Functional Characterization of Human Carbonic Anhydrase II Variants with Altered Zinc Binding Sites[†]

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ABSTRACT: Carbonic anhydrase II (CAII) contains a conserved His_3 zinc polyhedron which is essential for catalysis. Removal of any one of the His ligands by replacement with Ala decreases ($\approx 10^5$-fold), but does not abolish, zinc binding and increases the rate constant for zinc dissociation. CAII variants with a His ligand substituted with Cys, Asp, or Glu bind zinc only ≈10-fold better than a His_2 zinc polyhedron in CAII. The large decrease in zinc affinity (≈5 kcal/mol) in these variants compared to the wild-type His_3 site reflects mainly unfavorable compensatory protein structural rearrangements observed in the X-ray crystallographic structures of some of these CAII variants, described by Ippolito and Christianson (following paper in this issue). However, the zinc affinity of these sites is still higher than zinc polyhedra designed *de novo*. Substitution of the His zinc ligands with negatively charged amino acids both increases the pK_a of the zinc-bound water by ≥1.6 pH units, confirming that neutral ligands maintain the low zinc−water pK_a, and decreases the pH-independent k_{cat}/K_M for ester hydrolysis (3−30-fold) and CO_2 hydration ($\approx 10^3 - 10^5$-fold). Additionally, decreases in the dissociation constant ($\approx 10^2 - 10^5$-fold) for the transition state analog acetazolamide correlate with the decreased catalytic efficiency and increased pK_a of these CAII variants. These data indicate that the histidine ligands, although not essential for catalysis, are conserved to maximize electrostatic stabilization of both the ground-state zinc−hydroxide and the negatively charged transition state. These studies provide valuable insights into the functional consequences of engineering a catalytic zinc site in a metalloenzyme.

Carbonic anhydrase II (CAII)[1] (EC 4.2.1.1) catalyzes the reversible hydration of CO_2 to HCO_3^- and a proton with a second-order rate constant approaching the diffusion-controlled limit [reviewed by Silverman and Lindskog (1988)]. The three-dimensional structure of this enzyme, determined and refined at 1.54-Å resolution (Håkansson *et al.*, 1992), reveals a conserved zinc coordination polyhedron [reviewed by Hewett-Emmett and Tashian (1991)] that is optimized to provide catalytically active zinc−hydroxide at neutral pH. The zinc cofactor lies at the bottom of the active site cleft where it is coordinated to His94, His96, His119, and a hydroxide ion at physiological pH, in a tetrahedral geometry (Figure 1). Thr199 accepts a hydrogen bond from the zinc-bound hydroxide ion and donates a hydrogen bond to Glu106. CAII-catalyzed hydration proceeds through direct nucleophilic attack of the zinc-bound hydroxide ion on the carbonyl carbon of CO_2 to form zinc-bound bicarbonate (Silverman & Lundskog, 1988).

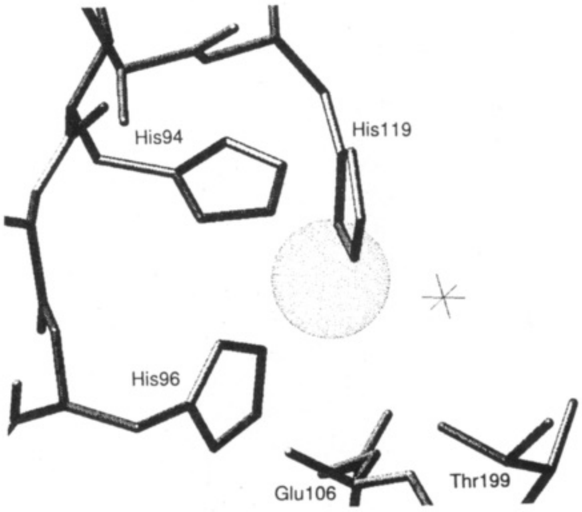

FIGURE 1: Structure of the active site of wild-type CAII taken from the crystal structure of Alexander *et al.* (1991), showing the zinc tetrahedrally coordinated to three imidazole ligands, His94, His96, and His119, and a solvent molecule. The residues involved in the conserved hydrogen bond network to the zinc−water, Thr199 and Glu106, are also depicted.

CAII has been used as a model for the *de novo* design of transition metal binding sites into a variety of protein scaffoldings including antibodies (Iverson *et al.*, 1990; Roberts *et al.*, 1990; Pessi *et al.*, 1993; Wade *et al.*, 1993; Stewart *et al.*, 1994, Bianchi *et al.*, 1994) and α-helical peptides (Handel & DeGrado, 1990; Regan & Clarke, 1990; Ghadiri & Choi, 1990; Ruan *et al.*, 1990; Suh *et al.*, 1991; Handel *et al.*, 1991). However, none of these designed metal sites approach the avid metal binding and catalytic activity of metalloenzymes observed in nature. Therefore, to probe the functional importance of the three histidine residues in CAII which coordinate zinc, we have substituted these amino

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[1] Abbreviations: CAII, human carbonic anhydrase II; Tris, tris(hydroxymethyl)aminomethane; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; PAR, 4-(2-pyridylazo)resorcinol; PNPA, *p*-nitrophenyl acetate.

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acids with Cys, Asp, Glu, and Ala and characterized the catalytic parameters and zinc and inhibitor binding of these variants. This work, in combination with the three-dimensional structures of these variants (Xue, 1992; Kiefer *et al.*, 1993a; Ippolito & Christianson, 1994), reveals that the metal binding site of CAII is sufficiently plastic to accommodate binding of one zinc ion when any one of the three His zinc ligands is replaced with an alternative ligand, but that this substitution drastically reduces zinc affinity and catalytic efficiency. Furthermore, these data indicate that the His_3 metal polyhedron, although not essential for catalysis of CO_2 hydration, facilitates high catalytic efficiency by electrostatic stabilization of both zinc−hydroxide to maintain high concentrations of this species at neutral pH and the negatively charged transition state. This work provides insight into the structure−function properties of metalloenzymes essential for the *de novo* design or redesign of transition metal sites with catalytic activity.

