Nature and by Design

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Ever since its discovery at the University of Pennsylvania[1] and the University of Cambridge[2] more than 60 years ago, the zinc enzyme carbonic anhydrase has occupied a prominent position at the frontiers of biochemistry, medicinal chemistry, and protein engineering. Seven genetically-distinct forms of this enzyme (known as isozymes I–VII) have evolved in numerous tissues and cellular locations, and each contains a catalytically-obligatory zinc ion.[3,4] This diversity reflects the ubiquitous biological requirement for rapid hydration of carbon dioxide to yield bicarbonate ion plus a proton.[5–9] Although a deceptively simple reaction, the chemical and structural aspects of this mechanism have only recently been delineated for an isozyme found in the red blood cell, carbonic anhydrase II (CAII) (Figure 1). The three-dimensional structure of CAII[10] has stimulated research probing the determinants of the substrate association site and the pathway the product proton traverses through the enzyme active site. On the basis of phylogenetic comparisons,[3,4] the substrate and zinc binding sites are mainly conserved among all catalytic carbonic anhydrase isozymes found in mammals. However, the trajectory of catalytic proton transfer to bulk solvent has diverged during the evolution of the seven isozymes.

We begin this Account with a brief review of the CAII mechanism, emphasizing recent developments (see previous Accounts for additional information[6,7]). Notably, more X-ray crystallographic and enzymological studies have been performed on CAII and its site-specific variants than any other metalloenzyme, and these studies uniquely illuminate the molecular details of catalysis. Importantly, this work provides an elegant example of how the complementary methods of molecular biology and structural biology can be used to probe the structure, function, and stability of a zinc binding site in a metalloenzyme. Additionally, these studies set a useful foundation for understanding the evolution of carbonic anhydrase into noncatalytic biological roles, such as signal transduction. For example, in the nervous system an extracellular, CAII-like domain of receptor protein tyrosine phosphatase β binds an axonal cell recognition molecule (contactin) important for neuronal development and differentiation.[11–15] Intriguingly, the putative zinc binding site of this domain has partially evolved away from that found in CAII.

We then review recent progress in the "directed evolution"—i.e., the structure-based redesign—of the CAII zinc binding site, following Nature's example. We describe novel structural determinants of protein–metal affinity and the chemical reactivity of zinc-bound solvent. Not only does this work represent the first molecular dissection of structure–function relationships in a protein–zinc binding site (and therefore serves as a paradigm for the design of *de novo* metal sites in other proteins), it also sets the foundation for the development and optimization of CAII as a metal ion biosensor. Remarkably, this ubiquitous metalloprotein can be engineered and exploited as a sensitive tool for analytical chemistry and biotechnology.

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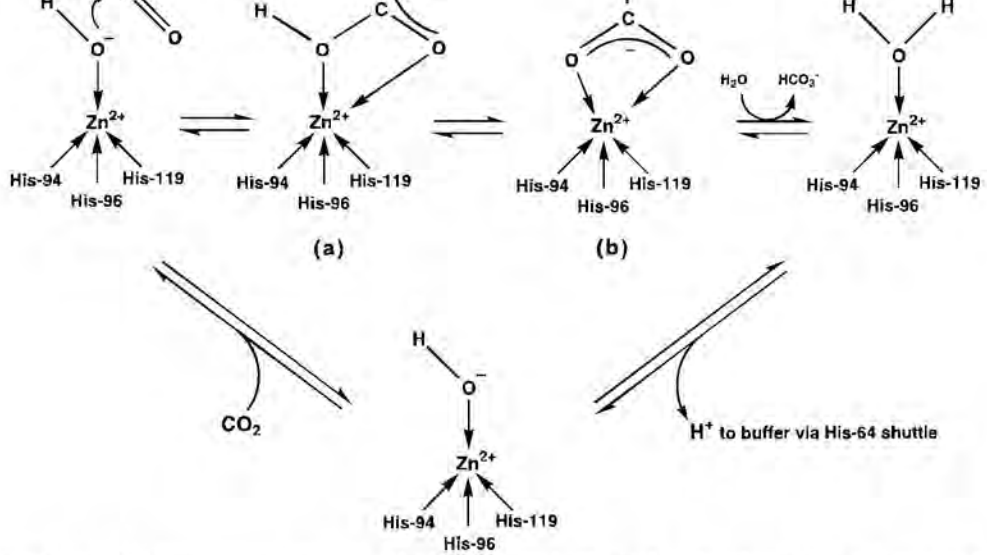

Figure 1. Summary of the CAII mechanism. Zinc-bound hydroxide attacks the carbonyl carbon of CO_2 to form zinc-bound bicarbonate. The initial mode of bicarbonate binding (a) may reflect the structure of either a discrete intermediate or the transition state. Bicarbonate may then isomerize (b), representing either a productive or a nonproductive complex. Following the exchange of a water molecule for zinc-bound bicarbonate, a proton is transferred from zinc-bound water to solvent via His-64 to regenerate the zinc hydroxide species.

