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Carbonic Anhydrase Activators. Activation of Isozymes I, II, IV, VA, VII, and XIV with L- and D-Histidine and Crystallographic Analysis of Their Adducts with Isoform II: Engineering Proton-Transfer Processes within the Active Site of an Enzyme

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Abstract: Activation of six human carbonic anhydrases (CA, EC 4.2.1.1), that is, hCA I, II, IV, VA, VII, and XIV, with L- and D-histidine was investigated through kinetics and by X-ray crystallography. L-His was a potent activator of isozymes I, VA, VII, and XIV, and a weaker activator of hCA II and IV. D-His showed good hCA I, VA, and VII activation properties, being a moderate activator of hCA XIV and a weak activator of hCA II and IV. The structures as determined by X-ray crystallography of the hCA II–L-His/D-His adducts showed the activators to be anchored at the entrance of the active site, contributing to extended networks of hydrogen bonds with amino acid residues/water molecules present in the cavity, explaining their different potency and interaction patterns with various isozymes. The residues involved in L-His recognition were His64, Asn67, Gln92, whereas three water molecules connected the activator to the zinc-bound hydroxide. Only the imidazole moiety of L-His interacted with these amino acids. For the D-His adduct, the residues involved in recognition of the activator were Trp5, His64, and Pro201, whereas two water molecules connected the zinc-bound water to the activator. Only the COOH and NH_2 moieties of D-His participated in hydrogen bonds with these residues. This is the first study showing different binding modes of stereoisomeric activators within the hCA II active site, with consequences for overall proton-transfer processes (rate-determining for the catalytic cycle). The study also points out differences of activation efficiency between various isozymes with structurally related activators, convenient for designing alternative proton-transfer pathways, useful both for a better understanding of the catalytic mechanism and for obtaining pharmacologically useful derivatives, for example, for the management of Alzheimer's disease.

Keywords: carbonic anhydrase · D-histidine · enzyme catalysis · kinetics · L-histidine · proton transfer

Introduction

Although activation of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) was reported[1] simultaneously with its inhibition,[2] further developments in these two fields had very different consequences for CA research.[3] While CA inhibitors (CAIs) have been constantly and extensively studied, as well as fruitfully exploited clinically for the prevention and treatment of several diseases,[4] CA activation became quite controversial soon after its initial reporting.[3] Consequently, no progress has been made in the research of CA activators (CAAs) for a long period and this topic received little attention till recently. In the last decade, by means of electronic spectroscopy, X-ray crystallography, and kinetic measurements, it has been proved that the activator molecule binds within the enzyme-active cavity at a site distinct from the inhibitor or substrate binding-sites,[3] participating thereafter in the rate-determining step of the catalytic cycle, that is, the proton-transfer processes between the active site and the environment.[4–8] Thus, the CA activator (A in Equation (1)) interferes directly in the proton-transfer step of the catalytic cycle, facilitating the intramolecular process by means of transient enzyme-activator complex(es).[3] Intramolecular reactions are faster than the intermolecular ones resulting in a significantly increased catalytic rate:[3]

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$$EZn^{2+}-OH_2 + A \rightleftharpoons [EZn^{2+}-OH_2 \cdots A \rightleftharpoons EZn^{2+}-OH^- \cdots AH^+] \rightleftharpoons EZn^{2+}-OH^- + AH^+ \text{enzyme-activator complexes} \quad (1)$$

It has been shown mainly by this group that a multitude of physiologically relevant compounds such as biogenic amines (histamine, serotonin, catecholamines), amino acids, oligopeptides, or small proteins among others, act as efficient CAAs for many of the 15 presently known human CA isozymes.[3] Ultimately, activation of some members of the α-CA family (of the four genetically unrelated CA gene families presently known, the α-CAs–δ-CAs)[4] was shown to contribute to a possible therapeutic approach for the enhancement of synaptic efficacy that may represent a conceptually new approach for the treatment of Alzheimer's disease, aging, and other conditions that require remedial achievement of spatial learning and memory therapy.[9,10] Despite this presently largely unexplored field, CAAs might lead to interesting pharmacological applications.[3] Sun and Alkon reported[9,10] that phenylalanine, an activator first investigated by our group,[11,12] when administered to experimental animals produces a relevant pharmacological enhancement of synaptic efficacy, spatial learning, and memory, proving that this class of unexplored enzyme modulators may be used for the management of conditions in which learning and memory are impaired.[9,10] It should also be mentioned that it was previously reported that the levels of several CA isozymes (such as CA I and CA II) are significantly diminished in the brain of patients affected by Alzheimer's disease,[13a] a fact strongly supporting the involvement of CA isozymes in cognitive functions.[9,10] Very recently, two important new studies showed[13b,c] by means of redox proteomics that isoforms CA I and CA II are oxidized with a very much decreased catalytic activity in the frontal cortex and hippocampus, respectively, of patients with Alzheimer's disease. Thus, in the brain of these patients the dysfunction of CA I and/or CA II activity leads to imbalances of the extra and intracellular pH triggering the aggregation of proteins, thus contributing to progression of the disease.[13b,c] It is clear that agents that may restore to a certain degree the catalytic activity of these particular isozymes (CA I and CA II), or increase that of other CA isozymes present in the brain (such as CA VA, CA VII, or CA XIV) of the CAA-type, might lead to conceptually novel approaches for the management of Alzheimer's disease.