MATERIAL AND METHODS

Preparation of CAII Variants. The CAII variants with substitutions in one of the three histidines which coordinate zinc (referred to as metal ligand variants) were produced using oligonucleotide-directed mutagenesis of the cloned human CAII gene in pCAM (Krebs & Fierke, 1993). The entire CAII gene of the variants was sequenced using the dideoxy method of Sanger *et al.* (1977) with Sequenase 2.0. The plasmids encoding the variant CAII's were transformed into the *Escherichia coli* strain BL21(DE3) (Hanahan, 1983). These cells were grown, and protein was induced and purified to ≥95% homogeneity by chromatography on DEAE-Sephacel and S-Sepharose resins, as previously described (Alexander *et al.*, 1993). Zinc sulfate (100 μM) and dithiothreitol (0.5 mM for His94Cys, His96Cys, and His119Cys) were included in the buffer throughout the purification. The concentration of the variant enzymes was calculated from absorbance using $\epsilon_{280} = 5.4 \times 10^4\ M^{-1}\ cm^{-1}$ as determined for native CAII (Tu & Silverman, 1982).

Catalytic Activity. The k_{cat}/K_M for CAII-catalyzed *p*-nitrophenyl acetate (PNPA) hydrolysis was measured at 25 °C in either 50 mM CHES (pH 8.7−9.6), 50 mM TAPS (pH 7.8−8.4), or 50 mM MOPS (pH 6.8−7.5), ionic strength = 0.1 M with sodium sulfate, 0.5 mM PNPA and 0.1−20 μM CAII by measuring the initial rate of absorbance increase at 348 nm ($\epsilon_{348} = 5000\ M^{-1}\ cm^{-1}$) (Armstrong *et al.*, 1966). Under the conditions of this assay, zinc sulfate was present in a slight molar excess to enzyme and His96Ala and His119Ala CAII bind greater than 1 mol of zinc per mol of enzyme. Background rates, measured by inhibiting CAII with acetazolamide (0.5 mM), were subtracted from the observed rates. The pK_a, pH-independent second-order rate constant and standard errors were calculated by a weighted fit of the pH dependence of k_{cat}/K_M obs to eq 1 using the program KaleidaGraph (Synergy Software).

$$k_{cat}/K_{M\ obs} = k_{cat}/K_M/(1 + 10^{(pK_a-pH)}) \qquad (1)$$

Initial rates of CO_2 hydration were measured in a KinTek stopped-flow apparatus at 25 °C by the changing pH-indicator method (Khalifah, 1971). The reaction was monitored at 578 nm in 50 mM TAPS buffer, pH 8.9, 25 μM *m*-cresol purple, 0.1 mM (ethylenedinitrilo)tetraacetic acid (EDTA), ionic strength = 0.1 M with sodium sulfate, 0.01−10 μM CAII, and a slight molar excess of zinc sulfate to CAII. Removal of the EDTA from the buffer or addition of excess zinc sulfate did not increase the observed activity. Under these conditions all of the CAII's likely bind no more than one zinc per molecule. The CO_2 concentration varied from 3−24 mM. Background rates, measured in the absence of CAII, were subtracted from the observed rates.

Zinc Analyses, Dissociation Constant, and Dissociation Rate Constant. All solutions were prepared in plasticware using deionized water (18 MΩ). Protein samples were chromatographed on PD-10 columns (Sephadex G-25M, 5 cm × 15 cm, Pharmacia) equilibrated with 15 mM potassium phosphate buffer, pH 7.0, to remove unbound zinc. Enzyme-bound zinc (E−Zn) was quantitated using the colorimetric 4-(2-pyridylazo)resorcinol (PAR) method of Hunt *et al.* (1984) and measuring the absorbance at 500 nm. A standard curve was determined for each experiment in 15 mM potassium phosphate buffer, pH 7.0, 4.0 M guanidine hydrochloride, and 100 μM PAR using either a zinc sulfate standard (Aldrich) (0−6 μM) or wild-type CAII (0−6 μM).

Apo-wild-type CAII was prepared as described (Kiefer *et al.*, 1993b). Variant apo-CAII's were prepared by a modification of the method of Hunt *et al.* (1977) using Amicon diaflow filtration against first 25 mM dipicolinate, pH 7.0 (0.65 mL/min, 100 min), and then 10 mM Tris sulfate, pH 7.0, followed by chromatography on a PD-10 column. Dithiothreitol (0.5 mM) was included during zinc removal in the case of His94Cys, His96Cys, and His119Cys but removed prior to dialysis by PD-10 chromatography. Dissociation constants for zinc were obtained by dialyzing apoenzyme for 4 h at 30 °C against excess zinc sulfate (0.35−350 μM) in 10 mM Tris sulfate, pH 7, purged with nitrogen, removing unbound zinc by chromatography on a PD-10 column, and measuring the protein concentration and bound zinc concentration in the eluant using the PAR assay (Hunt *et al.*, 1984). The concentration of free zinc in the dialysis buffer was calculated from the Tris−zinc stability constants (Dawson *et al.*, 1986). The dissociation constant was calculated using KaleidaGraph program with eq 2. The endpoint, *C*, ranged from 0.9 to 1.3 except for His96Ala CAII where the data are consistant with two independent zinc binding sites and *C* = 2.0.

$$[E-Zn]/[E]_{tot} = C/(1 + K_D/[Zn]_{free}) \qquad (2)$$

$$[E-Zn]/[E]_{tot} = A_0 e^{(-kt)} \qquad (3)$$

The rate constant for zinc dissociation was determined by diluting CAII into zinc-free 10 mM Tris sulfate, pH 7, containing EDTA (final concentration 40−50 μM CAII, 33−38 mM EDTA) to trap zinc dissociating from the enzyme. At various times after dilution, free zinc and EDTA were removed by chromatography on a PD-10 column and enzyme-bound zinc was quantitated using the PAR assay. The total time after dilution was calculated as the sum of the incubation time and the time to complete the PD-10 column (4 min). A_0 (which ranged from 0.85 to 1.1), the dissociation rate constant, and standard errors were calculated using KaleidaGraph with eq 2.

