Engineering a Cysteine Ligand into the Zinc Binding Site of Human Carbonic Anhydrase II†

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ABSTRACT: Substitution of cysteine for threonine-199, the amino acid which hydrogen bonds with zinc-bound hydroxide in wild-type carbonic anhydrase II (CAII), leads to the formation of a new His_3Cys zinc coordination polyhedron. The optical absorption spectrum of the Co^{2+}-substituted threonine-199→cysteine (T199C) variant and the three-dimensional structure [Ippolito, J. A., & Christianson, D. W. (1993) *Biochemistry* (following paper in this issue)] indicate that the new thiolate side chain coordinates to the metal ion, displacing the metal-bound solvent molecule. The engineered thiolate ligand *increases* zinc binding (4-fold) and decreases catalytic activity substantially ($\approx 10^3$-fold) but not completely. However, this residual activity is due to an active species containing a zinc-bound solvent ligand with the cysteine-199 side chain occupying an alternate conformation. The equilibrium between these conformers reflects the energetic balance between the formation of the zinc–thiolate bond and structural rearrangements in the Ser-197→Cys-206 loop necessary to achieve this metal coordination. This designed His_3Cys metal polyhedron may mimic the zinc binding site in the matrix metalloproteinase prostromelysin.

Zinc coordination polyhedra in proteins exhibit variations in ligand number, charge, structure, and amino acid composition depending upon the role of the metal ion (Christianson, 1991; Vallee & Auld, 1990). Utilizing these natural protein–zinc complexes as paradigms, transition metal sites have been designed *de novo* into a variety of protein scaffoldings including antibodies (Iverson et al., 1990; Roberts et al., 1990; Pessi et al., 1993; Wade et al., 1993), α-helical bundles (Handel & DeGrado, 1990; Regan & Clarke, 1990), trypsin (Higaki et al., 1990; McGrath et al., 1993), and thioredoxin (Hellinga et al., 1991). Protein engineering experiments on naturally occurring metal sites, which have been optimized by evolution, will provide further insights into the molecular architecture of these sites and lead to the rational design of zinc sites with high avidity and catalytic activity. Currently, we have augmented the zinc polyhedron of human carbonic anhydrase II by introduction of a fourth protein ligand.

Carbonic anhydrase II (CAII,[1] EC 4.2.1.1) is a zinc metalloenzyme that catalyzes the reversible hydration of CO_2 to HCO_3^- and a proton with a second-order rate constant approaching the diffusion-control limit (Silverman & Lindskog, 1988). The three-dimensional structure of this enzyme has been determined and refined at 1.54-Å resolution (Håkansson et al., 1992). The zinc cofactor lies at the bottom of the active site cleft where it is tetrahedrally coordinated to His-94, His-96, His-119, and a hydroxide ion at physiological pH. Thr-199 accepts a hydrogen bond from the zinc-bound hydroxide ion and donates a hydrogen bond to Glu-106. This zinc coordination polyhedron in CAII, conserved among the family of carbonic anhydrases (Hewett-Emmett & Tashian, 1991), is optimized to provide catalytically active zinc hydroxide at neutral pH. CAII-catalyzed hydration proceeds through direct nucleophilic attack of the zinc-bound hydroxide ion on the carbonyl carbon of CO_2 to form a zinc-bound bicarbonate intermediate followed by product dissociation, resulting in the zinc–H_2O form. Finally, the zinc hydroxide species is regenerated by proton transfer to solvent using the active site residue His-64 as a proton shuttle (Silverman & Lindskog, 1988; Tu et al., 1989). Recent structural–genetic analyses of CAII have delineated the functional roles of a conserved hydrophobic face in substrate binding and stability (Nair et al., 1991; Fierke et al., 1991; Krebs et al., 1993; Krebs & Fierke, 1993) and also illuminated the structural mobility of these regions (Alexander et al., 1991; Nair & Christianson, 1993).