Two X-ray crystallographic structures of the adducts of the main human isoform, hCA II, with activators are known at this moment: one with histamine,[14] and another with phenylalanine (a ternary complex in which azide is also bound to the Zn^{II} ion of the hCA II active site).[12] Both of them showed the activator molecule to be bound at the entrance of the active-site cavity (in a region different from the inhibitor binding site), where it is anchored by hydrogen bonds to amino acid side chains (Asn62, Asn67, and Gln92) and water molecules, and also leading to a complete reorganization of the hydrogen bond network within the active-site cavity. Positioned in such a favorable way, the activator facilitates the rate-limiting step of CA catalysis, that is, the proton-transfer reaction between the zinc-bound water molecule and the environment (see [Eq. (1)]), which in many CA isozymes (in the absence of activators) is assisted by the amino acid residue His64 situated in the middle of the active-site cavity, and also possessing a pH-dependent conformational mobility, gradually changing its orientation relative to the metal site through a 64° ring-flipping.[15–17] This proton-transfer reaction (in which either the imidazolic moiety of His64, or a protonatable moiety of the activator molecule participates) leads to the formation of the catalytically active nucleophilic species of the enzyme, with hydroxide coordinated to the zinc ion.[3,4]

In a recent preliminary communication,[18] we have reported the X-ray crystal structure of the adduct of hCA II with L-histidine, and have investigated the activation properties of this amino acid and some of its derivatives (esters and dipeptides) for the esterase activity of isoforms hCA I, II, and IV. Here we report a detailed activation study (for the CO_2 hydrase activity, that is, the physiological reaction catalyzed by these enzymes) of isoforms hCA I, II, IV, VA, VII, and XIV with L- and D-histidine, as well as the X-ray crystal structures of the two adducts with the main physiological isoform hCA II. Many of these isozymes (for example, CA VA, CA VII, or CA XIV) have never been investigated for their interaction with activators although some were recently shown to be abundant in the brain where they play important physiological roles, some of which have only now started to be understood.[19] Thus, our study may bring novel insights regarding the catalytic/activation mechanisms of several CA isozymes, and may also be relevant for the design of CAAs with possible pharmacological applications for obtaining agents against Alzheimer's disease, or agents useful in the treatment of CA deficiencies, genetic conditions in which one CA isoform is absent.[20]

Results

No detailed activation studies of other CA isozymes except hCA I and II are available in the literature at this moment. Kinetic data for steady-state conditions and activation constants of hCA isozymes I, II, IV, VA, VII, and XIV with L-histidine, and D-histidine, at 25 °C, for the CO_2 hydration reaction, are shown in Table 1, whereas an example of the activation curve of hCA II with L-His, in the concentration range of 10^{-8}–10^{-2} M is shown in Figure 1.

To rationalize the CA-activating properties of these two amino acids, L-His and D-His, the X-ray crystal structures of their adducts with the physiologically most relevant isoform (hCA II) have been resolved. The statistics for data collection and refinement of the two adducts, hCA II–D-His and hCA II–L-His, are summarized in Table 2, whereas the details for the binding of the two stereoisomeric activators within the enzyme cavity are shown in Figures 2–5.

Table 1. Activation of hCA isozymes I, II, IV, VA, VII, and XIV with L- and D-histidine, at 25 °C, for the CO_2 hydration reaction.

Isozyme	k_{cat}[a] [s^{-1}]	$(k_{cat})_{L\text{-His}}$[b] [s^{-1}]	$(k_{cat})_{D\text{-His}}$[b] [s^{-1}]	K_A[c] [μM] L-His	D-His
hCA I[d]	2.0×10^5	13.4×10^5	9.1×10^5	0.03	0.09
hCA II[d]	1.4×10^6	4.3×10^6	2.7×10^6	10.9	43.5
hCA IV[e]	1.2×10^6	4.3×10^6	3.8×10^6	7.3	12.3
hCA VA[f]	2.9×10^5	9.8×10^5	12.0×10^5	1.34	0.12
hCA VII[d]	9.5×10^5	16.7×10^5	15.4×10^5	0.92	0.71
hCA XIV[f]	3.1×10^5	11.4×10^5	8.5×10^5	0.90	2.37

[a] Observed catalytic rate without activator. K_M values in the presence and the absence of activators were the same for the various CA isozymes (data not shown). [b] Observed catalytic rate in the presence of 10 μM activator. [c] The activation constant (K_A) for each isozyme was obtained by fitting the observed catalytic increases as a function of the activator concentration.[3,14] Mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[27] Standard errors were in the range of 5–10% of the reported values. [d] Human recombinant isozymes. [e] Truncated human recombinant isozyme lacking the first 20 amino acid residues.[26b] [f] Full-length human recombinant isoforms.[24–26]

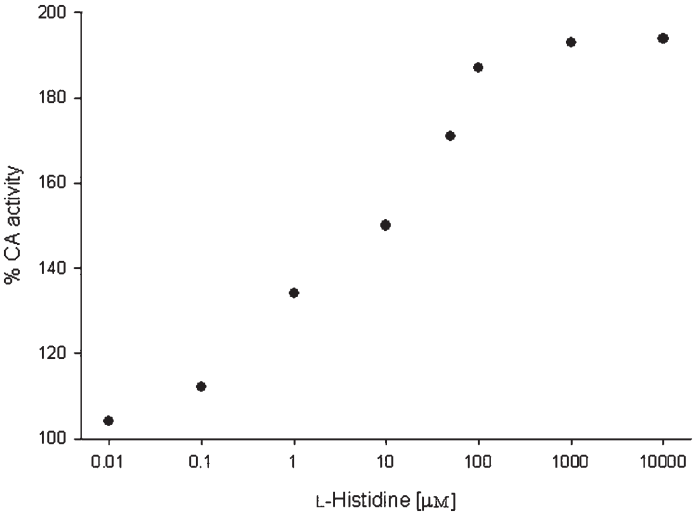

Figure 1. Activation of hCA II (10 nM) with L-histidine, in the concentration range of 10^{-8}–10^{-2} M, for the CO_2-hydration reaction at 25 °C, 10 mM Hepes buffer, pH 7.50. CA activity in the absence of activator was taken as 100%.

Discussion

Systematic studies of the activation of CA isoforms I and II with amino acids and related compounds, including natural/synthetic amino acids, their esters, *N*-alkyl, *N*-acyl, or pyridinium derivatives, have been reported by this group.[3,21] Such studies led to the following observations: 1) The most powerful CAAs in this series of compounds were L-histidine, L-proline, L-homoproline, together with aromatic amino acids, structurally related to L-phenylalanine (for example, compounds incorporating substituted phenyl rings, such as 4-halogeno-, 4-hydroxy-, or 4-amino-phenyl moieties, among others). In fact, X-ray crystallographic data (for the hCA II–histamine adduct, and the hCA II-azide-phenylalanine ternary complex)[12,14] clearly demonstrated that the aryl/hetaryl moieties present in the activator molecule increase the stability of the enzyme-activator complexes. 2) Derivatization of the amino or carboxylic groups generally diminished the activator efficiency, mainly due to decrease in the charge on the most electronegative atom of the molecule (through induced electronic effects), as it was later rationalized by means of QSAR calculations.[11] However, some *N*-derivatized or carboxy-derivatized amino acid derivatives still showed good CA-activation properties against isozymes hCA I and II.[3,21] 3) A strong correlation has been observed between the pK_a value of the activator molecule and its potency, with compounds possessing a pK_a in the range 6.5–8.0 for at least one deprotonatable moiety, leading to the best CA I and II activating properties.[3] All these data clearly showed that a CAA must possess specific steric and electronic requirements for good activity: First, it must fit within the restricted active-site cavity of the enzyme, but should also interact favorably with amino acid residues present in the activator binding pocket. Second, an ideal CAA should possess a moiety able to participate in proton-transfer processes, best suited with a pK_a in the range of 6.5–8.0 units.