Inhibitor Binding. A dissociation constant for the active site inhibitor, acetazolamide (Ac), was determined by incubating 0−954 μM CAII variant with 0.5−1.0 μM [^{3}H]-

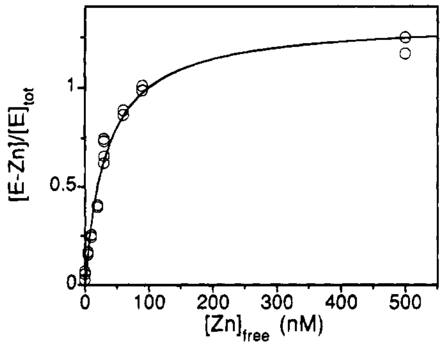

FIGURE 2: Measurement of a zinc dissociation constant for His119Asp CAII at pH 7. The zinc dissociation constant was obtained by dialyzing 0.5 mL of 60 μM apo-His119Asp CAII for 4 h at 30 °C against 1 L of zinc sulfate (0.0–0.35 mM) in 10 mM Tris sulfate, pH 7. Enzyme-bound zinc (E–Zn) was separated from free zinc (Zn_{free}) by chromatography on PD-10 columns and then quantitated using a colorimetric PAR assay (Hunt *et al.*, 1984). The $[Zn]_{free}$ was calculated from $[Zn]_{tot}$ as designated under Materials and Methods. The data were fit to eq 2 yielding a K_D = 25 ± 7 nM.

acetazolamide (^{3}H-Ac) in 50 mM TAPS, pH 8.0, ionic strength = 0.1 M with sodium sulfate, at 25 °C for 10 min. Enzyme bound ^{3}H-Ac (E-Ac) was separated from free ^{3}H-Ac by chromatography on a gel filtration centrifuge column. Sephadex G-25 (0.7 mL) (Pharmacia) equilibrated in the above buffer was placed into a SPIN-X filter unit (Costar) and centrifuged at 4000*g* for 30 s before and after 50 μL of sample was loaded (Penefsky, 1979). The column eluate was diluted into CytoScint scintillation fluid and radioactivity, reflecting E-Ac, was quantitated by scintillation counting. The dissociation constant was calculated using eq 4. The

$$[\text{E-Ac}]/[\text{Ac}]_{\text{tot}} = C/(1 + K_D/[\text{E}]) \qquad (4)$$

total concentration of acetazolamide ($[\text{Ac}]_{\text{tot}}$) was measured as the total number of tritium counts per minute loaded onto each centrifuge column, and the constant, *C*, varied from 0.8 to 0.9. The ^{3}H-Ac was synthesized (Kandel *et al.*, 1968; Roush, 1994) and generously provided by Eric Roush.

RESULTS

To probe the functional importance of the structural and chemical nature of the amino acids which coordinate zinc in CAII, the CAII metal ligand variants were characterized in terms of (1) metal binding, (2) esterase activity and pK_a of zinc-bound water, (3) hydrase activity, and (4) binding of a transition state analog, acetazolamide.

Metal Binding. If purified in the presence of 100 μM zinc sulfate, all of the CAII variants studied contain at least 1.0 mol of zinc per mol of enzyme as determined by quantitating the protein-bound zinc (E–Zn) using the PAR assay (Hunt *et al.*, 1984) after gel filtration chromatography to remove unbound zinc. However, these variants are isolated as apoenzyme if zinc is not added during the purification indicating a significant decrease in the zinc affinity. To further assess the effects of amino acid substitutions at each of the His ligands on zinc binding, a zinc dissociation constant (K_D) was measured by equilibrium dialysis at pH 7 for the purified CAII variants (Figure 2 and Table 1). Replacement of any one of the three His ligands with Cys, Asp, or Glu or removal of a ligand by replacement with Ala results in a ≈10^4-fold or a ≈10^5-fold decrease in zinc affinity, respectively. In most variants, the active site accommodates binding of only one zinc atom; however, His96Ala CAII binds 2.0 mol of zinc per mol of enzyme with $K_{D1} = K_{D2}$ and His119Ala CAII binds 2.7 mol of zinc per mol of enzyme at 1.0 μM zinc sulfate. To determine the effect of changing the pK_a of the metal ligands on the observed dissociation constants, the zinc K_D values were measured for the His94Asp and His94Cys CAII variants from pH 6.5 to 8.0 (data not shown). The pH dependence of the K_D for these variants is not significantly different from that of wild-type CAII (Lindskog & Nyman, 1964), indicating that the altered pK_a of the substituted amino acid is not responsible for the drastic decrease in zinc affinity.

Table 1: Zinc Dissociation Constants and Dissociation Rate Constants at pH 7.0 of Wild-Type and Metal Ligand CAII Variants

CAII	zinc K_D (nM)^a	zinc k_{off} (min^{-1})^b	$t_{1/2}$ (min)
wild-type	0.004 ± 0.001^c	(8.5 ± 0.5) × 10^{-5}	8200
His94Ala	270 ± 50	≥2.3^d	≥0.3^d
His94Cys	33 ± 7	0.0088 ± 0.0004	79
His94Asp	15 ± 5	0.011 ± 0.0004	63
His94Glu	14 ± 5	0.49 ± 0.24	1.4
His96Ala	100^e		
His96Cys	60 ± 20		
His119Ala	≤1000^f		
His119Cys	48 ± 10	0.09 ± 0.03	7.7
His119Asp	25 ± 7	0.17 ± 0.04	4.1

[a] K_D calculated using eq 2 as described in the legend of Figure 2. [b] k_{off} calculated using eq 3 as described in the legend of Figure 3. [c] Taken from Kiefer *et al.* (1993b). [d] Less than 0.01 mol of zinc/mol of enzyme bound after a 10-s incubation and subsequent PD-10 chromatography. [e] 2.0 mol of zinc per mol of enzyme bound with $K_{D1} = K_{D2}$ = 100 nM calculated using eq 2. [f] 2.7 mol of zinc per mol of enzyme bound at 1.0 μM zinc sulfate.

