Articles

Thermodynamics of Metal Ion Binding. 1. Metal Ion Binding by Wild-Type Carbonic Anhydrase†

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ABSTRACT: Understanding the energetic consequences of molecular structure in aqueous solution is a prerequisite to the rational design of synthetic motifs with predictable properties. Such properties include ligand binding and the collapse of polymer chains into discrete three-dimensional structures. Despite advances in macromolecular structure determination, correlations of structure with high-resolution thermodynamic data remain limited. Here we compare thermodynamic parameters for the binding of Zn(II), Cu(II), and Co(II) to human carbonic anhydrase II. These calorimetrically determined values are interpreted in terms of high-resolution X-ray crystallographic data. While both zinc and cobalt are bound with a 1:1 stoichiometry, CAII binds two copper ions. Considering only the high-affinity site, there is a diminution in the enthalpy of binding through the series Co(II) → Zn(II) → Cu(II) that mirrors the enthalpy of hydration; this observation reinforces the notion that the thermodynamics of solute association with water is at least as important as the thermodynamics of solute−solute interaction and that these effects must be considered when interpreting association in aqueous solution. Additionally, ΔC_p data suggest that zinc binding to CAII proceeds with a greater contribution from desolvation than does binding of either copper or cobalt, suggesting Nature optimizes binding by optimizing desolvation.

Association in aqueous solution, in either an intra- or intermolecular sense, lies at the heart of virtually all biological chemistry. Understanding the molecular basis for such associations is vital to our ability to rationally create defined motifs with predictable properties, specifically affinities and three-dimensional structures, in an aqueous milieu. Unfortunately, the unique properties of water as a solvent frustrate such an understanding. Water has both a large dipole and quadrupole, offers hydrogen bond donor and acceptor sites, and is exceptionally small. Armed with this set of characteristics, water satisfies almost all of the interactions typically invoked in discussions of association: hydrogen bonding, Coulombic stabilization of both ionic and dipolar species, and van der Waals stabilization through effective packing, despite a relatively low polarizability (*1*). The free energies of stabilization for complexation events in aqueous solution are *differential* stabilization energies between solute−solute and solute−solvent ensembles. Given then that binding free energies are very small differences between very large absolute values, it is perhaps not surprising that our ability to prepare motifs with predictable properties is, at best, rudimentary.

A significant proportion of all known enzymes, perhaps as high as one-third, are metalloproteins that require metal cofactors for their biological function. A 1996 survey of the 4048 protein crystal structures in the Brookhaven Protein Data Bank revealed zinc as one of the two most commonly bound transition metals, second only to iron (*2*). The binding of metal ions in biological systems is an especially challenging problem. The enthalpic stabilization of metal ions by water is immense—on the order of 50−200 kcal mol^{-1} (*3*)—and this enthalpy must be lost on transfer to a protein binding site. Furthermore, many metal ions are exceptionally insoluble, can alternate between multiple redox states, and exist at extraordinarily low concentrations. In response to these stringent demands, Nature has created a series of both high- and low-molecular weight metal binding species that provide affinities as high as 10^{40} M^{-1} (*4*). In many instances, these binding proteins tightly control metal ion homeostasis, as both metal deficiency and metal toxicity have deleterious cellular effects (*5*, *6*). Given the tremendous range of affinities, motifs, and functions, biological metal ion binding should serve as an important and instructive tool for elucidating the molecular basis of affinity in aqueous solution.

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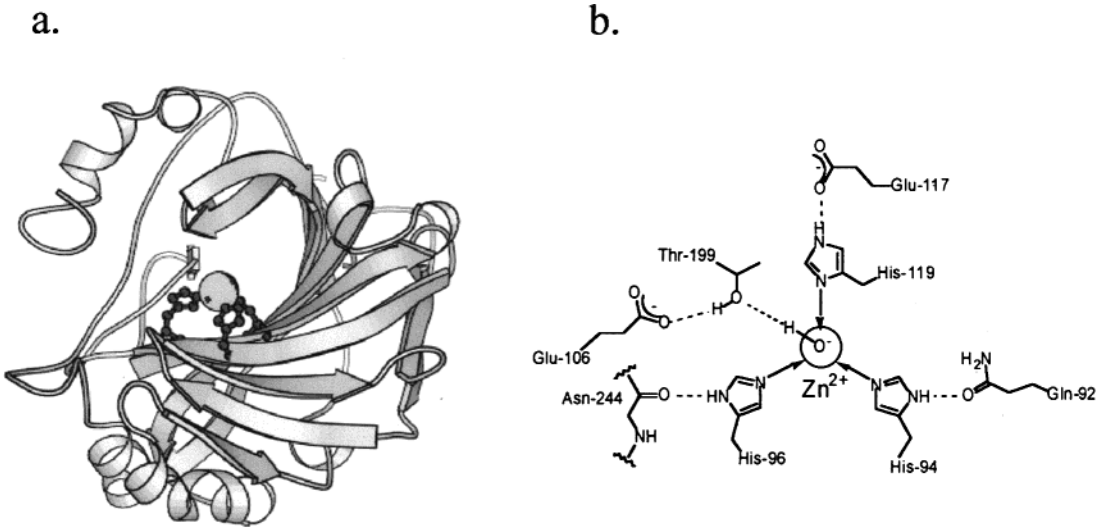

FIGURE 1: (a) Schematic drawing of the structure of human CAII (*11*). The α-helices and *β*-strands are indicated by ribbons and arrows, respectively. The imidazole side chains of the three direct histidine ligands are detailed as ball-and-stick structures, while the zinc ion is shown as a filled circle. (b) Schematic drawing of the direct and indirect metal ligands in the CAII zinc binding site.