We have begun characterizing modified metal binding sites in the prototypical zinc metalloenzyme, CAII, including substitution of cysteine for histidine ligands (Alexander et al., 1993), in order to probe the structural and functional importance of the metal ligands and the residues which they engage in hydrogen bond interactions. In this work we have engineered a fourth protein ligand into the zinc coordination polyhedron in CAII by replacing Thr-199 with cysteine. Spectroscopy and X-ray crystallography (Ippolito & Christianson, 1993) confirm the formation of a novel zinc–thiolate bond, displacing the zinc hydroxide nucleophile, which is accompanied by structural rearrangements in the Ser-197→Cys-206 loop. This inactive His_3Cys zinc site is in equilibrium with a small amount of an alternative, active conformer which maintains a zinc hydroxide ligand. This engineered His_3Cys metal polyhedron may mimic structural features of the zinc sites in β-lactamase II from *Bacillus cereus* (Sutton et al., 1987) and in matrix metalloproteinase proenzymes (Salowe et al., 1992; Holz et al., 1992).

MATERIALS AND METHODS

Preparation of the T199C Variant. The T199C CAII variant was produced by replacing the wild-type Thr-199 codon (ACC) with the codon for cysteine (TGT) using oligonucle-

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[1] Abbreviations: CAII, human carbonic anhydrase II; T199C, threonine-199→cysteine; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid.

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otide-directed mutagenesis (Stannsens et al., 1989) of the cloned human CAII gene in pCAM (Krebs & Fierke, 1993). The complete gene was sequenced using the dideoxy method of Sanger et al. (1977). The plasmid encoding T199C CAII was transformed into the *Escherichia coli* strain BL21(DE3), and cells were grown in induction media to A_{600} = 1 (Nair et al., 1991). CAII was induced by the addition of 0.25 mM isopropyl β-D-thiogalactopyranoside and 1 mM zinc sulfate and incubated for 5 h at 30 °C. Cells were pelleted and lysed with a lysozyme/EDTA procedure (Cull & McHenry, 1990), and T199C CAII was purified from this crude extract by DEAE-Sephacel and S-Sepharose ion-exchange chromatography, as described (Alexander et al., 1993), to ≥95% purity as assayed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). The concentration of T199C CAII was calculated from absorbance using $\epsilon_{280} = 5.4 \times 10^4$ M^{-1} cm^{-1} determined for wild-type CAII (Tu & Silverman, 1982).

Zinc Dissociation Constant. Apo-wild-type CAII was prepared using Amicon diaflow filtration against dipicolinate, as described (Alexander et al., 1993). Apo-T199C was prepared by incubation with a 40-fold molar excess of the thiol reagent *p*-(hydroxymercuri)benzenesulfonic acid for 10 min at 25 °C in 20 mM Tris–sulfate, pH 7.5 (Giedroc et al., 1986), followed by diafiltration against dipicolinate. The free thiol of Cys-199 was regenerated after diafiltration by the addition of 1 mM dithiothreitol. Dipicolinate was removed from the apoenzymes by chromatography on a PD-10 column (Sephadex G-25M, 5 cm × 15 cm, Pharmacia). Dissociation constants for zinc were obtained by dialyzing apoenzyme for 18 h at 30 °C against a zinc/dipicolinate buffer (0–0.75 mM total zinc/1 mM dipicolinate) in 10 mM Tris–sulfate, pH 7, purged with nitrogen; removing unbound zinc on a PD-10 column; and measuring the bound zinc concentration using the dithizone assay (Malmström, 1953). The concentration of free zinc was calculated from the dipicolinate–zinc stability constant (Sillén & Martell, 1964). $[E]_{tot}$ was determined from the bound zinc concentration at 0.75 mM zinc. The dissociation constant and asymptotic standard error were calculated using the SYSTAT (Systat, Inc.) curve-fitting program with eq 1.

$$[Zn^{2+}]_{bound}/[E]_{tot} = 1/(1 + K_D/[Zn^{2+}]_{free}) \quad (1)$$

Spectroscopy. Cobalt-substituted enzymes were prepared by adding a 4-fold molar excess of cobalt sulfate to freshly prepared apoenzyme. Optical absorption spectra were collected on enzyme solutions (50–100 μM) in 10 mM buffer (MES, pH 5.5 and 6.5; Tris, pH 7.5 and 8.5; CHES, pH 9.5) at 25 °C, using a Shimadzu 265 spectrophotometer. Spectra of the Co^{2+} chromophore in Co^{2+}-substituted protein were obtained using a reference cuvette containing the same concentrations of Zn^{2+}-CAII and cobalt sulfate to subtract the contributions from protein absorbance and scattering.