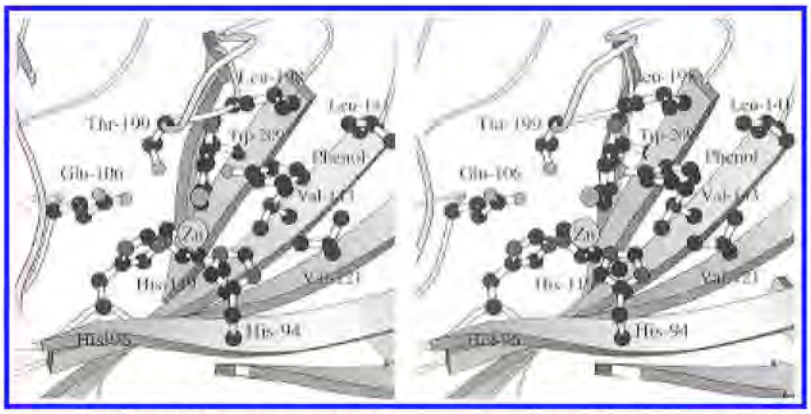

Figure 2. CAII active site, complexed with the competitive inhibitor phenol bound in the hydrophobic pocket.[31] Important active-site residues are labeled. Note that inhibitor (and therefore substrate) binding does not require the displacement of zinc-bound solvent (unlabeled gray sphere). Figure generated with MOLSCRIPT.[89]

Mechanism of Catalysis: Substrate Binding and Proton Transfer

The substrate association site of CAII is a hydrophobic pocket adjacent to zinc-bound hydroxide,[10,16] formed in large part by residues Val-143 at its base and Val-121, Trp-209, and Leu-198 at its neck (Figure 2). This pocket is highly conserved among all active isozymes on the basis of phylogenetic comparisons,[3,4] although in isozyme III the pocket is somewhat constricted by the bulky side chain of Phe-198.[17] The structure-based dissection of this pocket in CAII delineates the minimum size and shape of the pocket required for enzymatic activity; for instance, the occlusion of the pocket by the radical Val-143 → Phe substitution obliterates catalytic activity due to the loss of the substrate association site.[18,19] More moderate amino acid substitutions in the pocket (e.g., Val-143 → Gly or Val-121 → Leu) modestly affect the stability of the transition state for CO_2 hydration (rather than substrate affinity) and alter the structure of active site solvent, leading to slight changes in the rate constant for proton transfer from zinc-bound water to His-64 (see Figure 1). The hydrophobic pocket therefore has a minimum width[20–23] and depth[18,19] for efficient catalysis, and linear free energy relationships indicate that the volume of the amino

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ics simulations that point to the hydrophobic pocket as a substrate association site.[24−26] Experimental evidence supporting the catalytic role of the hydrophobic pocket comes from a variety of spectroscopic studies,[27,28] including Fourier transform infrared spectroscopy.[22,29] The shift of the asymmetric stretching vibration of CO_2 to a lower wavenumber upon association with CAII is consistent with the transfer of CO_2 from aqueous solution to a hydrophobic environment. These experiments also indicate that the affinity of CAII for CO_2 is low (~0.1 M), as required by the high turnover number of the enzyme which necessitates a rapid product dissociation rate constant. Finally, although the precatalytic enzyme−substrate complex is too short-lived to be observed by traditional X-ray crystallographic methods, the structure of the only known competitive inhibitor of CAII-catalyzed CO_2 hydration, phenol,[30] has been solved in complex with the enzyme:[31] phenol binds in the hydrophobic pocket and makes van der Waals contacts with Val-121, Val-143, Leu-198, and Trp-209 while its hydroxyl group hydrogen bonds with zinc-bound hydroxide (Figure 2). Since a competitive inhibitor must bind in the same location as the substrate, CO_2 must therefore bind in the hydrophobic pocket prior to catalysis. Importantly, CO_2 binding does not displace zinc-bound hydroxide, although long-range (i.e., >3 Å) weakly-polar interactions with zinc may contribute to substrate orientation.

