

Comparison between α - and β -carbonic anhydrases: can $\text{Zn}(\text{His})_3(\text{H}_2\text{O})$ and $\text{Zn}(\text{His})(\text{Cys})_2(\text{H}_2\text{O})$ sites lead to equivalent enzymes?[†]

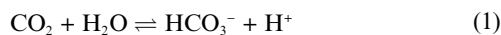
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Large models of α - and β -carbonic anhydrases were compared using DFT calculations. They indicate similar acidity of the coordinated water molecule and zinc affinity. This explains their similar mechanism of action, despite the wide difference in their first coordination sphere.

Carbonic anhydrase (CA) is a family of zinc enzymes which catalyze the reversible hydration of carbon dioxide.



CAs are ubiquitous throughout nature and are categorized into five different classes based on sequence homology.^{1–5} The active site of α -CA, a class which includes the well-characterized and most studied HCAII, contains a catalytically required zinc ion coordinated to three histidine residues and a water molecule. Its mechanism of action includes formation of a metal-bound hydroxide, the catalytic species which nucleophilically attacks the incoming carbon dioxide molecule. It is broadly accepted that the possibility of extracting a proton from the metal-bound water at physiological pH comes from the high Lewis acidity of a Zn^{II} surrounded by three neutral residues.^{6,7} By contrast, the two negatively charged Cys ligands of liver alcohol dehydrogenase inhibit deprotonation of the water bound to zinc (denoted $\text{H}_2\text{O}_{(\text{zn})}$).⁸ This hypothesis has also been supported by *ab initio* calculations,⁹ and by substitution of direct zinc ligands in HCAII with negatively-charged side chains.¹⁰

The structure of β -CAs active site, and of the recently discovered ζ -CA,⁵ show noticeable differences to that of α -CA.^{2,11} Indeed, the coordination sphere of zinc in the active form is $(\text{Cys})_2\text{His}(\text{H}_2\text{O})$ ^{12–15} with two negatively charged cysteinate ligands. This complex is generated from the $(\text{Cys})_2\text{HisAsp}$ resting state^{16–18} through a carboxylate shift mechanism, whereby the zinc-bound Asp is substituted by water.¹⁹ Based on similarities in the architecture of their active sites, it has been proposed that β -CAs share a common mechanism with α -CAs.¹² Despite the large difference in zinc coordination, both $\text{Zn}(\text{His})_3(\text{H}_2\text{O})$ and

$\text{Zn}(\text{Cys})_2\text{His}(\text{H}_2\text{O})$ sites may be able to be deprotonated to form a transient zinc-bound hydroxide ion.

In order to explain these conflicting results, we conducted a computational study at the DFT level on the influence of the protein environment of these sites on their structural and electronic properties. Starting from X-ray structures of representative members of the α -CA and β -CA classes (PDB 2CBA²⁰ and 1G5C¹³ respectively), we built active site models of increasing dimensions (Fig. 1).

We follow a “bottom-up” strategy²¹ starting with the most simple model of each CA, the zinc with its first coordination sphere (**1**). More complete models included the second coordination sphere around His/Cys Zn-ligands (**2**), the “gate-keeper” and its surrounding (**3**); and the remaining 2nd coordination sphere around Zn-bound water (**4**). Beside this step-by-step building, we also investigated, at the same DFT level, more extended models including all chemical groups located at a distance within 9 Å of Zn^{II} . This leads to models **α -5** and **β -5** which comprise 530 and 494 atoms respectively. For each model, geometry optimisation with fixed positions for C_α atoms, at the BP86/SVP level of calculation, has been achieved for the Zn-bound water (denoted **XH**, **X** = 1–5) and hydroxide (denoted **X[−]**) species (more detailed information on the models and computational aspects can be found in the ESI[†]).