Carbonic anhydrase (CA) is a prototypical zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide to bicarbonate at close to the diffusion-controlled limit (*7*). The enzyme is ubiquitous, present in all animals and photosynthesizing organisms investigated, as well as some nonphotosynthesizing bacteria (*8*−*10*). The most thoroughly studied isozyme, human CAII,[1] is the object of study here. This protein has been cloned and recombinantly expressed in *Escherichia coli*, and a crystal structure has been refined to 1.54 Å (Figure 1a) (*11*−*13*).

The active site of CAII, located in the central region of a 10-stranded, twisted *β*-sheet, is comprised of a cone-shaped cleft, 15 Å deep, with a tetrahedral Zn^{2+} ion at the bottom of the cleft. Three histidine ligands, at positions 94, 96, and 119, are directly coordinated to the metal center (Figure 1b). These direct ligands form hydrogen bonds to indirect ligands Gln-92, Asn-244, and Glu-117, respectively. Filling out the coordination sphere of zinc is a hydroxide ion that forms a hydrogen bond to Thr-199. With the exception of Asn-244, which is hydrogen bonded to His-96 through its main chain carbonyl oxygen, the direct and indirect metal ligands are invariant in all sequenced catalytically active α-carbonic anhydrases (*14*).

Although carbonic anhydrase is loaded with zinc in its physiologically relevant form, the protein binds a wide range of metal ions, including Co(II), Ni(II), Cu(II), Cd(II), Hg(II), and Pb(II), with varying affinities (*15*). Only the cobalt enzyme retains significant catalytic activity at neutral pH (*16*). Structures of metal-substituted enzymes reveal that the geometry of the metal site often varies with the identity of the metal. For example, copper binds to wild-type CAII in a trigonal bipyramidal geometry, accepting an additional water as a ligand (*13*). To further understand metal affinity and specificity, we report here calorimetric studies of the binding of carbonic anhydrase II to its native metal ligand Zn(II) and to Co(II) and Cu(II). These energetic studies, in conjunction with the currently available high-resolution structural data, facilitate an understanding of the intermolecular interactions relevant to high-affinity metal ion binding in aqueous solution.

[1] Abbreviations: CAII, human carbonic anhydrase II; WT, wild-type; ACES, *N*-(acetamido)-2-aminoethanesulfonic acid; DPA, dipicolinate; ESI-MS, electrospray mass spectrometry.

MATERIALS AND METHODS

Expression and Purification of CAII. The plasmid encoding CAII was transformed into *E. coli* BL21(DE3) cells, and CAII was induced by addition of 0.23 mM isopropyl *β*-D-thiogalactopyranoside during late log growth. The cells were incubated at 30 °C for 5−6 h, and CAII was purified by ion exchange chromatography on DEAE-Sephacel followed by SP-Sepharose Fast Flow (*17*, *18*). The concentration of CAII was determined by stoichiometric titration with acetazolamide (*19*). The concentration of the enzyme−acetazolamide complex was assayed by a decrease in fluorescence (excitation = 280 nm, emission = 470 nm) in the presence of dansylamide at concentrations of CAII (≥0.5 *μ*M) well above the K_D for acetazolamide (*20*).

Preparation of Apoenzyme. All buffers and solutions were prepared using deionized water and stored in plasticware previously treated with a 5 mM (ethylenedinitrilo)tetraacetate solution to remove trace metal ions. ACES was purchased from Research Organics.

Metal-free apo-CAII was prepared using Amicon diaflow filtration, washing first against 50 mM dipicolinate (DPA, Sigma or Acros), pH 7.0, and then against 10 mM ACES, pH 7.0. Excess DPA was removed by gel filtration chromatography on a Pharmacia Sephadex PD-10 column (*21*). The residual zinc concentration after apo preparation was determined using the colorimetric 4-(2-pyridylazo)resorcinol method (*22*).

Mass Spectrometry. Electrospray mass spectrometry (ESI) experiments were performed on a Perkin-Elmer Sciex API 150EX triple-quadrupole mass spectrometer. Individual sample solutions were introduced into the ESI source by direct infusion at 5 *μ*L min^{-1}, and the ion source was heated to 80 °C. The mass range of 500.0−3000.0 amu was scanned in 0.5 amu steps with a dwell time of 1.0 ms. Data were displayed using BioMultiView 1.3.1, and protein molecular masses were calculated with BioSpec Reconstruct software.