Product bicarbonate ion is formed by the nucleophilic attack of zinc-bound hydroxide at CO_2 immobilized in the hydrophobic pocket, and the binding mode of bicarbonate ion to zinc has been the subject of some controversy. Central to this controversy is the possible role of Thr-199 as a "doorkeeper".[32−34] That is to say, the side chain hydroxyl group of this residue is proposed to allow only anions capable of donating a hydrogen bond to Thr-199 access to zinc binding. X-ray crystallographic structures of Co^{2+}-substituted and Thr-199 → Ala CAIIs in complex with bicarbonate are consistent with this interpretation.[35,36] However, the binding of azide anion, a competitive inhibitor of bicarbonate dehydration, demonstrates that Thr-199 is *not* a doorkeeper all the time:[37,38] azide anion binds to zinc but does not hydrogen bond with Thr-199. likewise possible for product bicarbonate, as illustrated in Figure 1. Although such an interaction does not contribute to enzyme-product affinity, it is nevertheless likely to contribute to efficient catalysis by facilitating rapid product dissociation. Consistent with this interpretation are studies of Thr-199 variants showing that deletion of the Thr-199 hydroxyl group stabilizes bicarbonate binding[39,40] and alters the structure of the bound bicarbonate ion.[36] Therefore, the Thr-199 side chain promotes maximum catalytic efficiency by destabilizing the product complex to provide for rapid dissociation and by selecting for a catalytically competent bicarbonate−zinc complex (Figure 1). Thus, it is clear that the zinc binding site and its environment have evolved for optimal catalytic activity.

Following bicarbonate dissociation is the rate-determining step of proton transfer to regenerate the active catalyst, zinc-bound hydroxide (Figure 1).[5−9] The product proton is not transferred from zinc-bound water directly to bulk solvent; instead, it is first transferred to an intermediate "shuttle" residue, and then transferred to bulk solvent. Because of its greater exposure to solvent, the shuttle residue can more efficiently transfer a proton to a variety of acceptors with pK_a values higher than that of water.[7] Interestingly, the proton transfer pathway has divergently evolved among the carbonic anhydrase isozymes. In isozyme II, His-64 is the catalytic proton shuttle,[41,42] and it exhibits significant conformational mobility which accompanies its function.[43−45] This residue is too far from zinc-bound solvent to allow for direct proton transfer; instead, proton transfer is achieved across two intervening, hydrogen-bonded solvent molecules in the native enzyme (Figure 3). Isozymes IV, VI, and VII also contain His-64, which may similarly function as a proton shuttle (isozyme I contains His-64, but His-200 is the major proton shuttle group in this isozyme[46]). However, isozyme III contains Lys-64, and the main proton transfer pathway is direct transfer to bulk solvent.[47] Intriguingly, isozyme V contains Tyr-64, which plays no major role in proton transfer[48] due in part to steric effects arising from the bulky adjacent side chain of Phe-65.[49] The three-

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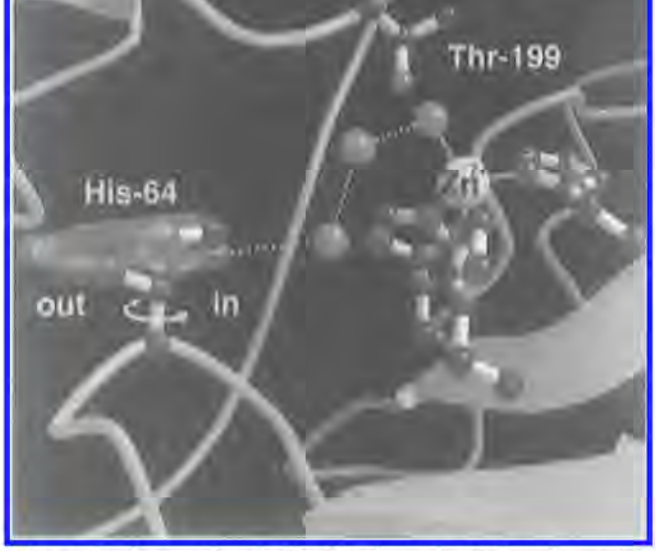