As expected, the Zn–O bond length (Table 1) is significantly larger in **β -1H** than in **α -1H** (2.093 and 2.406 Å respectively) confirming the strong difference between the naked $\text{Zn}(\text{His})_3(\text{H}_2\text{O})$ and $\text{Zn}(\text{Cys})_2\text{His}(\text{H}_2\text{O})$ sites. The competition between ligands to bind to the metal turns in favor of the electronically rich Cys groups. Including the second coordination sphere around Cys/His Zn-ligands reduces the difference between the $\text{Zn}(\text{His})_3(\text{H}_2\text{O})$ and $\text{Zn}(\text{Cys})_2\text{His}(\text{H}_2\text{O})$ sites as H-bonded His and Cys become respectively more and less able to donate electron density to the zinc.²² The changes in the Zn–ligand bond lengths is thus indicative of the electronic effect of the environment on the zinc site. In both cases, from **2** to **3** and **4**, we observe a shortening of the Zn–O bond length, mostly due to the O–H \cdots gatekeeper hydrogen bond. Less expected was the difference between **4** and **5**, indicating that even the most external groups play a significant role in the metal–ligand bond lengths. For our larger models **α -5H** and **β -5H**, the difference in the computed Zn–OH₂ bond lengths (0.124 Å) is significantly reduced compared to models **1** (0.313 Å) but remains large. On the other hand, the bending of the Zn-bound water molecules, reflecting the extent of electron transfer to the metal ion as well as hydrogen bonds networks, is similar in **α -5H** and **β -5H** (Table 1).

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[†] Electronic supplementary information (ESI) available: Computational methods; structural parameters and comparison with experiment, Cartesian coordinates and single point energies of the stationary points. See DOI: 10.1039/c0dt01454k

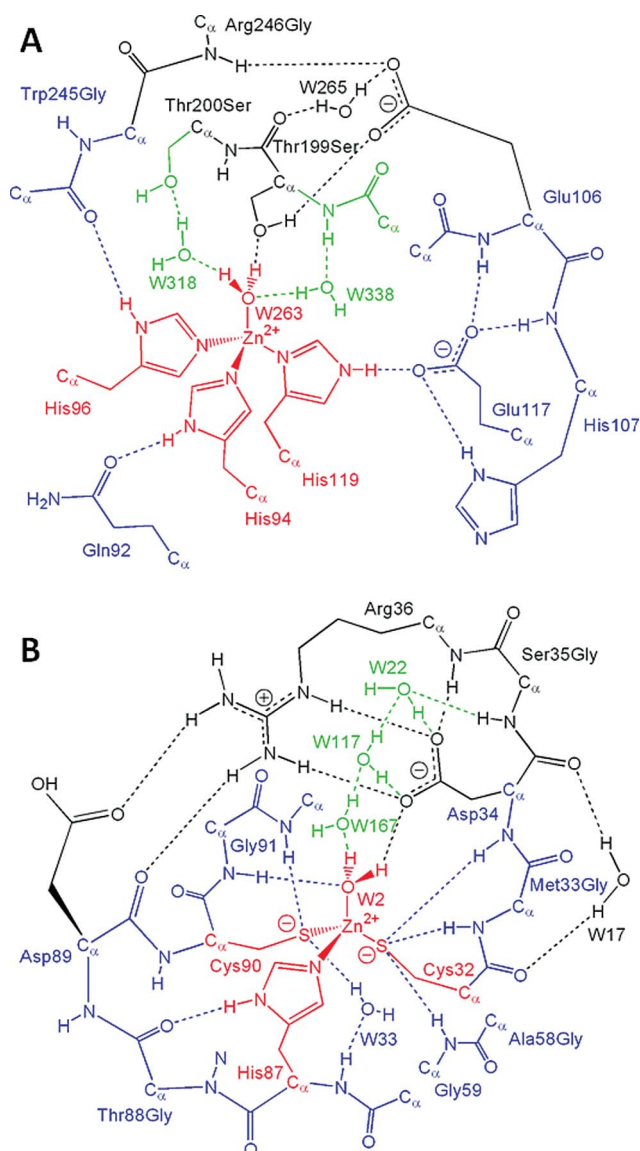


Fig. 1 Structures of the α -CA (A) and β -CA (B) models. Models **1** in red, models **2**, **3** and **4** add respectively the blue, black and green parts.

Thus inclusion of the protein environment moves structurally closer the active sites of α - and β -CAs without making them similar. We further evaluate their similarity by final energy calculations at the B3LYP/TZVPP level.