It must be noted that no D-amino acids have been investigated up to now for CA-activation effects, and no isoforms other than the red blood cell cytosolic hCA I and II have been included in such studies. Here we present activation data with L-His and D-His against six physiologically relevant human CA isozymes, that is, hCA I, II, IV, VA, VII, and XIV (Table 1 and Figure 1), for the physiological reaction catalyzed by them (CO_2 hydration to bicarbonate and a proton). Among the CA isoforms investigated in the present study, some are cytosolic (hCA I, II, and VII), others are extracellular (CA IV and CA XIV), and CA VA is mitochondrial.[4] Two of the cytosolic forms, hCA I and II, are ubiquitous in the human body, whereas isoform VII is restricted to the brain.[4] Both hCA IV and hCA XIV have an extracellular active site, being membrane-associated (CA IV) or transmembrane (CA XIV) isoforms, quite abundant in the brain (and other tissues too)[4,7] where they seem to play important physiological roles in pH homeostasis.[19] Finally, CA VA is a mitochondrial isozyme involved in several biosynthetic processes such as gluconeogenesis, ureagenesis, and lipogenesis, among others.[22,23] Inhibition of many of these CAs by means of sulfonamides or sulfamates was recently shown to lead to novel approaches for the development of pharmacological agents useful for the treatment of glaucoma, obesity, or cancer, among others.[23–26]

As reported previously,[14] the affinity constant (K_{aff}) of an activator for the corresponding CA isoform has been denominated the "activation constant (K_A)" in order to obtain a measure of the strength for the interaction between enzyme and activator, similarly with the inhibition constant (K_I), which defines the potency of an inhibitor in the enzyme-inhibitor (EI) complex.[3,4] By representing the catalytic increase as a function of activator concentration, a typical sigmoid curve is obtained, from which the affinity constant (K_A) may be estimated by nonlinear least-squares fitting. An example of K_A determination is provided in

Figure 1 for the activation of hCA II with L-His. It may be observed that L-His starts to increase the hCA II catalytic activity at concentrations as low as 0.1 μM (with an activation of 112%). At 1 μM a stronger activation (of 134%) is observed that increases at around 150% at 10 μM concentrations of L-His. By further increasing the concentration of activator, the catalytic increases are dose-dependent until a plateau is reached of around 190% at concentrations of 100 μM–10 mM of L-His (Figure 1). Fitting the observed catalytic increases shown in Figure 1 as a function of L-His concentration allows the estimation of the affinity constant of L-His for hCA II that is of the order of 10.9 μM. We obtained all the activation constants reported in Table 1 in a similar manner. It should be also mentioned that detailed kinetic measurements (data not shown) showed that the activator does not change the value of the Michaelis–Menten constant (K_M) that is the same in the absence or the presence of the two activators investigated here. On the contrary, the observed catalytic rate of the enzyme (k_{cat}) is increased in the presence of activators (Table 1), supporting our previous observations[3,14] that CAAs do not influence the binding of CO_2 to the CA active site, but intervene in the rate-determining step of the catalysis (the transfer of protons from the active site to the environment).

The data in Table 1 also show that concerning the catalytic activity for the CO_2 hydration reaction (at 25°C, under steady-state conditions), there are two types of CA isoforms: the low activity ones (CA I-like), including hCA I, hCA VA, and hCA XIV, among others (k_{cat} values in the range of $(2.0–3.1)\times10^5\ s^{-1}$), and the high activity ones (CA II-like), among which include hCA II and hCA IV (k_{cat} values in the range of $(1.2–1.4)\times10^6\ s^{-1}$). On the other hand, hCA VII is an intermediate case, being similar to the high-activity isoforms, with a k_{cat} of $9.5\times10^5\ s^{-1}$. Our data show that all six CA isoforms are activated by L- and D-His, but in a very different manner (Table 1). Thus, the high activity isoforms hCA II and hCA IV were the least-activated isozymes both by L- and D-His. In fact, both activators showed rather high activation constants, in the range of 7.3–10.9 and 12.3–43.5 μM for L-His and D-His, respectively. Thus, hCA II, one of the best catalysts known in nature,[5,7] is also the least-activatable isoform with these two amino acids. This is quite logical since the proton-transfer processes in hCA II, assisted by His64,[5–8,15–17] are already very efficient for assuring a high turnover of the catalytic cycle, and thus, quite high concentrations of activator are needed for supplementing these processes. However, it should be noted (Table 1) that even the low concentration of L-His of 10 μM, produces a threefold increase in k_{cat} that is remarkable for such an efficient enzyme (and hCA II is the least-activatable isoform among those investigated here). It may also be observed that L-His is a much more efficient hCA II activator (around four times) as compared to its stereoisomer D-His, a fact that will be rationalized shortly when the X-ray crystal structures of these two adducts will be discussed. The same is true for hCA IV (an isozyme also possessing His64 as a proton-shuttle residue),[8] but for this isoform the differences in the activation constants of the two amino acids are much smaller, with L-His being only 1.68-fold a better activator as compared to D-His.