Table 1: Summary of Thermodynamic Parameters for Metal Ion Binding to CAII at 25 °C[a]

metal	K_H[b] (M^{-1})	$\Delta G°_H$[c] (kcal mol^{-1})	ΔH_H (kcal mol^{-1})	$\Delta S°_H$ (eu)	ΔC_p (cal K^{-1} mol^{-1})	K_L (M^{-1})	$\Delta G°_L$ (kcal mol^{-1})	ΔH_L (kcal mol^{-1})	$\Delta S°_L$ (eu)
Co(II)	$(5 \pm 3.5) \times 10^7$	-10.5 ± 0.7	-18 ± 1	-25 ± 0.6	1 ± 33	–	–	–	–
Zn(II)	$(1.2 \pm 0.2) \times 10^{12}$	-16.4 ± 0.2	-8.6 ± 0.2	25 ± 0.7	-117 ± 10	–	–	–	–
Cu(II)	$(5.9 \pm 1.4) \times 10^{13}$	-18.8 ± 0.2	-5 ± 0.2	46 ± 0.5	-57 ± 8	1×10^5	-7 ± 0.6	-2.5 ± 0.4	15 ± 0.5
Zn(II)[d]	$(1.2 \pm 0.2) \times 10^{12}$	-16.4 ± 0.2	-6.4 ± 0.3	34 ± 0.8	–	–	–	–	–

[a] Subscripts H and L represent values for the high- and low-affinity sites, respectively. Data were collected in experiments performed at four or five different temperatures with 5.0 mM metal ion and 50−303 μM apo-CAII in 10 mM ACES, pH 7. Parameters were determined using the ORIGIN software with one- or two-site curve fitting. Parameters which are not applicable are represented by dashed lines. [b] From equilibrium dialysis (*24*, *41*). [c] From the expression $\Delta G° = -RT \ln K_{eq}$, using values of K_{eq} from equilibrium dialysis. [d] Values corrected for buffer protonation using the expression $\Delta H_{cal} = \Delta H_{binding} + N_{H^+}\Delta H_{ionization}$, where N_{H^+} is the number of protons released during binding (*28*).

Denatured enzyme (10 μM) was prepared in an aqueous solution containing 30% acetonitrile and 0.1% trifluoroacetic acid. For native ESI experiments, enzyme (30 μM) was exchanged into 10 mM ammonium acetate buffer using either gel filtration chromatography or dialysis.

Isothermal Titration Microcalorimetry. All thermodynamic data were collected on either an Omega or VP-ITC titration microcalorimeter (MicroCal, Inc., Northampton, MA); details of instruments and data reduction have been reported previously (*23*). The cell volumes of the calorimeters are 1.3678 and 1.4346 mL, respectively. All protein and metal ligand solutions were degassed under vacuum prior to the titration experiments. Zinc sulfate was purchased from Aldrich as the volumetric standard. Cobalt and copper were purchased from Aldrich as atomic absorption solutions dissolved in nitric acid. Samples consisted of apo-CAII (50−303 μM) in 10 mM ACES, pH 7, with buffer as the reference. After cell equilibrium was reached, a 2−5 mM solution of metal ($ZnSO_4$, $CuNO_3$, or $CoNO_3$) in the same buffer was injected using an injection schedule of 30−70 injections, 3 μL volume, 10 s duration, 5−10 min interval. For ΔC_p determinations, titrations were performed at four or five different temperatures; the cell temperature varied by <0.3 °C over the course of an experiment. Two titrations were performed at 25 °C in 10 mM PIPES, pH 7, where 249 μM apo-CAII was titrated with 5 mM $ZnSO_4$. Data analysis was carried out using the ORIGIN software from MicroCal. All binding enthalpies are reported after subtraction of the appropriate enthalpy of metal ion dilution.

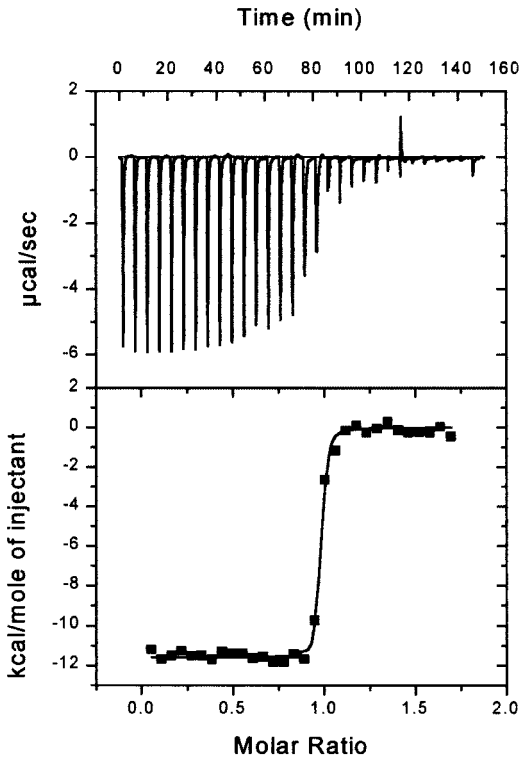

FIGURE 2: Isothermal titration calorimetry curve of zinc binding to apo-WT CAII fit to the single-site binding equation (280 μM wild-type CAII at 35 °C, 30 injections of 3 μL each of 5.0 mM $ZnSO_4$).

RESULTS

A representative thermogram for the titration of apo-CAII with $ZnSO_4$ is shown in the top panel of Figure 2. The bottom panel shows heat evolved per mole of titrant as a function of the molar ratio of total ligand to total enzyme (q_i/X_{tot} vs X_{tot}/M_{tot}), together with the fit to a single-site binding equation. The thermodynamic parameters derived from such titrations are listed in Table 1. The shape of the thermogram in calorimetric titration is dependent on the unitless parameter *c*, numerically equivalent to the product of the binding constant and the concentration of binding sites. The binding equation will fit curves where *c* ranges from 1 to 1000. Here, values of *c* exceed 1000, and the reported free energies of binding were derived from binding constants determined by equilibrium dialysis (*24*). The determination of $\Delta G°$ and ΔH from distinct experiments avoids the potential complication of correlated errors.