Catalytic Activity. Initial rates of CO_2 hydration and HCO_3^- dehydration were measured in a KinTek stopped-flow apparatus at 25 °C by the changing pH-indicator method (Khalifah, 1971). Buffer/indicator pairs were TAPS/*m*-cresol purple (pH 9, 578 nm), MOPS/*p*-nitrophenol (pH 7, 400 nm), and MES/chlorophenol red (pH 6.1, 574 nm). The buffer concentration was 50 mM, I = 0.1 M with sodium sulfate, and contained 0.1 mM EDTA. The assay was initiated by dilution of CAII (10 nM–20 μM final concentration) into substrate (3–24 mM CO_2 or 10–100 mM HCO_3^-). Solvent isotope effects were measured using solutions prepared with 94% D_2O. The solution pD was determined by adding 0.4 to the pH meter reading (Glasoe & Long, 1960). The kinetic parameters and standard errors were determined using the software SYSTAT (Systat, Inc.) by fitting the data to eq 2.

$$\text{rate} = k_{cat}[E][S]/(K_M + [S]) \quad (2)$$

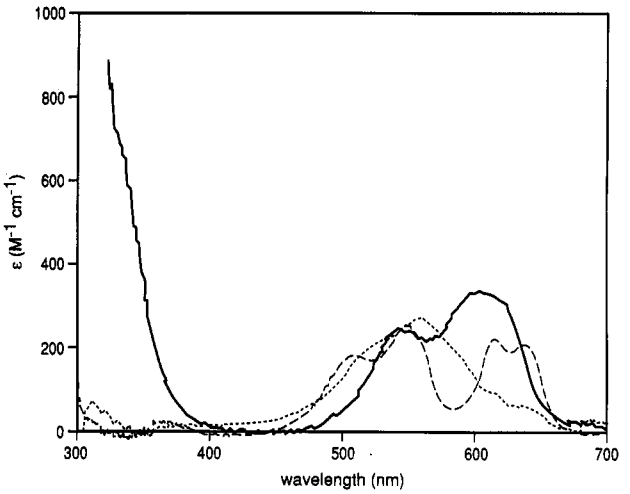


FIGURE 1: Optical absorption spectra of Co^{2+}-substituted CAII. The spectra of Co^{2+}-T199C in 10 mM Tris, pH 7.5 (—), and Co^{2+}-wild type in 10 mM MES, pH 5.5 (- - -), and in 10 mM CHES, pH 9.5 (– –), were collected at 25 °C using identical concentrations of Zn^{2+}–protein (50–100 μM) and cobalt sulfate (0.25–0.5 mM) in the reference cuvette. The molar absorptivities were calculated from the observed absorption and the molar concentration of CAII.

RESULTS AND DISCUSSION

Metal Binding. Substitution of Co^{2+} into the zinc binding site of CAII provides a useful spectroscopic probe of the composition and the geometric arrangement of ligands about the metal ion (Bertini & Luchinat, 1984; Vallee & Galdes, 1984). Figure 1 compares the optical absorption spectrum of Co^{2+}-substituted T199C CAII at pH 7.5 to the spectra of Co^{2+}-wild-type CAII at low and high pH. The pH dependence of the Co^{2+}-wild-type spectra reflects ionization of the metal-bound water (Lindskog, 1966). The shape of the spectrum of Co^{2+}-T199C CAII at pH 7.5 resembles the shorter wavelength pair of peaks in the high-pH spectrum of wild type, displaying broad ligand field absorption bands at 545 nm (ϵ = 289 M^{-1} cm^{-1}) and 605 nm (ϵ = 385 M^{-1} cm^{-1}); however, the longer wavelength peaks apparent in the high-pH spectrum of wild-type CAII were not observed. The absorption spectrum of Co^{2+}-T199C CAII most closely resembles the spectrum of Co^{2+}-wild-type CAII with hydrogen sulfide bound to the metal (Bertini et al., 1978). The intensity of the ligand field absorption bands in the spectrum of Co^{2+}-T199C CAII is consistent with four-coordinate geometry around the metal (Bertini & Luchinat, 1984; Vallee & Galdes, 1984), as observed in crystallographic studies of wild-type CAII at both high- and low-pH values (Håkansson et al., 1992; Nair & Christianson, 1991). Furthermore, there is no significant change in the Co^{2+}-T199C spectrum from pH 5.5 to 9.5 (data not shown), indicating that the majority of the enzyme population (≥85%) does not contain metal-bound water which ionizes under these conditions. In addition, a new shoulder is observed in the Co^{2+}-T199C spectrum at 330 nm (ϵ = 844 M^{-1} cm^{-1}), indicative of sulfur to Co^{2+} charge transfer and thiolate coordination (Bertini & Luchinat, 1984; Vallee & Galdes, 1984). The observed shift in the high-wavelength absorption envelope to longer wavelengths is also consistent with thiolate coordination, as indicated by the spectral effects of thiolate ligands in model compounds (Corwin