Since the CAII variants with three potential zinc ligands bind zinc more tightly (4.5–19-fold) than His94Ala which has only two potential zinc ligands, it can be inferred that all of the engineered metal binding sites coordinate zinc with three protein ligands. Indeed, this has been substantiated for the majority of these CAII variants (with the exception of His96Cys and, possibly, His119Asp) through observation of a Co^{2+}→sulfur charge transfer band and tetrahedral geometry in the optical absorption spectrum of Co^{2+}-substituted CAII (His94Cys) (Alexander *et al.*, 1993) and/or X-ray crystallography (Xue, 1992; Kiefer *et al.*, 1993a; Ippolito & Christianson, 1994).

To further investigate the properties of these novel zinc sites, a zinc dissociation rate constant (k_{off}) was measured for wild-type and the CAII variants by the time-dependent disappearance of the E–Zn complex after dilution into EDTA to trap the dissociated zinc (Figure 3 and Table I). Unlike dipicolinic acid which efficiently catalyzes the dissociation of zinc from wild-type CAII (Hunt *et al.*, 1977), EDTA is believed to act only by lowering the free metal ion concentration (Lindskog, 1982). The half-time for the dissociation of zinc from wild-type CAII at 25 °C and pH 7 ($t_{1/2}$ = 5.7 days) is slightly shorter than that measured previously for bovine CAII at 0 °C and pH 5 ($t_{1/2}$ ≈ 8 days) (Kindani & Hirose, 1977). As might have been predicted, the weakened zinc affinity in the CAII metal variants drastically increases the rate constant for zinc dissociation (k_{off}) relative to wild-type CAII; substitution of His94 with Cys or Asp had the smallest effect (≈100-fold faster) while the remaining substitutions had a larger effect (≈2500-fold) (Table 1). Deletion of the His94 side chain by replacement

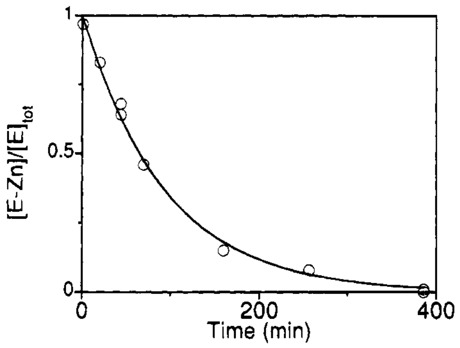

FIGURE 3: Measurement of a zinc dissociation rate constant for His94Asp CAII at pH 7. Zinc dissociation was initiated by diluting the enzyme into 10 mM Tris sulfate buffer, pH 7, containing EDTA (final concentration 40 μM His94Asp CAII, 35 mM EDTA). At various times, enzyme-bound zinc (E−Zn) was separated from EDTA and free zinc (Zn_{free}) by chromatography on PD-10 columns and quantitated using the PAR assay (Hunt *et al.*, 1984). The data were fit to eq 3 yielding a $k_{off} = (1.1 \pm 0.04) \times 10^{-2}$ min^{-1}.

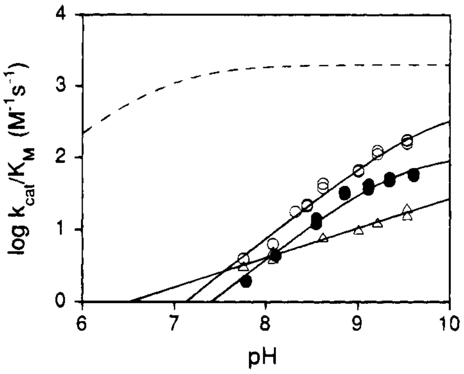

FIGURE 4: pH dependence of k_{cat}/K_M for the PNPA esterase activity of His94Asp (○), His94Cys (●), and His94Ala (△) CAII measured at 25 °C in either 50 mM CHES (pH 8.7−9.6), 50 mM TAPS (pH 7.8−8.4), or 50 mM MOPS (pH 6.8−7.5), μ = 0.1 M with sodium sulfate. The data for His94Asp and His94Cys were fit to eq 1 yielding the values listed in Table 2, and the data for H94A were fit to a line with a slope of 0.4 ± 0.02. The dotted line shows the pH dependence of k_{cat}/K_M for wild-type CAII (Fierke *et al.*, 1991).

Table 2: Steady-State Kinetic Parameters for PNPA Hydrolysis and Dissociation Constant for Acetazolamide of CAII Variants

CAII	k_{cat}/K_M[a] (M^{-1} s^{-1})	pK_a[a]	$K_{D\ Ac}$ (μM)[b]
wild-type	2500 ± 200[c]	6.8 ± 0.1[c]	$(6.1 \pm 0.4) \times 10^{-3}$[c] (5.7×10^{-3})[d]
His94Ala	18[e]	[f]	
His94Cys	117 ± 20	≥9.5	55 ± 8 (1.7)
His94Asp	365 ± 70	≥9.6	207 ± 50 (5.1)
His94Glu	≤2[g]		559 ± 74
His96Ala[h]	507 ± 90	8.4 ± 0.1	
His96Cys	90 ± 15	8.5 ± 0.1	
His119Cys	8 ± 1.2[i]	[j]	141 ± 30
His119Asp	830 ± 170	8.6 ± 0.2	2.4 ± 0.5 (0.5)

[a] pK_a and pH-independent k_{cat}/K_M calculated using eq 1 as described in the legend of Figure 4. [b] $K_{D\ Ac}$ measured in 50 mM TAPS, pH 8.0, μ = 0.1 M with sodium sulfate, as described in the legend of Figure 5, and calculated using eq 4. [c] Taken from Fierke *et al.* (1991). [d] The binding constant corrected for the pK_a of the zinc−water by $K_{D\ corr} = K_{D\ obs}/(1 + 10^{(pK_a - pH)})$ is shown in parentheses. [e] The value for k_{cat}/K_M at pH 9.5. [f] The pK_a could not be calculated as the data fit best to a line with a slope equal to 0.4. [g] PNPA hydrolysis was increased ≤10% over the background rate at 20 μM enzyme. [h] Binds more than 1 mol of zinc/mol of enzyme under assay conditions. [i] The value for k_{cat}/K_M at pH 8.5. [j] Too little activity to measure the pK_a.

with Ala yields a k_{off} that is increased by more than 3 × 10^4-fold relative to wild-type CAII. These data suggest that exchange of the protein metal ligands is at least partially rate-limiting in zinc dissociation; the weakened zinc affinity in the CAII variants facilitates exchange of the protein metal ligands with solvent, resulting in an increased rate constant for zinc dissociation. Indeed, exchange of the metal ligands is also suggested to be rate-imiting for zinc dissociation catalyzed by dipicolinate (Pocker & Fong, 1983).