Henkens and Sturtevant previously reported the enthalpy of zinc binding to bovine carbonic anhydrase as 8.5 kcal mol^{-1} (*25*). These experiments were carried out in phosphate buffer where precipitation of zinc phosphate was likely significant. Additionally, metal ion was added from water, rather than buffer, and dilution effects were apparently not corrected for. Here, we used ACES buffer to minimize protein−buffer and metal−buffer interactions. The exothermic enthalpy of zinc binding to carbonic anhydrase obtained at 25 °C (Table 1) is consistent with enthalpy changes determined for complexation of zinc by amino acids (*26*). Evaluation of the enthalpy of zinc binding to carbonic anhydrase as a function of temperature provides a change in molar heat capacity accompanying binding of −117 cal M^{-1} K^{-1} (Figure 3).

Our calorimetric measurements assume that the initial protein is entirely in the apoenzyme form while the final holoprotein is zinc-bound and in the native conformation, with no denaturation occurring during the experiment. To unequivocally demonstrate the validity of our assumption, we examined preparations of apo- and holoprotein by electrospray mass spectrometry (ESI-MS). Figure 4 shows

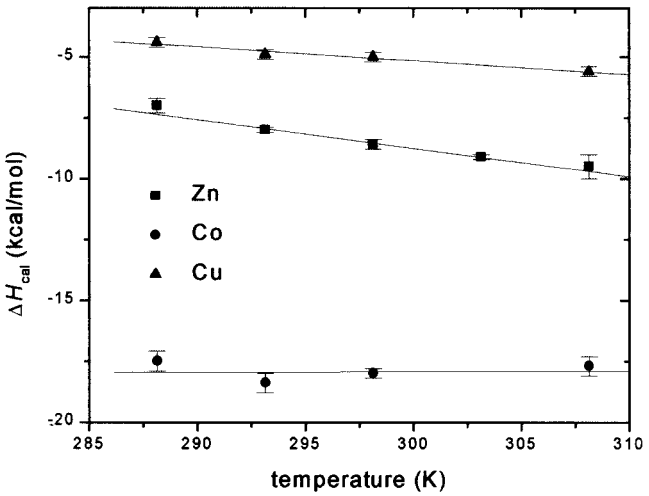

FIGURE 3: Temperature dependence of the enthalpy of binding of zinc, cobalt, and copper to apo-CAII. The derived ΔC_p values are -117 ± 10, 1 ± 33, and -57 ± 8 cal K^{-1} mol^{-1}, respectively.

the ESI-MS spectra of both denatured and native apo-CAII, as well as the corresponding spectrum of zinc-loaded holoenzyme. The sample of zinc-loaded holoenzyme shown was taken directly from the calorimeter cell following a standard titration. Panels A and B of Figure 4 demonstrate that native and denatured enzyme, respectively, are readily distinguishable by ESI-MS. Panel C shows the spectrum of the zinc-loaded CAII, and the final panel of Figure 4 shows a 1:1 mixture of holo- and apoprotein. Further study demonstrated that as little as 5% of holoenzyme is visible in a sample of apocarbonic anhydrase. Together, these spectra demonstrate that apoenzyme preparations do not contain any appreciable level of denatured or zinc-bound carbonic anhydrase. Furthermore, greater than 95% of the protein after a titration is zinc-bound, native CAII. The stoichiometry of the binding reaction in the titration reactions was typically 0.9−1.1, using the concentration of CAII determined by acetazolamide titration. As a result of these findings, we have fixed the stoichiometry of binding at unity during reduction of our data. We do so because, in the absence of other data, stoichiometric binding is the simplest model that is physically realistic. Finally, we note parenthetically that variation of the protein concentration—the practical result of fixing binding stoichiometries—has no impact on measured enthalpies of binding; these values are determined solely by the concentration of ligand.

Ligand binding is frequently coupled to the uptake or release of a proton; in such instances, the enthalpy of buffer protonation/deprotonation is included in calorimetrically derived binding enthalpies. To examine this possibility, we measured the enthalpy of zinc binding to CAII in ACES and PIPES buffers, the ionization enthalpies of which are 7.2 and 2.1 kcal mol^{-1}, respectively (*27*). The diminution in binding enthalpy in PIPES versus ACES indicates a net release of 0.3 protons to the buffer upon binding of the zinc ligand at pH 7 (*28*). The thermodynamic parameters corrected for buffer protonation are shown in Table 1.