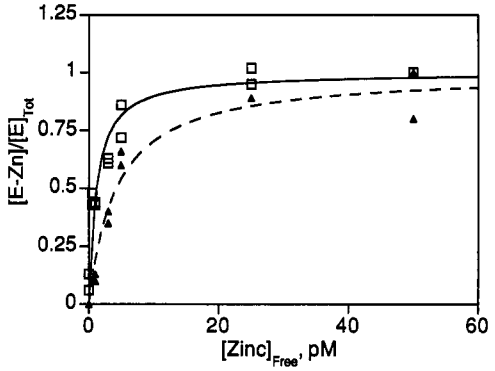


FIGURE 2: Measurement of a zinc dissociation constant for T199C and wild-type CAII at pH 7. Zinc dissociation constants were obtained by dialyzing 0.5 mL of 80 μM apo-T199C (□) or apo-wild-type CAII (▲) for 18 h at 30 °C against 1 L of zinc sulfate (0–0.75 mM) in 1 mM dipicolinate (10 mM Tris, pH 7) purged with nitrogen. Enzyme-bound zinc (E–Zn) was separated from free zinc (Zn) and dipicolinate by chromatography on PD-10 columns and then quantitated using a colorimetric dithizone assay (Malmström, 1953). The data were fit to eq 1 using the computer program SYSTAT (Systat, Inc.).

et al., 1987, 1988). Taken together, these data indicate that the geometry of the metal site in T199C is similar to that of the native enzyme (tetrahedral); however, the Cys-199 residue coordinates to the metal ion displacing the zinc–water ligand. This model of the metal coordination polyhedron of T199C is confirmed by the X-ray crystal structure of this variant (Ippolito & Christianson, 1993).

To determine the effect of an additional protein–zinc ligand on zinc binding affinity, we measured a zinc dissociation constant (K_D) by dialyzing apo-CAII against varying zinc concentrations and then determining the concentration of enzyme-bound zinc. The K_Ds for T199C and wild-type CAII at pH 7 of 1.1 ± 0.2 and 4 ± 1 pM, respectively, were calculated from a plot of $[E{\cdot}Zn^{2+}]/[E]_{tot}$ versus $[Zn^{2+}]_{free}$ as shown in Figure 2. Zinc affinity increases as the pH increases in wild-type and T199C CAII in a parallel manner (data not shown), likely due to ionization of a histidine ligand in the apoenzyme (Lindskog & Nyman, 1964; Pocker & Fong, 1983). As predicted, the T199C CAII variant has *increased* affinity for zinc although the magnitude of this effect (4-fold) is relatively modest. The X-ray structure of T199C CAII indicates that the conformation and stereochemistry of the engineered thiolate–zinc linkage are optimal (Ippolito & Christianson, 1993). However, energetically unfavorable rearrangements in the Ser-197→Cys-206 loop, necessary to achieve this metal coordination conformation, may mask the energetic gain of the thiolate–zinc interaction.

CO_2 Hydration. There is considerable evidence that hydration of CO_2 by wild-type CAII consists of two main steps as shown in eqs 3 and 4 (Silverman & Lindskog, 1988; Tu et al., 1989): (1) nucleophilic attack of zinc-bound hydroxide 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 (His-64) to regenerate the zinc–OH^- species. In wild-type CAII, hydration of CO_2 is the rate-limiting step at low substrate concentrations; however, proton transfer becomes rate limiting at high CO_2 concentrations, as indicated by a significant solvent isotope effect on k_{cat}.