The rate constant for zinc association (k_{on}) with wild-type CAII can be calculated from the zinc K_D and k_{off}, assuming a single association step, where $k_{on} = k_{off}/K_D = 3.5 \times 10^5$ M^{-1} s^{-1}; a slightly smaller value for k_{on} ($\approx 10^4$ M^{-1} s^{-1}) has been measured directly for bovine CAII (Henkens & Sturtevant, 1968). Similarly, the zinc association rate constants for the CAII variants can be calculated and are not significantly altered from that of wild-type CAII except for His94Cys, His94Asp, and His119Cys CAII, where k_{on} decreases 10−100-fold. The second-order rate constants for association of zinc with small ligands are slower than diffusion-controlled at 10^6−10^8 M^{-1} s^{-1}, implicating a two-step binding mechanism in which dissociation of metal-bound inner-sphere water molecules may be the rate-limiting step [reviewed by Eigen (1962), Eigen and Hammes (1963), and Eigen and Wilkens (1965)]. For CAII, the rate constant for zinc association may also be limited by desolvation or, perhaps, by a protein conformational change.

PNPA Hydrolysis and pK$_a$ of Zinc-Bound Solvent. To determine the effects of variation of the zinc ligands on ester hydrolysis and the pK_a of the zinc-bound solvent, the pH dependence of the second-order rate constant for CAII-catalyzed PNPA hydrolysis was measured for each of the CAII variants (Figure 4 and Table 2). Replacement of any of the three histidine ligands with an amino acid capable of zinc coordination decreases the pH-independent second-order rate constant; however, the magnitude of the decrease is variable (3−30-fold) and does not correlate with the position of the substituted amino acid.

Experimental evidence indicates that the pH dependence of the esterase activity for wild-type CAII directly reflects the ionization of the zinc-water moiety [reviewed by Silverman and Lindskog (1988)]. The pH dependence of PNPA hydrolysis for all of the variants, except His94Ala, is consistent with the ionization of a single enzymic group with maximal activity at high pH (Figure 4). The observed pK_a values are listed in Table 2. Replacement of any of the three neutral histidine ligands with a negatively charged Cys or Asp increases the pK_a of zinc-bound water by ≥1.6 pH units, with the greatest increases (≥2.7 pH units) occurring upon substitution at position 94.

In His94Ala CAII, the pH dependence of log (k_{cat}/K_M) for PNPA hydrolysis defines a line with a slope = 0.4 (Figure 4) suggesting a change in the catalytic mechanism of ester hydrolysis in this variant; perhaps zinc-hydroxide is no longer acting as a nucleophile. Additionally, the high k_{cat}/K_M for His96Ala CAII relative to variants with three zinc ligands and the fact that this enzyme binds 2.0 moles of zinc under the assay conditions suggest the possibility of a novel catalytic mechanism.

CO_2 Hydration. The steady-state kinetic parameters for CO_2 hydration catalyzed by the zinc ligand CAII variants were measured at pH 8.9 (Table 3). The pH-independent values for k_{cat}/K_M are also listed in Table 3 [calculated using the zinc−water pK_a determined from the pH dependence of PNPA hydrolysis (Table 2)]. Substitution of any one of the

Table 3. Steady-State Kinetic Parameters of CAII Variants for CO_2 Hydration at pH 8.9

CAII	k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)	k_{cat} ($\times 10^{-4}$) (s^{-1})	K_M (mM)
wild-type	110 ± 10^a $(110)^b$	100 ± 10^a	8.2 ± 1
His94Ala	0.012 ± 0.002	$\leq 0.12^c$	$\geq 100^c$
His94Cys	0.11 ± 0.01 (0.55)	1.2 ± 0.4	112 ± 45
His94Asp	0.11 ± 0.01 (0.66)	0.8 ± 0.38	75 ± 44
His94Glu	$\leq 0.01^d$		
His96Ala	$\leq 0.01^d$		
His96Cys	0.073 ± 0.002 (0.10)	$\leq 0.73^c$	$\geq 100^c$
His119Ala	0.12 ± 0.01	$\leq 1.2^c$	$\geq 100^c$
His119Cys	0.11 ± 0.01 (0.11)	1.1^c	$\geq 100^c$
His119Asp	3.8 ± 0.3 (5.7)	$\leq 38^c$	$\geq 100^c$

[a] Taken from Kiefer *et al.* (1993b). [b] The pH independent values for k_{cat}/K_M calculated using the zinc−water pK_a values in Table 2 are shown in parentheses. [c] No curvature was observed in a plot of rate versus $[CO_2]$ up to 24 mM. [d] CO_2 hydration was increased ≤10% over the background rate at 10 μM enzyme and 24 mM CO_2.