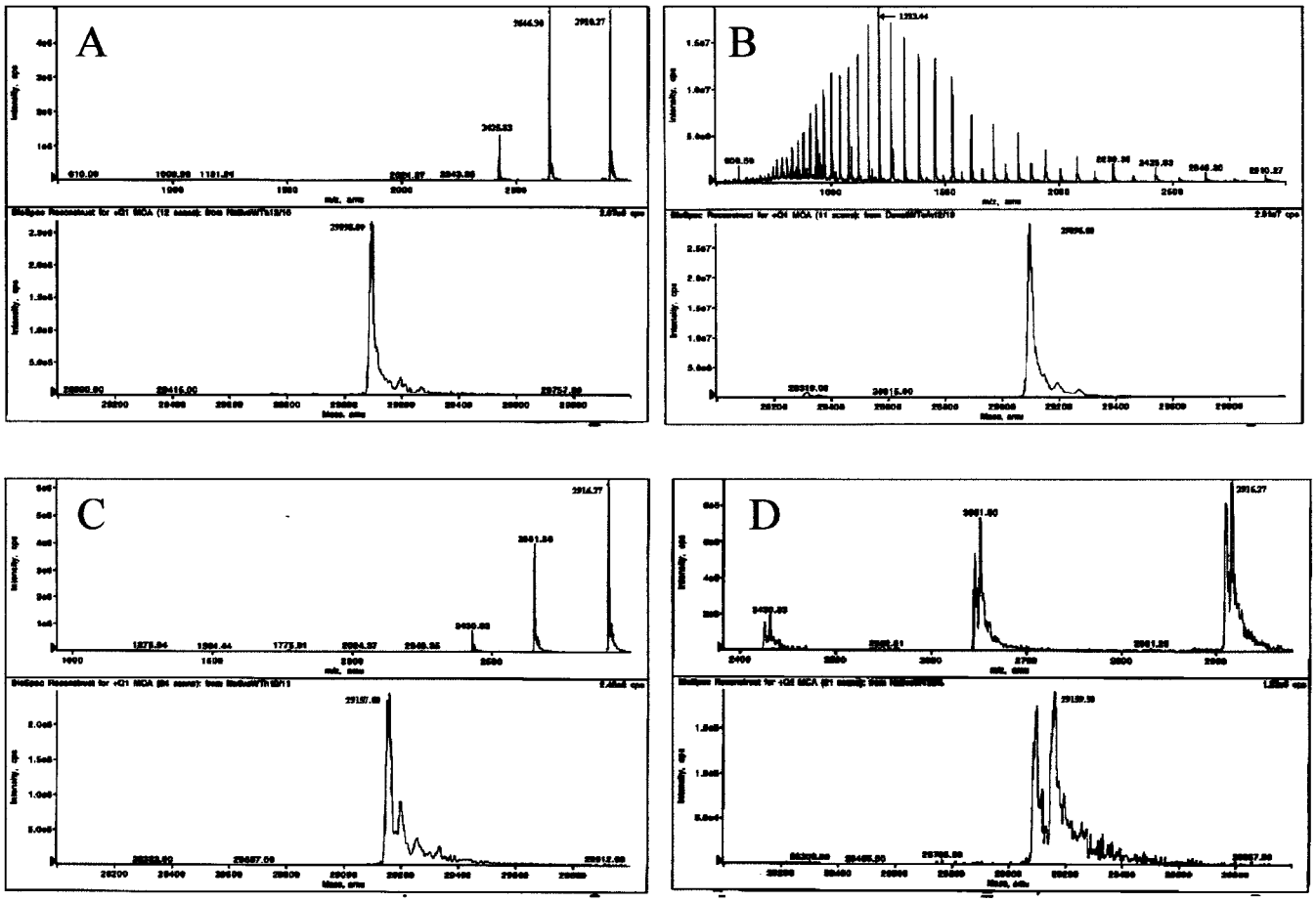

FIGURE 4: ESI-MS of denatured, apo- and holo-CAII: (A) apo-CAII, (B) denatured CAII, (C) zinc-loaded holo-CAII after isothermal titration calorimetry, and (D) 1:1 mixture of holo- and zinc-loaded CAII.

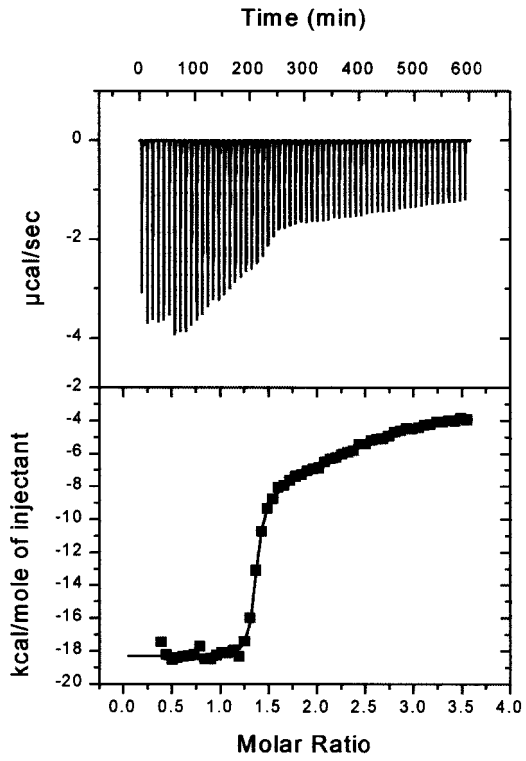

FIGURE 5: Isothermal titration curve of Co(II) binding to wild-type apo-CAII (275 μM apo-CAII at 20 °C, 60 injections of 3 μL each of 4.0 mM $ZnSO_4$). The curve is best fit by a model of two independent binding sites, a tight binding site with $K > 6 \times 10^6$ M^{-1}, $\Delta H = -18.4 \pm 0.4$ kcal mol^{-1}, and a weak binding site with $K \approx 1500$ M^{-1}.