Given this mechanism, replacing the zinc-bound solvent molecule with a cysteine ligand should completely inhibit the catalytic activity. In fact, k_{cat}/K_M for CO_2 hydration and

Table I: Catalytic Activity of CAII[a]

	wild-type	Thr-199→Cys
CO_2 hydration, MOPS buffer, pH 6.9		
k_{cat}/K_M ($\mu M\ s^{-1}$)	45 ± 2	0.037 ± 0.003
k_{cat} (ms^{-1})	600 ± 20	0.57 ± 0.07
K_M (mM)	13.4 ● 1.1	15.4 ● 3.6
CO_2 hydration, TAPS buffer, pH 9.0		
k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)	110 ± 10	0.11 ± 0.02
k_{cat} (ms^{-1})	1000 ● 100	1.1 ● 0.2
K_M (mM)	8.2 ± 1	8.2 ± 3.4
$(k_{cat}/K_M)^H/(k_{cat}/K_M)^D$ [b]	1.0 ± 0.2	1.0 ± 0.3
$(k_{cat})^H/(k_{cat})^D$ [b]	3.3 ± 0.2	3.5 ± 0.2
HCO_3^- dehydration, MES buffer, pH 6.1		
k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)	13 ± 1	0.014 ● 0.002
k_{cat} (ms^{-1})	560 ± 40	0.57 ± 0.08
K_M (mM)	45 ± 9	40 ± 12

[a] Activity measured as a function of substrate concentration in 50 mM buffer, 25 °C, 0.1 mM EDTA, I = 0.1 with sodium sulfate, using a pH-indictor assay (Khalifah, 1971). [b] k^D measured in 94% D_2O.

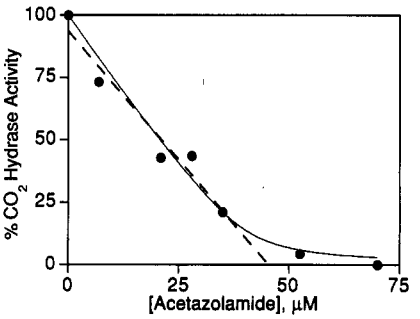


FIGURE 3: Acetazolamide inhibition of CO_2 hydrase activity of T199C CAII. Initial rates of CO_2 hydration were monitored using a pH indicator assay in TAPS buffer, pH 9 (Khalifah, 1971). Enzyme (35 μM) was preincubated with acetazolamide (up to 350 μM) in buffer and diluted 3.5-fold into dissolved CO_2 (final concentration = 24 mM). The data were fit either to a line (– –) or a quadratic equation (—) (Morrison, 1969), varying both K_I and $[E]_{tot}$.

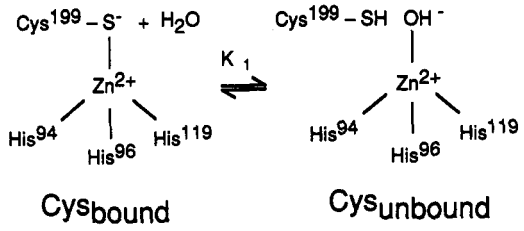
$$E\text{–}OH^- + CO_2 \leftrightarrow E\text{–}HCO_3^- + H_2O \leftrightarrow E\text{–}OH_2 + HCO_3^- \quad (3)$$

$$E\text{–}OH_2 \leftrightarrow {}^+H\text{–}E\text{–}OH^- + B \leftrightarrow E\text{–}OH^- + BH^+ \quad (4)$$

HCO_3^- dehydration catalyzed by T199C CAII (Table I) is decreased by a factor of 10^3 compared to wild-type CAII; however, some residual activity is observed with significant similarities to wild-type activity, including $(K_M)^{CO_2} \approx 15$ mM, $(K_M)^{HCO_3^-} \approx 42$ mM, solvent isotope effect on $(k_{cat})^{CO_2} \approx 3$, and catalysis of CO_2 hydration decreases 2–3-fold as the pH decreases from 9.0 to 6.9 (Table I). These data suggest that the active species of the T199C variant is functioning via a mechanism similar to that of the native enzyme.

To rule out the possibility that the residual activity is due to contamination by wild-type or oxidized Cys-199 CAII, we stoichiometrically titrated the CO_2 hydrase activity of T199C CAII with the active site inhibitor acetazolamide (Vidgren et al., 1990), using enzyme concentrations significantly higher than the observed K_I (Figure 3). Either a linear or quadratic fit (varying both $[E]_{tot}$ and K_I (Morrison, 1969)) of these data indicates that 44 ± 4 or 40 ± 5 μM acetazolamide, respectively, is required to completely inhibit 35 μM T199C CAII. This result indicates that the observed activity is a property of the entire enzyme population, assuming that the T199C variant binds acetazolamide at least 2-fold weaker than wild-type CAII [K_D = 0.01 μM (Fierke et al., 1991)]. This assumption is supported by (i) an estimation of $K_I \approx 0.5$ μM from the quadratic fit of CO_2 hydrase activity as a function of

Scheme I



acetazolamide concentration at 7 μM T199C CAII and (ii) a greater than 100-fold increase in the T199C K_D for a structurally similar inhibitor, dansylamide, compared to wild-type CAII (data not shown). Therefore, the observed catalytic activity is associated with the T199C variant of CAII.