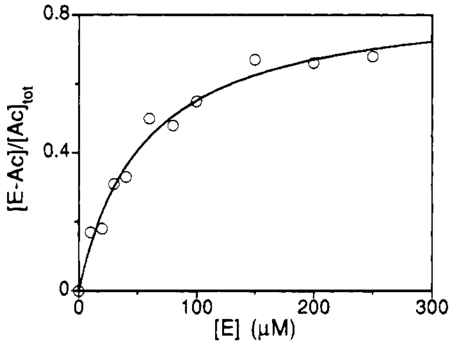

FIGURE 5: Measurement of the acetazolamide dissociation constant ($K_{D\ Ac}$) for His94Cys CAII at pH 8.0. His94Cys CAII (0−250 μM) was incubated with 1.0 μM [^{3}H]acetazolamide (^{3}H-Ac) in 50 mM TAPS, pH 8.0, $\mu = 0.1$ M with sodium sulfate, for 10 min at 25 °C. Enzyme-bound ^{3}H-Ac (E-Ac) was separated from free ^{3}H-Ac by chromatography on Sephadex G-25 centrifuge columns and quantitated by scintillation counting of the column eluant. The dissociation constant was determined from fitting this data to eq 4, yielding a $K_{D\ Ac} = 55 \pm 8$ μM.

three His ligands with Cys, Asp, or Glu results in a decrease in the pH-independent k_{cat}/K_M of 20−10000-fold and k_{cat} of 3−1000-fold with His94Glu and His119Asp CAII being the least and most active, respectively. Removal of a ligand decreases k_{cat}/K_M 10^3−10^4-fold and k_{cat} 80−1000-fold. The values for K_M have increased drastically (≈9−20-fold) for all of the variants, so much so that in some cases no curvature in a plot of rate versus CO_2 concentration was observed. The high errors in k_{cat} and K_M occur because the highest concentration of CO_2 achievable under the assay conditions was at least 3-fold lower than the K_M.

Sulfonamide Binding. To probe the effects of varying the structure of the zinc ligands on the transition state structure, a dissociation constant for acetzolamide, a transition state analog, was measured at pH 8 for selected CAII variants (Figure 5 and Table 2). Replacement of the His ligand at position 94 and 119 with Cys, Asp, or Glu results in large decreases in affinity for acetazolamide (4×10^2−9×10^4-fold). The 1.9-Å resolution crystal structure of wild-type CAII complexed with acetazolamide reveals that the sulfonamide group coordinates to the zinc ion replacing the zinc-bound solvent (Vidgren *et al.*, 1990). Sulfonamide binding to wild-type CAII is pH-independent from 7 to 9 with decreased binding at both higher and lower pH. This pH dependence is consistent with the formation of the bound complex by ionized inhibitor binding to the zinc−water form of CAII or the equivalent reaction between the unionized inhibitor and the zinc−hydroxide form of the enzyme (Kernohan, 1966; Lindskog & Thorslund, 1968). The increased pK_a of the zinc−water for the variants relative to wild-type CAII should alter the pH dependence of the acetazolamide K_D ($K_{D\ Ac}$); therefore, the values listed in parentheses in Table 2 have been corrected for these varied pK_as.

DISCUSSION

CAII variants, with an amino acid substitution in one of the three conserved His zinc ligands, were constructed and the metal binding and catalytic parameters were characterized in order to ascertain the functional consequences of alteration of the metal site. These data are interpreted in light of the structural effects of these substitutions determined by X-ray crystallography (Xue, 1992; Kiefer *et al.*, 1993a; Ippolito & Christianson, 1994). These correlations between structure and function are essential for determining rules for the engineering of catalytically active transition metal sites in proteins.

Metal Binding. Deletion of the side chain of His94 diminishes zinc affinity significantly, incurring a net free energy cost of 6.6 kcal/mol,[2] but *not completely*. In fact, this His_2 metal binding site actually binds zinc 10^2−10^3-fold more tightly than similar designed sites (Higaki *et al.*, 1990; Arnold & Haymore, 1991).

In wild-type CAII, His94 and His96 coordinate zinc through the Nϵ atom, as do the majority of His ligands found in zinc protein structures (Chakrabarti, 1990), while His119 coordinates zinc through the Nδ atom. Therefore, we predicted that replacement of His94/96 and His119 with amino acids with the same distance between C_α and the atom capable of coordinating zinc, i.e., Glu and Asp, respectively, would perturb zinc binding the least. However, this was not observed; zinc affinity in the variants with one His zinc ligand substituted by Glu, Asp or Cys (His_2X metal polyhedron) was not greatly dependent on either the side chain structure or position. The energetic advantage of insertion of an amino acid that could ligand with zinc (compared to the respective Ala variant) is modest, ≈1.5 kcal/mol, and less favorable than interaction with histidine by ≈5 kcal/mol. Nevertheless, all of these His_2X variants bind zinc more tightly than similar *de novo* designed metal polyhedra ($K_D \approx 1.0$−100 μM at pH 7;[3] Iverson *et al.*, 1990; Pessi *et al.*, 1993; Wade *et al.*, 1993; Stewart *et al.*, 1994; Handel *et al.*, 1993).

Despite the decreased affinity, the crystal structures of these variants demonstrate that the engineered zinc ligands in His94Cys and His94Asp CAII coordinate the metal ion with optimal stereochemistry, although significant protein structural rearrangements accompany these interactions (Kiefer *et al.*, 1993a; Ippolito & Christianson, 1994). In contrast, the varied ligand in His94Glu (Xue, 1992), His119Cys, and His119Asp (Ippolito & Christianson, 1994) CAII interacts with the metal suboptimally. Since the stability constants for various small molecule− and enzyme−zinc complexes containing nitrogen and sulfur or oxygen

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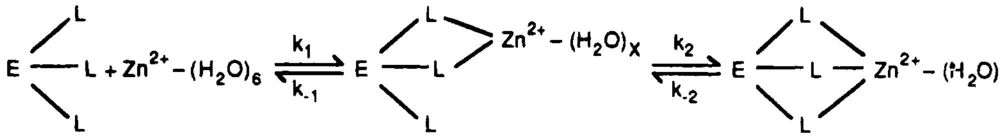
[2] $\Delta\Delta G = -2.303RT \log (K^{\text{variant}}/K^{\text{wild-type}})$

[3] The K_D values determined at pH 6 were divided by 10 to roughly correct for pH effects.

Scheme 1



ligands (Sillen & Martell, 1964; Coleman & Vallee, 1961) indicate that the decrease in binding energy is not due to the replacement of a nitrogen ligand with a sulfur or oxygen ligand, the decreased zinc avidity in the variants at position 94 must be dictated mainly by the energetically unfavorable protein structural arrangements necessary for metal–ligand coordination (Kiefer *et al.*, 1993a; Ippolito & Christianson, 1994).