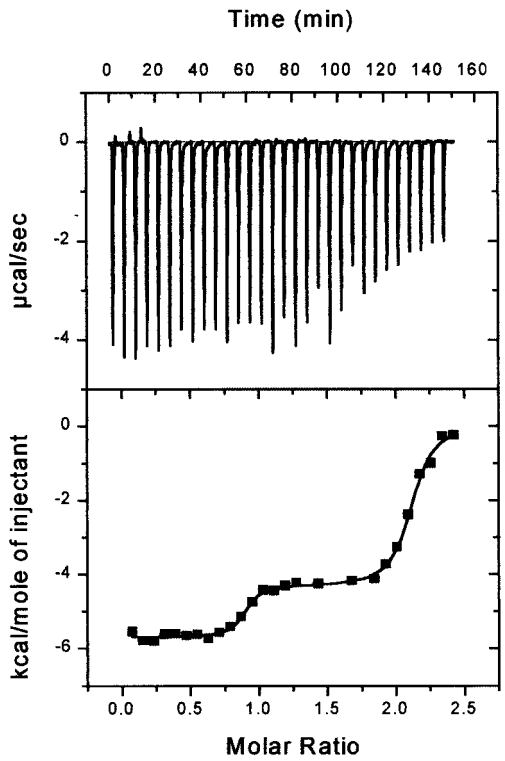

FIGURE 6: Isothermal titration calorimetry curve of C(II) binding to apo-WT CAII fit to the two-site binding equation. Binding to the tight binding site occurs with an association constant of $(5.9 \pm 1.4) \times 10^{13}$ M^{-1} (determined by equilibrium dialysis) and an enthalpy of -5 ± 0.1 kcal mol^{-1}. The weaker binding site has an association constant of 10^5 M^{-1} and an enthalpy of -2.5 ± 0.4 kcal mol^{-1} (303 μM wild-type CAII at 25 °C, 30 injections of 3 μL each of 5.0 mM $CuCl_2$).

Cobalt and copper were chosen for binding to CAII because their affinities and on rates bracket those of zinc; copper binds more rapidly than zinc and with a 50-fold higher affinity, while cobalt binds more slowly and with a 10^4-fold decrease in affinity (*24*). This difference in zinc and cobalt affinities is typical of tetrahedral protein zinc sites (*29*) and may be due to the loss of ligand field stabilization energy when cobalt changes from the octahedral coordination in solvent to tetrahedral coordination in a protein metal site (*30*). Because of its fully occupied d-shell, zinc is not subject to ligand field stabilization effects and incurs little or no energy loss upon changing from octahedral to tetrahedral geometry (*30*). However, both the catalytic activity and structure of Co(II)-bound CAII mimic that of zinc. The X-ray crystal structure of WT CAII bound to cobalt reveals tetrahedral geometry with little alteration (<1 Å) in the positions of the coordinating histidines from those positions in zinc-bound CAII (*31*). Calorimetry of cobalt binding to WT CAII should then provide intrinsic binding enthalpies, with little contribution from conformational changes of the protein.

The binding of Co(II) proceeds in a fashion similar to that of zinc, although with a substantially larger enthalpy of binding and a greatly diminished change in molar heat capacity (Table 1). However, at higher enzyme concentrations, two binding sites for cobalt are observed (Figure 5). In contrast, binding of Cu^{2+} yields a markedly different thermogram at lower enzyme concentrations, requiring a two-site fit and indicating that two distinct metal ions bind with markedly differing affinities (Figure 6). Evaluation of the binding as a function of temperature yields a ΔC_p value for the high- and low-affinity sites of -57 cal K^{-1} mol^{-1} and near zero, respectively. The location of this second binding site is not known definitively, although a second copper binding site has been previously observed in both inhibition studies (*32*) and metal binding studies (*33*). Furthermore, the crystal structure of copper-substituted CAII reveals two bound copper ions, one replacing the active site zinc and a second copper coordinating two histidine side chains, His-4 and His-64, at the mouth of the active site cleft (*13*).

A significant decrease in the calorimetric peak width is observed for copper binding relative to zinc. The calorimeter used here measures power, or the rate of heat generation in the sample cell as a function of time. As a result, the peaks contain kinetic information providing the processes are slow on the time scale of the instrument (10 s^{-1}) (*34*). The association rate constant for zinc is $\sim 10^5$ M^{-1} s^{-1}, significantly lower than the diffusion-controlled rate constant. A proposed mechanism of zinc ion association with CAII, consistent with mutagenesis data, involves a rapid association with two of the three histidines, followed by a slow rearrangement of the third direct ligand to provide a tight, tetrahedral metal polyhedron (*35*). However, the association rate for copper ion binding to CAII has been extrapolated to occur at the diffusion-controlled rate constant of $\sim 10^9$ M^{-1} s^{-1} (*24*). The differences in the association rate constants for zinc and copper provide a rational for the decreased peak width in the copper titrations relative to other metal binding titrations.

Table 2: Thermodynamics of Metal Ion Hydration and Binding to CAII[a]

ion	ΔH(hydration)[b] (kcal mol^{-1})	ΔH(binding)[c] (kcal mol^{-1})	ΔC_p(hydration)[b] (eu)
Cu(II)	−520	−5	−42.1
Zn(II)	−507	−6.4	−42.5
Co(II)	−503	−18	−43.7

[a] All values determined at 25 °C. [b] Taken from ref *3*. [c] Taken from Table 1. The enthalpy for Zn(II) binding is the value corrected for buffer protonation.