For the low-activity isoforms (including here also hCA VII, which, as mentioned earlier, shows an intermediate behavior between low and high activity CAs), the activation constants with the two amino acids investigated here were much lower, in the range of 0.03–1.34 and 0.09–2.37 μM for L-His and D-His, respectively (Table 1). The least active isoform (for the CO_2 hydration reaction), hCA I, was also the one most sensitive to activation by L- and D-His showing nanomolar affinity for binding within its active site, and an increase of 6.7-times of the k_{cat} value in the presence of 10 μM of L-His for example (see Table 1). In fact, although hCA I has been known for a long time,[3] its catalytic mechanism is less well understood as compared to that of the high-activity isoform hCA II.[5] In contrast to hCA II, isoform I has several more His residues within the active-site cavity (His64, His67, His200, and His243) that might participate in proton-transfer processes between the zinc-bound water molecule and the external reaction medium.[14] However, as shown by detailed kinetic, site-directed mutagenesis, and crystallographic studies of Lindskog's group,[5] none of them has the appropriate pK_a or steric arrangement for efficiently assisting such proton-transfer processes that are critical for the catalytic cycle.[14] As a consequence, hCA I has a much lower catalytic activity as compared to hCA II, although the sequence homology between these two ubiquitous cytosolic isoforms is quite high.[4] This may also explain why in the presence of an external activator, such as L- or D-His investigated here, the catalytic efficiency of hCA I is greatly improved, and the activators show high binding affinity for its active site.

The other investigated isoforms, hCA VA, hCA VII, and hCA XIV, also showed activation constants in the low micromolar range for the two activators (K_A values of 0.90–1.32 and 0.12–2.37 μM for L-His and D-His, respectively). It should be noted that among these isoforms, hCA VII and XIV have the His unit in position 64, which is thought to be involved in the proton-transfer processes between the active site and the environment,[15] whereas hCA VA has a Tyr unit in this position,[17] an amino acid that cannot take part in such processes in the pH range at which the experiments have been conducted.[14] However, isoforms VII and XIV are those best activated by L-His (after hCA I), whereas hCA VA shows a slightly higher activation constant with this amino acid. The opposite is true for D-His that is a better hCA VA activator than L-His by approximately 11-times, with an activation constant comparable to that shown towards hCA I (0.12 μM for CA VA as opposed to 0.09 μM for CA I). The activation constant of D-His for hCA VII is also in the submicromolar range (0.71 μM), whereas that for hCA XIV is 2.37 μM.

These data clearly suggest at least three important facts: 1) The least catalytically efficient CA isozymes (such as CA I and VA, among others) are generally the most activatable by L- and D-His, whereas the very active ones (such as CA

II and IV) show higher activation constants for these two amino acids. 2) There are important differences in activating efficacy of the two stereoisomers, L- and D-His. Some isoforms, such as CA I, II, IV, and XIV, are better activated by the L-amino acid, whereas others (such as CA VA and CA VII) show a better activation profile with D-histidine. This fact is quite important as it points out that the diverse CA isozymes probably possess different activator binding sites on one hand, and on the other hand, such structural differences may lead to the discovery (or the development) of isozyme-selective CAAs. 3) By selecting different CA isozymes and diverse CAAs among the many classes of such derivatives already reported,[3,4a,21] it is possible to engineer proton-transfer processes between the active site of the enzyme and the reaction medium, increasing the catalytic efficiency of a particular isoform. Such phenomena are critical for better understanding the catalytic mechanisms of enzymes for which the rate determining step is a proton-transfer reaction, as well as in this particular case, for the design of more efficient CAAs, some of which may show biomedical applications for the management of CA deficiencies or Alzheimer's disease, among others.[21]

The first structure obtained by X-ray crystallography for a CA-activator complex was that of the adduct between hCA II and histamine.[14] This structure has confirmed our previous hypothesis on the activation mechanism,[3] revealing that histamine binds in the hydrophilic region located at the entrance of the active site, establishing through the nitrogen atoms of the imidazole ring several hydrogen bonds with water molecules and polar amino acids residues, whereas the aliphatic amino group was exposed freely into the solvent. The supplementary hydrogen bond pathways generated by the binding of the activator have two key consequences for the rate-determining step of catalysis: 1) In the stabilization of the His64 "in" conformation—a steric requirement for the proton-shuttling.[15] 2) By offering adjacent routes for the proton transport from the zinc-bound water molecule to the external medium. Furthermore, histamine shows few contacts with the enzyme, limited to the imidazole ring that interacts with three protein amino acid residues (Asn62, Asn67, Gln92). This interaction is favorable for the CA-histamine complex dissociation in the last step of the activation mechanism, in the same way in which the conformational flexibility of His64 confers to this residue the ability to easily shuttle protons between the deep part and the rim of the active-site cavity during the catalytic cycle, thus facilitating the overall proton-transfer processes. It can thus be concluded that the activator actually acts as an efficient second proton shuttle, besides the native one, His64. It should be also mentioned that hCA II binds histamine with the displacement of at least three water molecules from the active-site cavity, followed by a substantial rearrangement of the hydrogen bond network within the cavity.[14] The entropic contribution to the histamine free energy of binding provided by the release of water molecules energetically favors the CA-activator complex formation, in addition to its kinetic and steric stabilization through the new hydrogen bond bridges. By using histamine binding to hCA II as a model system,[14] we decided to investigate by means of X-ray crystallography the binding of L- and D-histidine to this physiologically relevant isoform.

To assess the molecular basis responsible for the activating properties of L-His and D-His towards hCA II, and presumably also other isoforms (as the active-site residues that differ among the various α-CA isozymes are rather limited),[3,7,8] we solved the crystal structures of these complexes, which were prepared and crystallized as previously reported for other CA–activator adducts.[12,14,18] These three-dimensional structures were analyzed by difference Fourier techniques, the crystals being isomorphous to those obtained for the native enzyme (see the Experimental Section for details).

The structure refinement allowed us to evidence the spatial arrangement of the activators within the enzyme active site, with the omit electron density maps shown in Figure 2. It may be observed that one molecule of L-His or D-His is bound to the entrance of the active-site cavity, similar to the binding of histamine[14] and phenylalanine[12] to this isozyme. However, it may already be observed that the two stereoisomers bind differently to the enzyme (Figure 3).