These data are most consistent with a model (Scheme I) in which the majority of the T199C enzyme is inactive due to the cysteine thiolate displacing the zinc–solvent ligand ("Cys_{bound}"); however, this is in equilibrium ($K_1 = [Cys_{unbound}]/[Cys_{bound}]$) with an active species containing a zinc-bound hydroxyl ligand ("$Cys_{unbound}$"). If the catalytic properties of the $Cys_{unbound}$ conformer are similar to those of wild type, then equilibration between the two conformers must be slow relative to turnover to account for the similarity of the K_Ms and isotope effects between T199C and wild-type CAII. For example, if the two conformers rapidly interconvert, K_M for bicarbonate should increase significantly due to competition between binding bicarbonate and forming Cys_{bound}. A minimum value for K_1 of 0.001 can be inferred by the 10^3-fold decrease in k_{cat}/K_M for the Cys-199 variant, assuming that the specific activity of the $Cys_{unbound}$ species is not larger than that of wild type. A maximum value for K_1 of 0.25 can be set since the $Cys_{unbound}$ conformer is not readily detected in the X-ray structure of T199C CAII (Ippolito & Christianson, 1993). Additionally, the pH independence of the Co^{2+}-substituted T199C CAII spectra indicates that a zinc–water species does not accumulate (≤20% of E_{tot}) at pH 5.5, implying either that its pK_a has decreased significantly from the wild-type value of 6.8 (Silverman & Lindskog, 1988) or that $K_1 \leq 0.01$ (calculated from $[E\text{–}H_2O]/[E]_{tot} = 1/(1 + [(K_A/[H^+])(1 + 1/K_1)]) \leq 0.2$). This latter equilibrium constant is more likely since numberous substitutions at position 199 cause significant increases in the pK_a for zinc–water (Krebs, 1992; Liang et al., 1993). The equilibrium constant relating the $Cys_{unbound}$ and Cys_{bound} conformers is significantly larger than predicted from the stability constants of zinc–thiolate complexes (Sillén & Martell, 1964); however, this equilibrium also reflects the energetic balance between the formation of the zinc–thiolate bond and structural rearrangements. Possible explanations for this large equilibrium constant include that the zinc–solvent ligand is stabilized significantly compared to small molecule zinc complexes and/or that the conformational changes in the loop are very energetically unfavorable. Additional structural modifications in the T199C CAII variant which would stabilize the new conformation of the Ser-197→Cys-206 loop should both increase zinc binding and decrease residual catalytic activity.

The structure of the $Cys_{unbound}$ species can be modeled from the structure of T199C CAII in the presence of acetazolamide (Ippolito & Christianson, 1993), which reveals that the Cys-199 side chain rotates away from the zinc ion to accommodate coordination of the sulfonamide group with zinc. In this conformer the cysteine side chain does not form the hydrogen bond with the nonprotein zinc ligand observed with Thr-199 in the wild-type structure (Håkansson et al., 1992). Removal of this hydrogen bond by substitution of alanine or valine for threonine at position 199 causes 30–100-fold decreases in reactivity (Krebs, 1992; Liang et al., 1993), consistent with the estimated specific activity of the $Cys_{unbound}$ conformer. However, the observed catalysis may also arise from a small population of an additional $Cys_{unbound}$ conformer that hydrogen bonds with the zinc-bound hydroxide and has activity similar to that of wild type.

The mobility of the Ser-197→Cys-206 loop in CAII allows a cysteine substituted at position 199 to form a zinc–thiolate ligand and displace the catalytically essential zinc–solvent molecule. We are currently investigating whether similar rearrangements occur upon substitution at position 199 of other amino acids with high affinity for zinc, such as histidine, glutamic acid, and aspartic acid. Indeed, the plasticity of other metalloproteins, including thioredoxin (Hellinga et al., 1991) and ferredoxin (Martin et al., 1990), also allows rearrangement of the protein to optimize the metal coordination in structural variants. However, in naturally occurring zinc proteases, such as carboxypeptidase A (Christianson & Lipscomb, 1989) and thermolysin (Matthews, 1988), a catalytically important glutamate residue forms a stable hydrogen bond with the zinc–water ligand. In both cases, the glutamate residue is situated in a region of defined secondary structure with low thermal motion, as indicated by the crystallographic B factor (Rees et al., 1983; Holmes & Matthews, 1982). This immobility is clearly an essential design feature of catalytically active zinc polyhedra.