Similar results were obtained with the CAII variant Thr199Cys, which forms a novel His$_3$Cys zinc polyhedron (Kiefer *et al.*, 1993b); in this case, movement of the loop flanking Cys199 allows the thiolate side chain to coordinate the metal ion, displacing the metal-bound solvent molecule (Ippolito & Christianson, 1993). However, the observed increase in zinc affinity is small, reflecting the energetic balance between the formation of the zinc–thiolate bond and unfavorable structural rearrangements (4–5 kcal/mol). Likewise, in His96Cys CAII thiolate coordination is not observed (Ippolito & Christianson, 1994) because the energetic cost of the necessary β-sheet rearrangement is larger than the energetic gain of thiolate coordination. Indeed, the zinc K_D for His96Cys is less than 2-fold lower than that for His96Ala CAII. In all cases, there appears to be a trade-off between conformational changes and metal coordination.

Finally, structural changes in the apoenzyme forms of all of the CAII variants may also contribute to decreased protein–zinc affinity. For example, the X-ray structure of apo-His94Cys CAII reveals no hydrogen bond contact between Cys94 and Gln92, as is observed in wild-type CAII (Alexander *et al.*, 1993; Håkansson *et al.*, 1992); instead, Cys94 hydrogen bonds with His96 and His119. The hydrogen bonds between the protein and the zinc ligands are believed to orient the ligands for optimal metal coordination, thus lowering the entropic barrier to metal binding (Argos *et al.*, 1978), and CAII variants lacking one of these hydrogen bonds bind zinc 3–10-fold less tightly than wild-type (L. L. Kiefer, S. A. Paterno, and C. A. Fierke, unpublished data). Therefore, the increased entropic cost of orienting the protein ligands upon metal binding in His94Cys CAII could also decrease the observed metal affinity.

Association rate constants slower than the diffusion-control limit for the binding of zinc to CAII or to some small molecule ligands implicate a two-step mechanism for binding (Eigen, 1962; Eigen & Hammes, 1963); one possible mechanism is depicted in Scheme 1. In this model, zinc is coordinated to two protein ligands in an initial complex prior to formation of the tetrahedral complex containing three protein ligands. Sinc His94Ala CAII can only undergo the first half of the reaction, a value for $k_{-1} \geq 2.3$ min^{-1} can be estimated from the rate constant for dissociation of zinc from this variant. Additionally, a value for k_1 approaching the diffusion-control limit (2.5×10^7 M^{-1} s^{-1}) can be approximated from the rate constant for zinc association with a CAII variant in which the hydrogen bond between Glu117 and His119 is altered (L. L. Kiefer, S. A. Paterno, and C. A. Fierke, unpublished result). Given these assumptions, the measured values of k_{off} and K_D (Table 1) suggest that the rate-limiting step for zinc association and dissociation is ligand exchange between a solvent molecule and the third protein ligand ($k_{\text{on}} = k_1k_2/k_{-1}$ and $k_{\text{off}} = k_2$). Indeed, the smallest increases in k_{off} and the largest decreases in k_{on} were observed for the two variants, His94Cys and His94Asp CAII, which have the most favorable metal coordination and the most extensive structural rearrangements (Kiefer *et al.*, 1993a; Ippolito & Christianson, 1994).

Ground State and Transition State Stabilization. The large increases in the pK_a of the zinc-bound water in CAII variants with a negatively charged amino acid (Asp, Glu, or Cys) substituted for one of the histidine ligands clearly demonstrate that neutral ligands are important for maintaining the low zinc–water pK_a due to electrostatic stabilization of the ground state zinc–hydroxide relative to zinc–water (Christianson & Alexander, 1989). In fact, the increases in pK_a correlate with increased negative charge near the zinc ion, as observed in the crystal structures (Kiefer *et al.*, 1993a; Ippolito & Christianson, 1994). For the variants with the highest pK_a, the thiolate–zinc distance is only 2.3 Å (His94Cys) or the carboxylate–zinc interaction is *syn*-oriented (His94Asp). Furthermore, a smaller increase in pK_a is observed for the His96Cys CAII variant where an uncharged water molecule displaces the cysteine ligand to form a His$_2$(H$_2$O)$_2$ metal polyhedron. These results are consistent with the observation that enzymes containing a metal polyhedron with one or more negatively charged ligands, such as thermolysin, carboxypeptidase A, and adenosine deaminase (Matthews, 1988; Christianson & Lipscomb, 1989; Wilson *et al.*, 1991), catalyze hydrolysis by a general base activated zinc-bound water rather than nucleophilic attack by a stabilized zinc–hydroxide.

Similarly, substitution of the His ligand at position 94 or 119 decreases the binding energy for the CAII inhibitor, acetazolamide, by 2.6–4.0 kcal/mol. Crystallographic studies of the acetazolamide–CAII complex reveal that this inhibitor mimics the catalytic transition state; the sulfonamido anion displaces the zinc–solvent to form an inner sphere ligand and hydrogen bonds form between the amide proton and the Oγ atom of Thr199 and between a sulfonamide oxygen and the main chain amide hydrogen of Thr199 (Vidgren *et al.*, 1990). The linear correlation between log ($K_{D\ \text{Ac}}$) and pK_a ($R = 0.99$, slope = 1.5, plot not shown) for the metal ligand variants indicates that factors which stabilize zinc–hydroxide relative to zinc–water stabilize the zinc-bound sulfonamide anion even more strongly. This implicates interactions between CAII variants and the sulfonamide moiety, especially decreased electrostatic interactions between the sulfonamide anion and the zinc, as the major factors in the decreased acetazolamide binding.