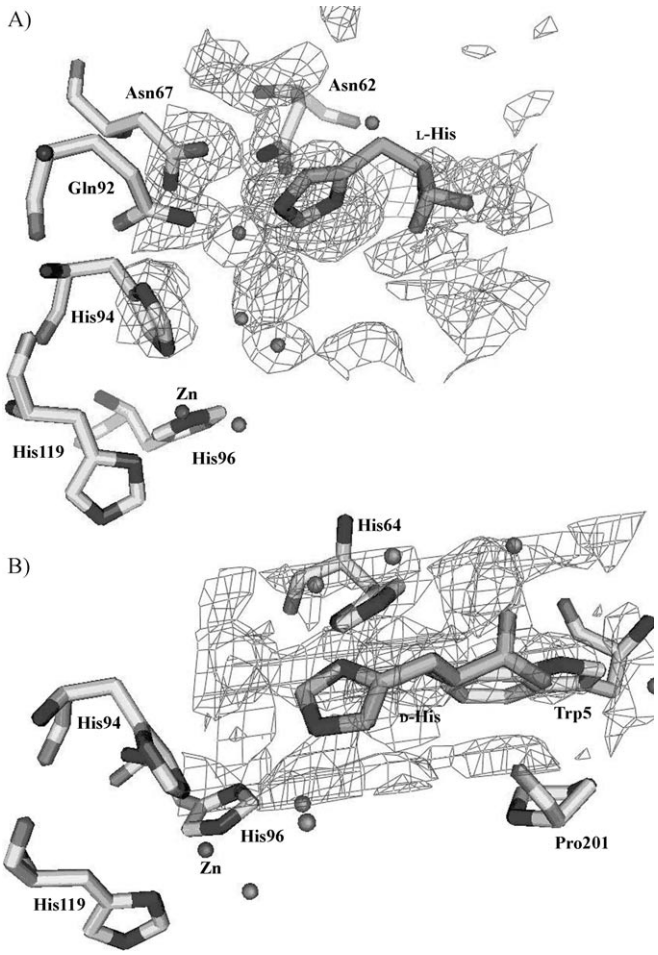

Figure 2. Omit electron density maps of hCA II in complex with L-His (A) and D-His (B) at 2.5 σ.

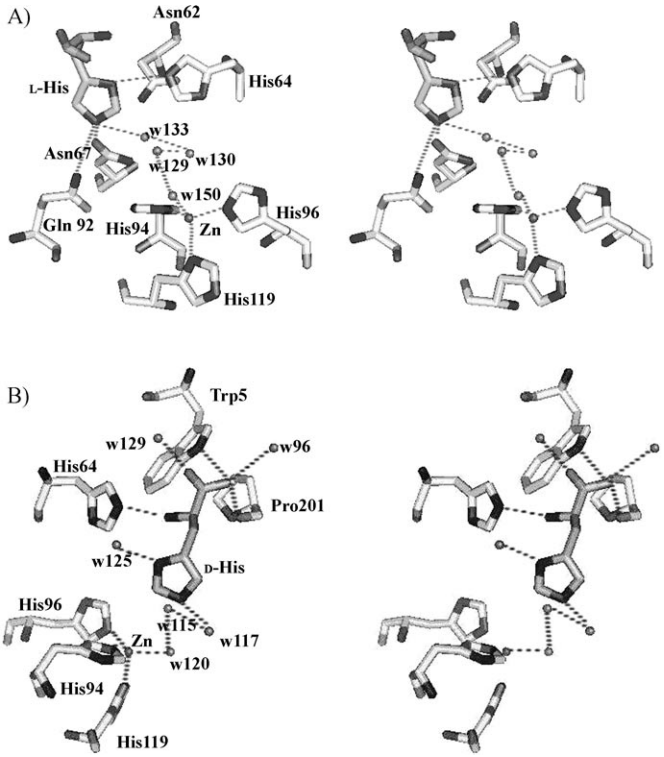

Figure 3. Stereoview of the hCA II–L-His (A) and hCA II–D-His (B) adducts, with amino acid residues participating in recognition of the activator molecule evidenced. Hydrogen bonds, water molecules involved in the binding of the activators, and the active-site Zn^{II} ion coordination are also shown.

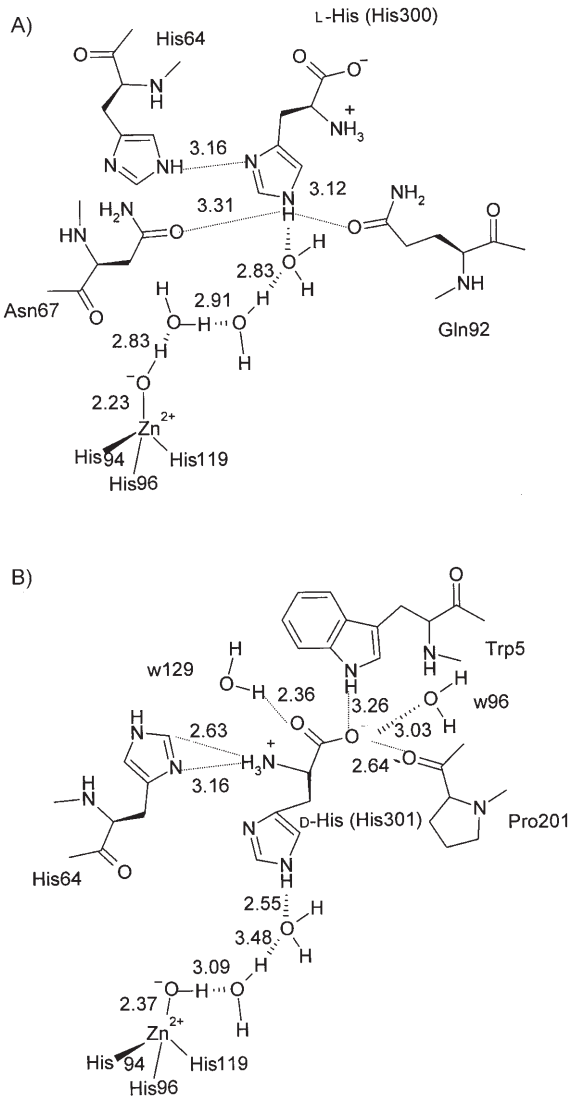

Figure 4. Schematic representation for the binding of L-His (numbered as His300) (A) and D-His (numbered as His301) (B) to the hCA II active site. The Zn^{II} ligands and hydrogen bonds connecting the Zn^{II} ion and the activator molecules through a network of several water molecules are shown, as well as the hydrogen bonds (dotted lines) between the activator molecules and amino acid residues involved in their binding (values represent distances in Å).