Figure 3. Proton shuttle His-64 adopts the "in" conformation in native CAII and engages in a hydrogen-bonded solvent network (red) with zinc-bound solvent at pH 8.5.[32] Proton transfer across this network regenerates the reactive zinc-bound hydroxide species in catalysis (Figure 1). Upon protonation at lower pH values, His-64 swings away from the active site to the "out" conformation.[44] Thus, His-64 is conformationally mobile, as indicated. Figure generated with MOLSCRIPT.[89]

dimensional structure of isozyme V suggests that a residue in the vicinity of Tyr-131 (e.g., the phenolic side chain of this residue or one of three flanking lysines) may serve as a proton shuttle group,[49] but further experiments are necessary to confirm this speculation. Rate constants for proton transfer in the carbonic anhydrases are dependent on the difference in pK_a between zinc-bound water and the proton shuttle group, and are limited by organization of the intervening hydrogen-bonded solvent molecules.[9,45,50] Individual components of the zinc binding site may therefore affect catalytic proton transfer by modulating the pK_a of zinc-bound water.

Structure-Based Protein Engineering: Redesigning the Zinc Binding Site

As summarized in the preceding section and in Figure 1, the zinc ion in the CAII active site plays a central role in the mechanism of CO_2 hydration. The principal role of zinc is that of an electrostatic catalyst, since it stabilizes the negatively-charged transition state leading to bicarbonate formation. It also depresses the pK_a of its bound water molecule to provide an ample supply of nucleophilic hydroxide ion for catalysis at neutral pH values. Logically, the protein environment of zinc is critical for optimizing the electrostatic properties of the metal ion for catalysis, and this environment includes not only the direct metal ligands but also the residues hydrogen bonding with these ligands (i.e., "indirect" or "second shell" ligands) (Figure 4). By combining the techniques of molecular and structural biology, we have dissected the determinants of affinity and catalysis in the zinc binding site of CAII.

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examples are structurally characterized by atomic resolution X-ray crystallographic methods to provide ultimate proof-of-design.[58,59] Indeed, examples characterized by other biophysical techniques sometimes illustrate the limitations of current structure-based design approaches despite the best attempts of computational structure analysis—for example, where "designed" metal ligands do not coordinate to the target metal ion,[60,61] or where zinc affinity is at least 4 orders of magnitude weaker[57] than that measured for CAII.[62,63] Furthermore, none of the designed sites function as efficient catalysts. Hence, there is much yet to be learned regarding structure–activity and structure–stability relationships in protein–metal binding sites, and the zinc binding site of CAII is a universally-recognized and easily-characterized paradigm. We note that there is a novel application of redesigned CAII zinc binding site variants in analytical chemistry and biotechnology: a CAII-based metal ion biosensor.[64] Following Nature's example in the evolution of carbonic anhydrase for different biological functions, the "directed evolution", or structure-based redesign, of the CAII zinc binding site allows for the optimization of its properties[65] in the development of a zinc biosensor.

Altering the Direct Zinc Ligands. A thorough understanding of three factors must precede the molecular dissection and redesign of direct metal ligands in any protein–metal binding site. First, the chemical nature of the target metal must be considered. It could be "hard", like the small, highly-charged magnesium ion, it could be "soft", like the large, highly-polarizable mercury ion, or it could be intermediate between these two extremes (i.e., "borderline").[66] An optimized metal binding site in a protein molecule will exhibit hardness complementary to that of its target metal ion. Since zinc is a metal of borderline hardness, it is satisfactorily coordinated by the harder ligands aspartate and glutamate, the borderline ligand histidine, and the softer ligand cysteine. Second, the conformations of the amino acid side chains that comprise the metal coordination site