This designed His_3Cys zinc site in CAII may be related to postulated His_3Cys sites in β-lactamase II from *B. cereus* (Sutton et al., 1987) and in prostromelysin (Holz et al., 1992; Salowe et al., 1992). Indeed, the activation of prostromelysin to mature stromelysin is proposed to involve replacement of the thiolate ligand by a water molecule to form a catalytically active zinc site (Holz et al., 1992; Salowe et al., 1992; Van Wart & Birkedal-Hansen, 1990). This "cysteine switch" in prostromelysin is reminiscent of the equilibration between the Cys_{bound} and $Cys_{unbound}$ conformers in T199C CAII; however, in prostromelysin the active form is achieved by reaction of the thiol with organomercurials, oxidants, or other sulfhydryl reagents or by removal of the cysteine with proteolytic cleavage (Springman et al., 1990). In a similar fashion, it is possible that the activity of T199C CAII could be regulated by reversible oxidation of the cysteine at position 199 or by engineering the Ser-197→Cys-206 loop such that its mobility is pH dependent. As indicated by this work, engineering the CAII metal binding site will yield insight into the construction of novel metal binding sites designed to carry out specific functions.

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REFERENCES

Alexander, R. S., Nair, S. K., & Christianson, D. W. (1991) *Biochemistry 30*, 11064–11072.

Alexander, R. S., Kiefer, L. L., Fierke, C. A., & Christianson, D. W. (1993) *Biochemistry 32*, 1510–1518.

Bertini, I., & Luchinat, C. (1984) *Adv. Inorg. Biochem. 6*, 71–111.

Bertini, I., Canti, G., Luchinat, C., & Scozzafava, A. (1978) *J. Am. Chem. Soc. 100*, 4873–4877.

Christianson, D. W. (1991) *Adv. Protein Chem. 42*, 281–355.

Christianson, D. W., & Lipscomb, W. N. (1989) *Acc. Chem. Res. 22*, 62–69.

Corwin, D. T., Jr., Fikar, R., & Koch, S. A. (1987) *Inorg. Chem. 26*, 3079–3080.