Inspection of the electron density maps at various stages of the crystallographic refinement, showed features compatible with the presence of one L-His/D-His molecule bound to the active site, as clearly illustrated in Figure 3. The binding of the activator to the enzyme did not significantly perturb the enzyme structure, even in close proximity of the ligand. As a matter of fact, the root-mean-square of the deviation-(rmsd), calculated over the entire Cα atoms of hCA II–L/D-His complexes with respect to the unbound enzyme was 0.35 Å. Interactions between the protein and Zn^{II} ion were entirely preserved in these adducts (Figure 3). Thus, the Zn^{II} ion retained a tetrahedral geometry, being coordinated to the imidazolic moieties of His94, His96, and His119, as well as to a water molecule (zinc–oxygen distance of 2.23 and 2.37 Å for the L-His adduct and D-His adduct, respectively) (Figures 3 and 4).

As seen in Figure 3 A and Figure 4 A, L-His binds at the entrance of the hCA II active site, in the activator binding site previously discovered by us in the histamine–hCA II adduct,[12,14] participating in three strong hydrogen bonds with Nε of His64 (distance of 3.16 Å), the carbonyl oxygen of Gln92 (distance of 3.12 Å), and Oδ of Asn67 (distance of 3.31 Å). In addition, the activator molecule participates in an extended network of hydrogen bonds that involve its Nε moiety, three water molecules (w133, w130, and w129), as well as the zinc-bound water molecule (w150). All these hydrogen bonds are strong, the distances between two adjacent oxygen atoms being in the range of 2.83–2.91 Å (Figure 4 A). They are probably also involved in the proton-transfer processes by which activators facilitate the rate-determining step of the catalytic cycle. Again it should be emphasized that in hCA II, in the absence of activators, it is the imidazole moiety of His64, which acts as a proton shuttle between the active site and the environment.[3,4–8,14–17] It must also be mentioned that similarly to histamine,[14] the amino group (in fact the ammonium moiety, as the amino acids are probably in their zwitterionic form at the pH values at which the experiments have been performed) of L-His does not participate in any contacts with the enzyme. The same is true for the carboxylate moiety of L-His, and

this may also explain why some L-His derivatives in which either the amino or the carboxy moieties have been modified, still possess CA activatory properties, which in some cases were even enhanced as compared to those of L-His[18] (data not shown). Indeed, the methyl ester of L-His or the dipeptide β-alanyl-histidine (carnosine) showed enhanced CA II activatory properties as compared to L-His,[18,21] probably because the modified carboxy/amino moieties participate in more favorable contacts with amino acid residues in the neighborhood of the activator binding site, some of which (such as Leu198, Pro202, Pro201, and Phe131) are shown in Figure 3 A.

The binding of D-His to hCA II is on the other hand quite different as compared to that of its stereoisomer L-His discussed above (Figures 3 B and 4 B). Thus, similarly to L-His, also D-His is anchored at the entrance of the enzyme active-site cavity by interacting with three amino acid residues present there. However, these amino acid residues are different, being Trp5, Pro201, and His64. Furthermore, the imidazole moiety of D-His (unlike the corresponding moiety of L-His) does not participate in any such hydrogen bond with the above-mentioned amino acid residues involved in the anchoring. On the contrary, just the ammonium and carboxylate moieties of D-His participate in such interactions, a situation again completely different from that observed in the hCA II–L-His adduct, in which the ammonium and carboxylate moieties of the activator did not hydrogen bond with amino acid residues (or water molecules) from the active site (Figures 2 A and 3 A). Thus, the COO^- group of D-His makes four hydrogen bonds with Pro201, Trp5, and two water molecules (w96 and w129). Practically, the O^- of the COOH moiety of D-His makes a strong hydrogen bond of 2.64 Å length with the carbonyl oxygen of Pro201, a weaker hydrogen bond (of 3.03 Å) with w96, as well as an even weaker one (of 3.26 Å) with the endocyclic NH moiety of Trp5. The carbonyl oxygen of the COO^- moiety of D-His is on the other hand involved in a very strong hydrogen bond (of 2.36 Å) with w129 (Figures 3 B, 4 B). The ammonium moiety of D-His is also involved in two hydrogen bonds, one with the imidazolic nitrogen of His64 (of 3.16 Å), and a second with the CH moiety of the imidazolic ring situated between the two nitrogen atoms (of 2.63 Å). It should be also noted that a weak hydrogen bond has been evidenced between another water molecule (w123) and the CH_2 moiety of the activator molecule (data not shown in Figure 4 B).

Another difference between the two adducts reported here is related to the number of water molecules connecting the zinc-bound water to the activator, that, as shown above, involves three water molecules for the L-His adduct, but only two water molecules in the case of the D-His adduct (Figures 3 and 4). This relay of water molecules is involved in the proton-transfer processes between the active site and the environment.[14,15] For the D-His adduct discussed here, it is possible to see that the two water molecules involved make longer (weaker) hydrogen bonds (in the range of 2.55–3.48 Å) as compared to the corresponding relay observed for the three water molecules present in the L-His adduct (distance in the range of 2.83–2.91 Å) (Figure 4).

Although L-His and D-His are structurally very similar, their binding to hCA II is rather different, excepting the fact that they bind in the same region of the active site. A superposition of the two adducts is shown in Figure 5. Thus, although the two stereoisomeric amino acids both bind to the entrance of the active site, their orientation is quite different, with L-His penetrating deeper into the cavity, and interacting with amino acids Asn67, Gln92, and His64 by means of its imidazolic moiety, whereas D-His is positioned more towards the external part of the active site, where it interacts only by means of its carboxy and amino moieties with different amino acid residues, that is, Pro201, Trp5, and His64. In fact only this last amino acid, His64 (which, as stressed throughout this paper, is critically important for the catalytic cycle) interacts both with L- as well as with D-His, but in a very different manner. These results also explain the difference in the activating efficiency of the two amino acids towards hCA II. As shown above (Table 1), L-His is approximately a fourfold better activator as compared to its stereoisomer, D-His. This is probably due to the fact that L-His binds in a more favorable way for assisting intramolecular proton-transfer processes between the active-site cavity and the environment, as compared to the D-amino acid. Thus, the present findings constitute an interesting example of designing proton-transfer pathways within the active site of an enzyme, on the one hand leading to an enhanced catalytic activity, and on the other hand, that might be exploited for the design of pharmacologically useful compounds.