As for the structure, the proton affinity (PA) of Zn-bound hydroxide (Table 2) is greatly dependent upon the nearby residues. In the gas phase, the most significant changes are observed from α -1 to α -2 and α -3 and from β -1 to β -2. They can be related, respectively, to the inclusion of anionic Glu117 and Glu106 residues in α -CA, as already observed previously,^{23,24} and to the hydrogen bond network around the coordinated thiolates in β -CA. The protein environment thus reduces very strongly the difference between the $\text{Zn}(\text{His})_3(\text{OH})$ and $\text{Zn}(\text{Cys})_2\text{His}(\text{OH})$ sites ($\Delta\text{PA}_{\text{gas}} = 657$ and 17 kJ mol^{-1} for models **1** and **5** respectively). The same trend is observed when the models are embedded in water, treated as a continuum (see Table 2). However the PA variations are not only smaller, but they also are due to all the parts of the proteins. Consequently, even the environment distant from the zinc atom

Table 1 Main structural parameters for compounds **1–5**^a

	Zn–O ^b	Zn–L ₁ ^{b,c}	Zn–L ₂ ^{b,c}	Zn–L ₃ ^{b,c}	$\Sigma(\text{X–O–X})^d$
α -1H	2.093	2.001	2.010	2.007	359.7
α -2H	2.118	1.995	2.007	1.973	340.8
α -3H	1.996	2.057	2.003	2.037	322.4
α -4H	1.992	2.041	2.001	2.022	342.4
α -5H	2.014	2.013	2.007	1.999	331.2
β -5H	2.138	2.316	2.057	2.310	330.8
β -4H	2.068	2.311	2.092	2.328	319.9
β -3H	2.119	2.309	2.071	2.324	327.7
β -2H	2.274	2.293	2.057	2.295	318.9
β -1H	2.406	2.282	2.069	2.255	261.0

^a Geometry optimisation at the BP86/SVP level. ^b bond lengths (Å). ^c L₁ = N(His94)/S(Cys32); L₂ = N(His96)/N(His87); L₃ = N(His119)/S(Cys90) for α -/ β -CA respectively. ^d Sum of the Zn–O–H and H–O–H angles (°).

Table 2 Energetic properties for compounds **1–5**^a

	PA_{gas}^b	$\text{PA}_{\text{solv}}^b$	$\text{BE}(\text{H}_2\text{O}_{(\text{Zn})})^c$	$\text{BE}(\text{Zn})^c$
α -1	804	1207	104	1787
α -2	1065	1174	82	2389
α -3	1335	1218	232	2896
α -4	1285	1201	251	2871
α -5	1321	1262	251	2811
β -5	1338	1257	127	2817
β -4	1337	1215	159	2846
β -3	1330	1244	140	2809
β -2	1272	1207	48	2765
β -1	1461	1265	38	3109

^a Final energy computed at the B3LYP/TZVPP level. Values in kJ mol^{-1} .

^b Proton affinity for the reaction $\alpha/\beta\text{-X}^- + \text{H}^+ \rightarrow \alpha/\beta\text{-HX}$ in the gas phase and in solution ($\epsilon = 78.4$), respectively. ^c Bonding energy of $\text{H}_2\text{O}_{(\text{Zn})}$ and the zinc dication, respectively, to the enzyme.

plays a significant role in the relative stabilization of the Zn–OH₂ and Zn–OH species. This parallels the long-range solvent effects on water acidity observed for zinc in pure water.²⁵

Because of the neglect of entropic effects due to geometry optimisation with fixed positions for C_α atoms, only relative pK_a values could be reasonably estimated.²⁶ The $\text{H}_2\text{O}_{(\text{Zn})}$ of β -1H has a pK_a value 10.2 units higher than that of α -1H due to its considerably more electron-rich coordination environment. From this value, inclusion of successive parts of the environment of the active sites continuously decreases the gap between α - and β -CA sites, from 5.9 between β -2H and α -2H to 4.4, 2.4 and –0.8 for models **3**, **4** and **5**, respectively. The pK_a of $\text{H}_2\text{O}_{(\text{Zn})}$ is believed to be 6.8 for HCAII,²⁷ whereas large uncertainty remains for β -CA with measured pK_a values between 6.0 and 8.8.¹¹ Our calculations fit into this range of values and indicate that, despite its considerably more electron-rich coordination environment, the zinc-bound water molecule in β -CA has a pK_a value which is comparable to that of α -CA. The similar proposed mechanism of action for α - and β -CA through the nucleophilic zinc-bound hydroxide thus seems justified and explained by the whole environment of both enzymes.