DISCUSSION

Enthalpies of ligand binding are, of course, state functions and necessarily include contributions from changes in the protonation state of the final complex relative to the initial, unbound state. Zinc binding to carbonic anhydrase results in the diminution of the zinc-bound water pK_a, from 9 to 10 in bulk solution to 6.8 in the bound complex (*36*). Our calorimetric experiments were conducted at pH 7; roughly six-tenths of a proton are calculated to be released to buffer during zinc binding solely due to ionization of the zinc-bound water. However, this value will be attenuated if protonation of the zinc-bound water affects the pK_a values of nearby groups, such as His-64 (*37*). Evaluation of the enthalpy of zinc binding as a function of buffer ionization enthalpy yielded a net release of 0.3 protons. Accordingly, enthalpy values of zinc binding have been corrected for buffer protonation. The metal−water pK_a of Co(II)-CAII (*38*), and likely Cu(II)-CAII, is higher than that of the zinc enzyme, suggesting that the release of protons, and the corresponding enthalpic correction, is smaller. In this instance, perturbation of His acidities should not affect overall binding enthalpies. The pK_a's of the amino acids that coordinate zinc (His-94, His-96, and His-119) in carbonic anhydrase are estimated at less than 6 in the apoenzyme (*15*). Because metal binding will perturb imidazole pK_a values down, no significant change in protonation is expected from these amino acids upon Cu(II) or Co(II) coordination at pH 7.

Two aspects of metal binding to CAII bear comment: the decreasing enthalpy of binding through the series Co(II) → Zn(II) → Cu(II) and the striking difference in ΔC_p values between Zn(II) and the other two metal ions. In the former instance, the dominant factor is almost surely differences in the energetics of hydration of the metal ions themselves. Hydration is defined as the transfer of a solute particle from a fixed position in the ideal gas to a fixed position in water (*3*). The solution is defined at infinite dilution, and the position of the solute is fixed, so that the complicating factors of solute−solute interaction and translational degrees of freedom are precluded. The process therefore describes the net changes in thermodynamics due only to interaction of the hydrating solute in the aqueous environment. Table 2 indicates that the enthalpies of metal ion hydration decrease across the series copper, zinc, and cobalt. To the extent that the metal ion itself is desolvated during binding, the enthalpy of binding should be inversely proportional to the enthalpy of hydration; this trend is indeed observed.

A range of secondary factors also contribute to overall enthalpies of metal ion binding. Thus, for example, the crystal structure of the Cu^{2+}-loaded enzyme reveals trigonal bipyramidal coordination geometry about copper, with two coordinating water molecules (*13*). Direct ligands in the CAII binding site are optimally positioned for tetrahedral metal coordination geometry, and it is reasonable to suppose that electrostatic interactions with Cu^{2+} and hydrogen bonds to indirect ligands are weakened in the altered geometry. Consistent with this proposal, mutations in hydrophobic residues beneath the zinc binding site both decrease constraints on the tetrahedral geometry and enhance copper binding by several orders of magnitude (*24*).

The increment in ΔC_p for binding of the three metals is noteworthy. The change in molar heat capacity is primarily a measure of solvent reorganization that occurs during ligand binding (*39*). In general, the desolvation of hydrophobic surface area provides a negative contribution to ΔC_p, while the desolvation of ionic surface area provides a negligible or positive contribution to ΔC_p. Here, binding of the native zinc ion proceeds with a significantly negative heat capacity increment; such behavior is typical for ligand binding events (*40*). In contrast, binding of both Cu(II) and Co(II) proceeds with a greatly diminished change in molar heat capacity. Two possible explanations rationalize this phenomenon. First, the *negative* contribution to ΔC_p from the desolvation of hydrophobic surface area might be diminished in the copper and cobalt complexes relative to the zinc species, as a result of less efficient binding site desolvation. Second, the *positive* contribution to ΔC_p from metal ion desolvation might be enhanced in the copper and cobalt complexes relative to zinc, either from a larger inherent ΔC_p for metal ion desolvation or from a more efficient shielding of the metal ion from solvent in the bound complex. The partial molal heat capacities of divalent metal ions vary little across the series (Table 2) (*3*), suggesting this latter explanation can likely be discounted. On the other hand, it is clear that there is significantly greater desolvation of both the metal ion and the binding site for the zinc complex than for the copper complex due to the optimal organization of ligands for zinc complexation. This larger desolvation could in principle provide both a positive and a negative contribution to ΔC_p; that the increment is negative suggests that the desolvation of nonpolar surface area in the binding site makes the more significant contribution. Whether this contribution arises simply from the larger inherent contribution to heat capacity changes of nonpolar surface area than of ionic surfaces or whether the bound complex retains sufficient water to avoid a large heat capacity increment from metal ion desolvation is unclear.

Under circumstances of high protein concentration (above 250 μM) additional metal binding sites are occasionally observed. In some instances such titrations are well fit by a two-binding site equation and most likely represent second lower-affinity metal binding sites on a discrete protein surface. On the other hand, in some cases second metal sites saturate below stoichiometric metal ion concentration and are accompanied by protein precipitation. We interpret these latter observations as evidence for intermolecular metal ion binding, resulting in aggregation and, ultimately, precipitation. These observations underscore the importance of considering multiple models of ligand binding and unambiguous identification of the final, bound state prior to interpretation of calorimetric data.

In summary, the thermodynamics of metal ion binding to carbonic anhydrase in aqueous solution have been deter-

mined. As expected, the overall affinity and enthalpy of binding are determined by a complex interplay of solute−solute and solute−solvent interactions both prior to and following association. Desolvation, both of the metal ion and of the binding site, makes large—perhaps the most significant—contributions to the overall thermodynamics of association. Our results again emphasize the necessity of considering the interaction of solutes with solvent prior to association, when considering the origins of affinity in aqueous solution.