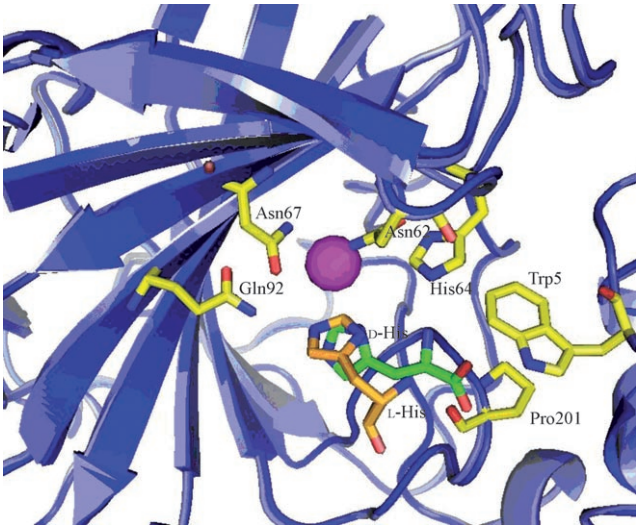

Figure 5. Superposition of the two hCA II adducts with L-His (orange) and D-His (green), with the zinc ion (magenta sphere) and amino acid residues present in the activator binding site (in yellow) evidenced.

Conclusion

Activation of six CA isoforms, hCA I, II, IV, VA, VII, and XIV, with L- and D-histidine has been investigated by kinetic and X-ray crystallographic methods. L-His behaved as a potent activator of isozymes I, VA, VII, and XIV (activation constants in the range of 0.03–1.34 μM), and a weaker activator of isoforms II and IV (K_A of 7.3–10.9 μM). D-His showed good hCA I, VA, and VII activatory properties (K_A in the range of 0.09–0.71 μM), being a moderate hCA XIV (K_A of 2.37 μM), and a weak hCA II and IV activator (K_A in the range of 12.3–43.5 μM). The X-ray crystallographic structure of the hCA II–L-His/D-His adducts showed the activators to be anchored at the entrance of the active-site cavity, participating in an extended network of hydrogen bonds with different amino acid residues and water molecules that may explain their different potency as well as interaction patterns with various CA isozymes. The His64, Asn67, and Gln92 amino acid residues were involved in L-His recognition, whereas three water molecules connected the activator to the zinc-bound water. Only the imidazole moiety of L-His interacted with these amino acids. For the D-His adduct, the amino acid residues involved in recognition of the activator molecule were Trp5, His64, and Pro201, and two water molecules connected the zinc-bound water to the activator in an extended network of hydrogen bonds. Furthermore, only the COOH and NH_2 moieties of the activator participated in hydrogen bonds with these amino acid residues. This is the first study showing the very different binding mode for stereoisomeric activators within the CA II active site, with consequences for the overall proton-transfer processes that are rate-determining for the catalytic cycle. It also points out the differences of activation efficiency between various isozymes with structurally related activators, that may be exploited for designing alternative proton-transfer pathways, useful both for a better understanding of the catalytic mechanism as well as for obtaining pharmacologically useful derivatives, mainly for the management of Alzheimer's disease, a condition in which the dysfunction of CA II activity has recently been evidenced.

Experimental Section

Materials: Zinc sulfate, sodium sulfate, ammonium sulfate, L-histidine, D-histidine, glutathione, ampicillin, chloramphenicol, yeast extracts, tryptone, thrombin, Tris-HCl, Hepes, *p*-aminomethylbenzenesulfonamide-agarose and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma–Aldrich, Milan, Italy. The prepacked glutathione sepharose 4B column was from Amersham. The various CA isozyme expression cells BL21 codon plus DE3(RIL) were from Stratagene, La Jolla, CA. All other chemicals were of reagent grade and were used without further purification.

Expression and purification of the recombinant human carbonic anhydrase isozymes I, II, IV, VA, VII, and XIV: The expression vector pMA-5-8 containing the hCA I or hCA II genes (at the Sma I and BamH I restriction cleavage sites) was a gift from Dr. Sven Lindskog. The corresponding plasmid was transformed into *E. coli* BL21 codon plus DE3-(RIL) host cells by following the standard molecular biology protocol.[6] The transformed cells were grown in LB medium, supplemented with 100 μg of ampicillin per milliliter, 50 $\mu g\,mL^{-1}$ of chloramphenicol, and 60 $\mu g\,mL^{-1}$ of $ZnSO_4$ at 37 °C until the A_{600} was 0.6. The expression of hCA I/hCA II was induced by addition of 400 μM IPTG and 400 μM $ZnSO_4$. The cells were incubated further at 25 °C overnight, and then centrifuged at 5000 *g* for 15 min. The pellet was washed in 20 mM Tris-HCl, pH 8.7, and was resuspended in the same buffer containing 0.5 mM EDTA. A working concentration of 1 mM PMSF in 2-propanol was added prior to sonication. The cells were sonicated for a total time of 10 min in a Branson bath sonifier utilizing 40% duty cycle in an ice-cold bath. The sonicated extract was centrifuged at 15000 rpm for 30 min, and the supernatant (crude extract) was collected for further purification. The enzyme activity of the recombinant hCA I or II was determined by spectrophotometric measurements by the stopped-flow method with CO_2 as substrate.[27] The protein concentration was determined according to the Bradford method, utilizing BSA as the standard protein. The recombinant form of human carbonic anhydrase II was purified from the crude extract by using a *p*-aminomethylbenzenesulfonamide-agarose column.[28] The purified enzymes were subjected to the SDS–PAGE analysis to confirm the homogeneity of the enzyme.