In view of this pK_a similarity between $\text{Zn}(\text{His})_3(\text{H}_2\text{O})$ and $\text{Zn}(\text{Cys})_2\text{His}(\text{H}_2\text{O})$ sites when including a large part of their enzymatic environment, we have further examined other chemical properties, namely the ability of $\text{H}_2\text{O}_{(\text{Zn})}$ to be displaced by an

incoming substrate/inhibitor and the ability of the protein to bind tightly the zinc cation (Table 2).

The bonding energy of the coordinated water molecule includes not only the zinc–water binding but also the hydrogen-bonds involving $\text{H}_2\text{O}_{(\text{Zn})}$ as well as other long-range electrostatic interactions. As expected, however, its trend parallels approximately that of the Zn–OH₂ bond length (Tables 1 and 2). Accordingly, $\text{H}_2\text{O}_{(\text{Zn})}$ is significantly more tightly bound in minimal models of $\text{Zn}(\text{His})_3(\text{H}_2\text{O})$ sites (like **α -1**) than in $\text{Zn}(\text{Cys})_2\text{His}(\text{H}_2\text{O})$ ones, but this is also the case for extended models. This shows that the environment of the enzyme, even if it can modulate the bonding energy of $\text{H}_2\text{O}_{(\text{Zn})}$, does not induce a closer similarity between the two kinds of sites. Thus, we postulate that an electron-rich coordination environment around zinc, as in $\text{Zn}(\text{Cys})_2\text{His}(\text{H}_2\text{O})$ sites, induces a higher lability of $\text{H}_2\text{O}_{(\text{Zn})}$ than less electron-rich sites like $\text{Zn}(\text{His})_3(\text{H}_2\text{O})$, allowing an easier catalytic mechanism through displacement of the coordinated water molecule by the substrate, as observed for liver alcohol dehydrogenase. On the other hand, the mechanism of catalysis by CAs includes the displacement of bicarbonate from the zinc ion by a molecule of water. The calculated bonding energy of $\text{H}_2\text{O}_{(\text{Zn})}$ indicates that this step should be more difficult for β -CA than for α -CA.

β -1H binds zinc almost twice as strongly as **α -1H**. This agrees with the larger Zn–thiolate binding energy compared to Zn–imidazole.²⁸ However, including the protein environment induces a decrease (an increase, resp.) of the Zn binding energy to the $(\text{Cys})_2\text{His}(\text{H}_2\text{O})$ ($(\text{His})_3(\text{H}_2\text{O})$, resp.) site. As a consequence, **α -5H** and **β -5H** bind zinc with almost the same strength. It is well known that Zn can be extracted or substituted by other transition metals in HCAII.²⁹ Even if, to the best of our knowledge, such metal extraction or substitution experiments have not been reported with β -CA,³⁰ we anticipate that this could also be achieved with an electron-rich coordination environment. It should however be noted that the metal substitution mechanism could differ between α - and β -CA. Indeed, cysteine, which is not present in the α -CA active site, could facilitate metal exchange due to its high lability³¹ or through the formation of metal–thiolate–metal bridges.³²

Conclusions

From reliable models of the active sites of α - and β -CA, we have shown that the zinc-bound water molecule has almost the same $\text{p}K_{\text{a}}$ for both enzymes, despite large electronic difference in their first coordination sphere. As a consequence, the mechanism of action of β -CA, which is postulated to proceed through a nucleophilic metal-bound hydroxide, is justified and explained by the influence of the environment. More generally, this shows that the deprotonation of the coordinated water molecule, which is one of the three modes of action of the majority of zinc enzymes, is not controlled by the first coordination sphere as previously proposed in the literature. The zinc affinity follows the same trends. On the contrary, compared to histidine, cysteine residues appear to facilitate the displacement of the zinc-bound water molecule regardless of surrounding details.

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