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REFERENCES

1. Franks, F. (1973) *Water A Comprehensive Treatise*, Plenum Press, New York.
2. Holm, R. H., Kennepohl, P., and Solomon, E. I. (1996) *Chem. Rev. 96*, 2239−2314.
3. Marcus, Y. (1994) *Biophys. Chem. 51*, 111−127.
4. Albrecht-Gary, A.-M., and Crumbliss, A. L. (1998) *The Coordination Chemistry of Siderophores: Thermodynamics and Kinetics of Iron Chelation and Release*, Vol. 35, Marcel Dekker, New York.
5. Labbe, S., and Thiele, D. J. (1999) *Trends Microbiol. 7*, 500−505.
6. Nelson, N. (1999) *EMBO J. 18*, 4361−4371.
7. Alberty, R. A., and Hammes, G. G. (1958) *J. Phys. Chem. 62*, 154−159.
8. Stadie, W. C., and O'Brien, H. (1933) *J. Biol. Chem. 103*, 521−529.
9. Meldrum, N. U., and Roughton, F. J. W. (1933) *J. Physiol. 80*, 113−142.
10. Lindskog, S. (1997) *Pharmacol. Ther. 74*, 1−20.
11. Forsman, C., Behraven, G., Osterman, A., and Jonsson, B.-H. (1988) *Acta Chem. Scand. B42*, 314−318.
12. Liljas, A., Kannan, K. K., Bergsten, P.-C., Waara, I., Fridborg, K., Strandberg, B., Carlbom, U., Jarup, L., Lovgren, S., and Petef, M. (1982) *Nat. New Biol. 235*, 131−137.
13. Håkansson, K., Carlsson, M., Svensson, L. A., and Liljas, A. (1992) *J. Mol. Biol. 227*, 1192−1204.
14. Hewett-Emmett, D., and Tashian, R. E. (1996) *Mol. Phylogenet. Evol. 5*, 50−77.
15. Lindskog, S., and Nyman, P. O. (1964) *Biochim. Biophys. Acta 85*, 462−474.
16. Kogut, K. A., and Rowlett, R. S. (1987) *J. Biol. Chem. 262*, 16417−16424.
17. Nair, S. K., Calderone, T. L., Christianson, D. W., and Fierke, C. A. (1991) *J. Biol. Chem. 266*, 17320−17325.
18. Krebs, J. F., and Fierke, C. A. (1993) *J. Biol. Chem. 268*, 948−954.
19. Nair, S. K., Krebs, J. F., Christianson, D. W., and Fierke, C. A. (1995) *Biochemistry 34*, 3981−3989.
20. Chen, R. F., and Kernohan, J. C. (1967) *J. Biol. Chem. 242*, 5813−5823.
21. Alexander, R. S., Kiefer, L. L., Fierke, C. A., and Christianson, D. W. (1993) *Biochemistry 32*, 1510−1518.
22. Hunt, J. B., Neece, S. H., Schachman, H. K., and Ginsberg, A. (1984) *J. Biol. Chem. 259*, 14793−14803.
23. Wiseman, T., Williston, S., Brands, J. F., and Lin, L.-N. (1989) *Anal. Biochem. 179*, 131−137.
24. Hunt, J. A., Ahmed, M., and Fierke, C. A. (1999) *Biochemistry 38*, 9054−9062.
25. Henkens, R. W., Watt, G. D., and Sturtevant, J. M. (1969) *Biochemistry 8*, 1874−1878.
26. Stack, W. F., and Skinner, H. A. (1967) *Trans. Faraday Soc. 63*, 1136−1145.
27. Grime, J. K. (1985) *Analytical Solution Chemistry*, J. Wiley, New York.
28. Gómez, J., and Freire, E. (1995) *J. Mol. Biol. 252*, 337−350.
29. Maret, W., and Vallee, B. L. (1993) *Methods Enzymol. 226*, 52−71.
30. Huheey, J. E. (1983) *Inorganic Chemistry*, Harper and Row, New York.
31. Håkansson, K., Wehnert, A., and Liljas, A. (1994) *Acta Crystallogr. D50*, 93−100.
32. Tu, C., Wynns, G. C., and Silverman, D. N. (1981) *J. Biol. Chem. 256*, 9466−9470.
33. Thompson, R. B., Maliwal, B. P., and Fierke, C. A. (1999) *Anal. Biochem. 267*, 185−195.
34. Williams, B. A., and Toone, E. J. (1993) *J. Org. Chem. 58*, 3507.
35. Kiefer, L. L., Paterno, S. A., and Fierke, C. A. (1995) *J. Am. Chem. Soc. 117*, 6831−6837.
36. Silverman, D. N., and Lindskog, S. (1988) *Acc. Chem. Res. 21*, 30−36.
37. Simonsson, I., and Lindskog, S. (1982) *Eur. J. Biochem. 123*, 29−36.
38. Tu, C., and Silverman, D. N. (1985) *Biochemistry 24*, 5881.
39. Chervenak, M. C., and Toone, E. J. (1994) *J. Am. Chem. Soc. 116*, 10533−10539.
40. Oas, T. G., and Toone, E. J. (1997) *Adv. Biophys. Chem. 6*, 1−52.
41. Hunt, J. A., and Fierke, C. A. (1997) *J. Biol. Chem. 272*, 20364−20372.

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