Corwin, D. T., Jr., Gruff, E. S., & Koch, S. A. (1988) *Inorg. Chim. Acta 151*, 5–6.
Cull, M., & McHenry, C. S. (1990) *Methods Enzymol. 182*, 147–153.
Fierke, C. A., Calderone, T. L., & Krebs, J. F. (1991) *Biochemistry 30*, 11054–11063.
Giedroc, D. P., Keating, K. M., Williams, K. R., Konigsberg, W. H., & Coleman, J. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8452–8456.
Glasoe, P., & Long, F. (1960) *J. Phys. Chem. 64*, 188–190.
Håkansson, K., Carlsson, M., Svensson, L. A., & Liljas, A. (1992) *J. Mol. Biol. 227*, 1192–1204.
Handel, T., & DeGrado, W. F. (1990) *J. Am. Chem. Soc. 112*, 6710–6711.
Hellinga, H. W., Caradonna, J. P., & Richards, F. M. (1991) *J. Mol. Biol. 222*, 787–803.
Hewett-Emmett, D., & Tashian, R. E. (1991) in *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics* (Dodgson, S. J., Tashian, R. E., Gros, G., & Carter, N. D., Eds.) pp 15–32, Plenum Press, New York.
Higaki, J. N., Haymore, B. L., Chen, S., Fletterick, R. J., & Craik, C. S. (1990) *Biochemistry 29*, 8582–8586.
Holmes, M. A., & Matthews, B. W. (1982) *J. Mol. Biol. 160*, 623–639.
Holz, R. C., Salowe, S. P., Smith, C. K., Cuca, G. C., & Que, L., Jr. (1992) *J. Am. Chem. Soc. 114*, 9611–9614.
Ippolito, J. A., & Christianson, D. W. (1993) *Biochemistry* (following paper in this issue).
Iverson, B. L., Iverson, S. A., Roberts, V. A., Getzoff, E. D., Tainer, J. A., Benkovic, S. J., & Lerner, R. A. (1990) *Science 249*, 659–662.
Khalifah, R. G. (1971) *J. Biol. Chem. 246*, 2561–2573.
Krebs, J. F. (1992) Investigation of the Roles of Hydrophobic Residues in Human Carbonic Anhydrase II Using Mutagenesis and Kinetic Analysis, Doctoral Thesis, Duke University, Durham, NC.
Krebs, J. F., & Fierke, C. A. (1993) *J. Biol. Chem. 268*, 948–954.
Krebs, J. F., Rana, F., Dluhy, R. A., & Fierke, C. A. (1993) *Biochemistry 32*, 4496–4505.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Liang, Z., Xue, Y., Behravan, G., Jonsson, B.-H., & Lindskog, S. (1993) *Eur. J. Biochem. 211*, 821–827.
Lindskog, S. (1966) *Biochemistry 5*, 2641–2646.
Lindskog, S., & Nyman, P. O. (1964) *Biochim. Biophys. Acta 85*, 462–474.
Malmström, B. G. (1953) *Arch. Biochem. Biophys. 46*, 345–363.
Martin, A. E., Burgess, B. K., Stout, C. D., Cash, V. L., Dean, D. R., Jensen, G. M., & Stephens, P. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 598–602.
Matthews, B. W. (1988) *Acc. Chem. Res. 21*, 333–340.
McGrath, M. E., Haymore, B. L., Summers, N. L., Craik, C. S., & Fletterick, R. J. (1993) *Biochemistry 32*, 1914–1919.
Morrison, J. F. (1969) *Biochim. Biophys. Acta 185*, 269–286.
Nair, S. K., & Christianson, D. W. (1991) *J. Am. Chem. Soc. 113*, 9455–9458.
Nair, S. K., & Christianson, D. W. (1993) *Biochemistry 32*, 4506–4514.
Nair, S. K., Calderone, T. L., Christianson, D. W., & Fierke, C. A. (1991) *J. Biol. Chem. 266*, 17320–17325.
Pessi, A., Bianchi, E., Crameri, A., Venturini, S., Tramontano, A., & Sollazzo, M. (1993) *Nature 362*, 367–369.
Pocker, Y., & Fong, C. T. O. (1983) *Biochemistry 22*, 813–818.
Rees, D. C., Lewis, M., & Lipscomb, W. N. (1983) *J. Mol. Biol. 168*, 367–387.
Regan, L., & Clark, N. D. (1990) *Biochemistry 29*, 10878–10883.
Roberts, V. A., Iverson, B. L., Iverson, S. A., Benkovic, S. J., Lerner, R. A., Getzoff, E. D., & Tainer, J. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6654–6658.
Salowe, S. P., Marcy, A. I., Cuca, G. C., Smith, C. K., Kopka, I. E., Hagmann, W. K., & Hermes, J. D. (1992) *Biochemistry 31*, 4535–4540.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Sillén, L. G., & Martell, A. E. (1964) in *Stability constants of metal ion complexes*, Special Publication No. 17, p 546, The Chemical Society, London.
Silverman, D. N., & Lindskog, S. (1988) *Acc. Chem. Res. 21*, 30–36.
Springman, E. B., Angleton, E. L., Birkedahl-Hansen, H., & Van Wart, H. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 364–368.
Stanssens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M., & Fritz, H.-J. (1989) *Nucleic Acids Res. 17*, 4441–4454.
Sutton, B. J., Artymiuk, P. J., Cordero-Borboa, A. E., Little, C., Phillips, D. C., & Waley, S. G. (1987) *Biochem. J. 248*, 181–188.
Tu, C. K., & Silverman, D. N. (1982) *Biochemistry 21*, 6353–6360.
Tu, C. K., Silverman, D. N., Forsman, C., Jonsson, B. H., & Lindskog, S. (1989) *Biochemistry 28*, 7913–7918.
Vallee, B. L., & Galdes, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 283–430.
Vallee, B. L., & Auld, D. S. (1990) *Biochemistry 29*, 5647–5659.
Van Wart, H. E., & Birkedal-Hansen, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5578–5582.
Vidgren, J., Liljas, A., & Walker, N. P. C. (1990) *Int. J. Biol. Macromol. 12*, 342–344.
Wade, W. S., Koh, J. S., Han, N., Hoekstra, D. M., & Lerner, R. A. (1993) *J. Am. Chem. Soc. 115*, 4449–4456.