Isoforms hCA IV, VA, VII, and XIV were obtained by using the GST-fusion protein approach, as described earlier by our group.[24–26] The various constructs reported earlier by this group,[24–26] were transfected into the *E. coli* strain BL21 for production of the GST-hCA fusion proteins. Following induction of the protein expression by adding 1 mM IPTG, the bacteria were harvested and sonicated in PBS. The cell homogenate was incubated at room temperature for 15 min and homogenized twice with a Polytron (Brinkmann) for 30 s each at 4 °C. The homogenate was centrifuged at 30000 *g* for 30 min affording the supernatant containing the soluble proteins. The supernatant was then applied to a prepacked glutathione sepharose 4B column. The column was extensively washed with buffer and then the GST-hCA fusion protein was eluted with a buffer consisting of 5 mM reduced-glutathione in 50 mM Tris-HCl, pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin.[24–26] The advantage of this method is that the hCA isoform is purified easily and the procedure is quite simple. The obtained hCA was then further purified by sulfonamide affinity chromatography,[28] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[27]

CA activation assay: An Applied Photophysics stopped-flow instrument was used for assaying the CA-catalyzed CO_2 hydration activity.[27] Phenol Red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining a constant ionic strength), was used for following the CA-catalyzed CO_2 hydration reaction. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction were used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activator (10 mM) were prepared in distilled–deionized water and dilutions up to 0.1 nM were performed thereafter with distilled-deionized water. Activator and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–A complex. The affinity constants (K_{aff}) of the activator for the enzyme were obtained by fitting the observed catalytic increases as a function of the activator concentration by nonlinear least-squares methods by using PRISM 3, as reported earlier by Briganti and co-workers[14] and represent the mean from at least three different determinations.[24–26] The activation constant (K_A) is defined as: $K_A = K_{aff}^{-1}$, in order to obtain values comparable with the inhibition constants (K_I) of CA inhibitors.

X-ray crystallography: Crystals of hCA II–L-His complex were obtained by co-crystallization at 4 °C in hanging drops, by using a vapor diffusion technique, as previously described.[7] The crystals were mounted in a sealed glass capillary with a droplet of mother liquid. Diffraction data were collected on a Rigaku AFC5R four-circle diffractometer by using a Rigaku RU200 rotating anode, operating at 50 V, 180 mA, in ω scan mode with $Cu_{K\alpha}$ radiation (1.5418 Å). The unit cell dimensions were de-

termined to be $a=42.70$, $b=41.79$, $c=72.83$ Å, and $\alpha=\gamma=90$, $\beta=104.47°$ in the space group $P2_1$. Data were collected by using a Mar300 Image-Plate Detector from MarReasearch and processed with the software DENZO.[29] A total of 15867 unique reflections were obtained with a completeness of 95.0% to a resolution of 2.0 Å. The structure was analyzed by the difference Fourier technique, by using the PDB file 1AVN as the starting model for refinement.[14] Electron density maps ($2F_o-F_c$) and (F_o-F_c) were calculated with the CNS program[30,31] and displayed by using the graphic program O.[31] The final model had an R factor of 0.18, R-free 0.218, for 14294 reflections at $F>2$ sigma (F_o) in the resolution range 8–2.0 Å with an rms deviation from standard geometry of 0.005 Å in bond lengths and 1.34° in bond angles. The hCA II–L-His model was derived by including 147 solvent molecules. Comparison of the refined temperature factors showed the activator molecule had an occupancy of 100%, unlike the histamine–hCA II adduct previously reported,[14] for which a partial occupancy of 30–40% was observed for histamine. Crystallographic parameters and refinement statistics are summarized in Table 2.

Table 2. Crystallographic parameters and refinement statistics for the hCA II–D-His and hCA II–L-His adducts.

Paramater	Value	
	hCA II–D-His	hCA II–L-His
X-ray source	rotating anode	rotating anode
wavelength [Å]	1.54	1.54
cell parameters		
a [Å]	42.06	42.70
b [Å]	41.43	41.79
c [Å]	72.52	72.83
β [°]	104.40	104.47
space group	$P2_1$	$P2_1$
no of unique reflections	13398	15867
completeness [%][a]	93.5 (89.0)	95.0 (90.1)
no of reflections [$>2\sigma(F_o)$]	13056	14294
$<I/\sigma(I)>$ [a]	10.23 (4.7)	22.1 (5.1)
R factor	0.249	0.182
R free [b]	0.300	0.218
rmsd of bonds from ideality [Å]	0.0016	0.0050
rmsd of angles from ideality [°]	1.34	1.34

[a] Values in parentheses relate to the highest resolution shell, 2.07–2.00. [b] Calculated by using 5% of the data.

The hCA II–D-His complex was also co-crystallized at 4°C by the hanging-drop vapor-diffusion method. Drops containing 5 µl of 10–20 mg mL^{-1} hCA II in 50 mM Tris-HCl buffer, pH 7.7–7.8, were mixed with 5 µL of precipitant buffer (2.4–2.5 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl, pH 7.7–7.8, and 1 mM sodium 4-(hydroxymercury)benzoate) with an added 50 mM of D-His, and equilibrated over a reservoir of 1 mL of precipitant buffer. Crystals were transferred into a cryoprotectant solution (20% ethylene glycol), mounted in a nylon loop and exposed to a cold (100 K) nitrogen stream. Diffraction data were collected on a CCD detector KM4 CCD/Sapphire by using $Cu_{K\alpha}$ radiation (1.5418 Å). The unit cell dimensions were determined to be $a=42.06$, $b=41.43$, $c=72.52$ Å, and $\alpha=\gamma=90$, $\beta=104.40°$ in the space group $P2_1$. Data were processed with the Mosfilm[32] and CCP4 suite.[33] A total of 13398 unique reflections were obtained with a completeness of 93.5% to a resolution of 2.0 Å. The structure was analyzed by the difference Fourier technique, by using the PDB file 1AVN[14] as the starting model for refinement. Electron density maps ($2F_o-F_c$) and (F_o-F_c) were calculated with the CNS program[30] and displayed by using the graphics program O.[31] The final model had an R factor of 0.249, R-free 0.300 (due to the lower quality of the crystals as compared to the L-His adduct), for 13056 reflections at $F>2$ sigma (F_o) in the resolution range 8–2.0 Å with a rms deviation from standard geometry of 0.0016 Å in bond lengths and 1.34° in bond angles. The number of solvent molecules used to build the hCA II–D-His model was 127, and the occupancy of the activator was of 100% as for the L-His adduct. Crystallographic parameters and refinement statistics are summarized in Table 2. Both structure coordinates have been deposited in the Brookhaven protein database (PDB ID 2ABE for the L-His adduct, and 2EZ7 for the D-His adduct, respectively).

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