, glycine; dmGly, *N,N*-dimethylglycine; IDA, iminodiacetic acid; mIDA, methyliminodiacetic acid; tmEDA, *N,N,N′,N′*-tetramethylethylenediamine; EDA, ethylenediamine; EDDA, ethylenediamine-*N,N′*-diacetic acid; GA, glutaric acid; SA, suberic acid; ITC, isothermal titration calorimetry.

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Supporting Information Available: Details of data reduction protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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