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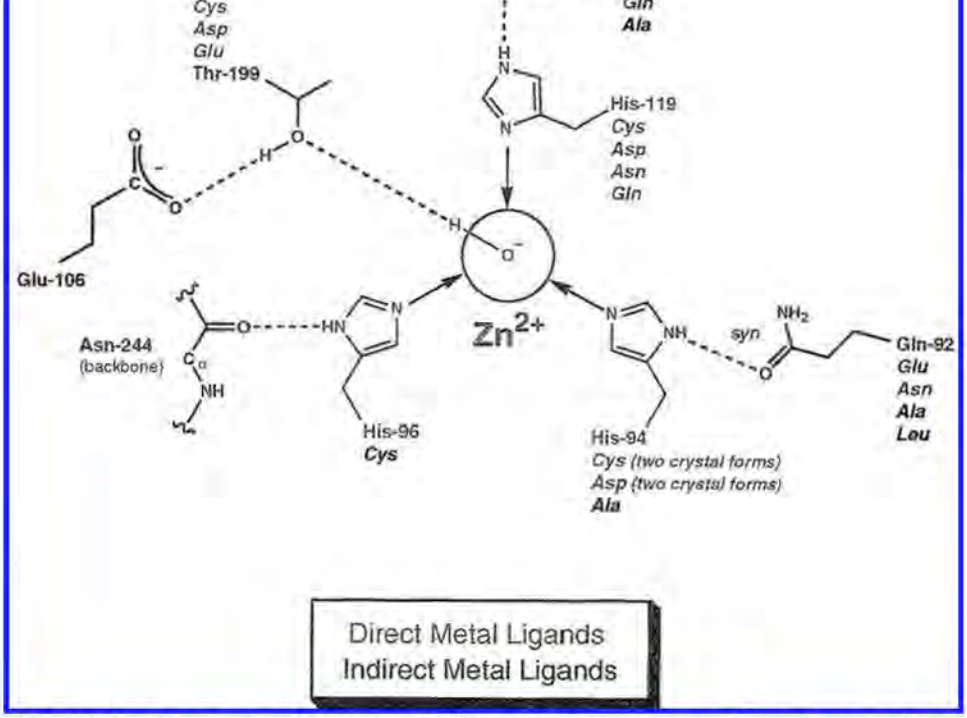

Figure 4. Scheme of the CAII zinc binding site. Variants with characterized properties and three-dimensional structures are indicated by italics at the site of substitution.

Table 1. Properties of Selected CA Variants with Altered Zinc Binding Sites[a]

variant	k_{cat}/K_M (μM^{-1} s^{-1})	pK_a	zinc K_d (nM)
wild type	110	6.8	0.004
H94D	0.11	≥9.6	15
H94C	0.11	≥9.5	33
H96C	0.073	8.5	60
H119C	0.11	nd	50
H119D	3.8	8.6	25

[a] Data from ref 75. nd = no data.

must be favorable.[54,67] If an engineered ligand must incur too high an energetic cost to coordinate to a metal ion, it will not do so. Even so, certain regions of a protein structure—e.g., loops, α-helices, or β-strands—can be sufficiently pliable to allow for modest conformational changes or segmental shifts which optimize metal coordination by an engineered ligand. Finally, and perhaps most importantly, the separation and stereochemistry of ligand−metal coordination must be reasonable. The geometric preferences of carboxylate,[68,69] imidazole,[70] and thiolate[71] ligands for protein−metal ion coordination have been outlined, and the results of these studies provide structural criteria by which metal binding site designs can be evaluated and optimized.

The alteration of direct zinc ligands in CAII yields important structural and functional insight as the properties of selected variants (Table 1) are interpreted in light of their three-dimensional structures.[72−76] As a point of reference, recall that the protein ligands to zinc in wild-type CAII are His-94, His-96, and His-119 (Figures 2 and 4). A notable feature in the CAII zinc binding site is that metal binding to the histidine ligands is very cooperative: the deletion of any one protein ligand by a histidine → alanine substitution decreases the zinc affinity[75] by a factor of ~10^5. This is much larger than the 10−100-fold decrease in metal affinity observed for removing one ligand from Cys_2His_2 zinc binding sites, including a zinc finger peptide[77] and a *de novo* designed zinc coordination polyhedron in the 4α-helical bundle protein designated $Z\alpha_4$.[78]

Similarly, substitution of a neutral histidine ligand by the negatively-charged side chains of aspartate, glutamate, or cysteine decreases the zinc affinity ~10^4-fold.[75] The structure of His-94 → Asp CAII reveals that this loss of protein−metal affinity arises primarily from the 0.9 Å movement of zinc that accommodates the shorter side chain of the ligand. Intriguingly, the structure of His-94 → Cys CAII demonstrates that the zinc ion occupies the same site in the His-94 → Asp and His-94 → Cys variants;[75,76] additionally, a 1 Å deformation of the β-strand containing Cys-94 ensures that this shortest ligand side chain coordinates to zinc (Figure 5). Notably, this β-strand

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