Catalysis. There is considerable evidence that hydration of CO$_2$ by wild-type CAII consists of two main steps as shown in eqs 5 and 6 (Silverman & Lindskog, 1988; Tu *et al.*, 1989): (1) nucleophilic attack of zinc-bound hydroxide

$$\mathrm{E{-}OH^- + CO_2} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \mathrm{E{-}HCO_3^- + H_2O} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} \mathrm{E{-}OH_2 + HCO_3^-} \quad (5)$$

$$\mathrm{E{-}OH_2} \underset{k_{-3}}{\overset{k_3}{\leftrightarrow}} \mathrm{{}^{+}H{-}E{-}OH^- + B} \underset{k_{-4}}{\overset{k_4}{\leftrightarrow}} \mathrm{E{-}OH^- + BH} \quad (6)$$

on CO_2 to form enzyme-bound HCO_3^- followed by product dissociation resulting in the zinc−H_2O form of the enzyme; and (2) proton transfer to solvent via a proton shuttle (His64) to regenerate the zinc−OH^- species. For wild-type CAII, the second−order rate constant for CO_2 hydration measures a combination of the CO_2 association and hydration steps ($k_{cat}/K_M \approx k_1$). At high CO_2 and buffer concentrations, intramolecular proton transfer between zinc−water and His64 becomes the rate-limiting step ($k_{cat} \approx k_3$). Therefore, K_M reflects this change in rate-limiting steps ($K_M \approx k_3/k_1$), not CO_2 affinity. A dissociation constant of 0.1 M for CO_2 binding to wild-type CAII has been estimated using infrared spectroscopy (Krebs *et al.*, 1993b; Riepe & Wang, 1968). In light of this, the substantial increases in K_M for the zinc ligand CAII variants are interpretable; the large decreases in k_{cat}/K_M require a higher concentration of CO_2 to affect the change in rate-limiting steps. Alternatively, for these CAII variants the K_M may actually reflect CO_2 dissociation ($K_M \approx k_{-1}/k_1$).

Alteration of the metal polyhedron in CAII decreases k_{cat}/K_M for both the esterase and hydrase activities. Removal of any one of the CAII His ligands by substitution with Ala causes large decreases in k_{cat}/K_M for CO_2 hydration ($>10^3$-fold), similar to the decreased activity of adenosine or cytosine deaminase variants with one of the zinc ligands replaced with Ala (Smith *et al.*, 1994; Bhaumik *et al.*, 1993). However, the second-order rate constant for CO_2 hydration and ester hydrolysis catalyzed by the CAII variants with three potential metal ligands (except His94Glu) are still 10^3-fold and 10^2-fold faster, respectively, than comparable second-order rate constants catalyzed by small molecule zinc and cobalt complexes (Kimura *et al.*, 1990; Woolley, 1975; Caffee *et al.*, 1973). Therefore, these metal ligand variants retain many of the features of the wild-type enzyme required for transition state stabilization, demonstrating that a His_3 metal polyhedron is not essential for catalysis of CO_2 hydration. In fact, amino acid sequence comparisons in the homologous bacterial and plant carbonic anhydrases indicate the absence of three conserved histidines, suggesting a novel zinc polyhedron in this class of enzymes (Guilloton *et al.*, 1992).

A linear correlation between log $(k_{cat}/K_M)^{\mathrm{hydrase}}$ and log $(1/K_{D\ \mathrm{Ac}})$ ($R = 0.95$, slope $= 0.65$) is consistent with identification of acetazolamide as a transition state analog (Wolfenden, 1969). In addition, the rough inverse proportionality between log (k_{cat}/K_M) for CO_2 hydration and the zinc−water pK_a for both the CAII variants with three protein zinc ligands indicates that the interactions which destabilize zinc-hydroxide relative to zinc−water similarly destabilize the transition state for CO_2 hydration relative to zinc−hydroxide. These correlations suggest that the dominant factor destabilizing zinc−hydroxide, sulfonamide binding, and the negatively charged transition state for CO_2 hydration is the increased electron density on the zinc ion as a result of replacement of a neutral ligand with a negatively charged ligand in these variants. Furthermore, the crystallographic data of the metal polyhydron of these variants support this conclusion (Ippolito & Christianson, 1994). In the most active variant, His119Asp CAII, the zinc ion experiences the least negatively charged environment as the carboxylate−zinc interaction is long (2.8 Å) and occurs with *anti* stereochemistry (Ippolito & Christianson, 1994) while the activity is decreased in variants with optimal metal−ligand distance and stereochemistry (i.e., His94Cys and His94Asp CAII).

Implications for the Rational Design of Metal Binding Sites. The functional and structural characterization of CAII variants with altered metal binding sites has yielded insight into structure−function relationships potentially useful for the design of metal binding proteins capable of zinc−hydroxide catalyzed hydration and hydrolysis. First, this work demonstrates that the decreased zinc affinity of altered metal sites in CAII reflects a combination of energetically unfavorable protein conformational changes and suboptimal metal coordination geometry. This suggests that the modest zinc affinity of designed metal sites with three protein ligands in antibodies and α-helical bundles (Iverson *et al.*, 1990; Pessi *et al.*, 1993; Wade *et al.*, 1993; Stewart *et al.*, 1994; Handel *et al.*, 1993) reflects suboptimal positioning of the protein ligands for metal coordination. In fact, these designed metal sites bind zinc at least 10-fold more weakly than His94Ala CAII, which contains only two protein ligands.

Second, this work indicates that the neutral charge on the His ligands plays a large role in increasing the electrostatic stabilization of both the zinc-bound hydroxide and the negatively charged transition state for CO_2 hydration. This stabilization is important for maintaining the low pK_a of the zinc−water molecule and for lowering the activation energy of CO_2 hydration which are both essential for maximal activity at neutral pH. In zinc proteases that contain a metal polyhedron with a negatively charged ligand (His_2Glu), such as thermolysin and carboxypeptidase A (Matthews, 1988; Christianson & Lipscomb, 1989), the loss of electrostatic stabilization is circumvented by insertion of a general base to activate the zinc−water for nucleophilic attack and a positively charged electrophilic residue to stabilize the negatively charged transition state.

In conclusion, elucidation of the relationships between structure and function in well-characterized paradigms, such as CAII, is a step toward conferring new catalytic functions on CAII, as well as facilitating the *de novo* design of metalloproteins capable of zinc−hydroxide catalyzed hydration and hydrolysis. Our current focus is the (re)design of CAII variants with more finely tuned functional properties using information gleaned from this work to target positions for a second round of protein structural changes.

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