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Carbonic anhydrase activators: The first X-ray crystallographic study of an adduct of isoform I☆

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Abstract—The X-ray crystallographic structure for the adduct of an activator with human carbonic anhydrase isozyme I (hCA I) is reported. L-Histidine binds deep within the enzyme active site, participating in a network of hydrogen bonds involving its carboxylate moiety and the zinc-bound water molecule, as well as the imidazole of His200, being in van der Waals contacts with Thr199, His200, His64, and His67. This binding is very different from that to the other major cytosolic isozyme hCA II.

Unlike carbonic anhydrase (CA, EC 4.2.1.1) inhibitors, widely clinically used for the treatment or prevention of a multitude of diseases,[1–3] activators of these enzymes were much less investigated.[4,5] By means of electronic spectroscopy on Co(II)-substituted enzyme, X-ray crystallography and kinetic measurements, it has been recently proved that CA activators (CAAs) bind within the enzyme active cavity (in the case of the physiologically most important isoform, human CA II, hCA II) at a site distinct of the inhibitor or substrate binding-sites,[4] 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] In this way, the activator behaves as a general base assisting deprotonation of the zinc-bound water molecule, with an enhanced generation of the basic form of the enzyme, containing hydroxide coordinated to Zn(II), which is the catalytically effective species of CA.[4–8]

A multitude of physiologically relevant compounds such as biogenic amines (histamine, serotonin, and catecholamines), amino acids, oligopeptides or small proteins among others, act as efficient CAAs for many of the 16 presently known human CA isoforms.[4,5] 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 constitute a possible therapy for the enhancement of synaptic efficacy, which may represent a conceptually new approach for the treatment of Alzheimer's disease, aging, and other conditions in which it is necessary the achievement of spatial learning and memory therapy.[9] In addition, the levels of several CA isozymes, including hCA I, are diminished in patients affected by Alzheimer's disease or in the older population.[10]

Few X-ray crystallographic structures of adducts of the main human isoform, hCA II, with activators are known at this moment: one with histamine,[11] one with L-histidine,[12a] and those with L- and D-phenylalanine,[12b] but no X-ray crystal structures of other CA isozymes with activators are available at this moment. All of them showed the activator molecules to be bound at the entrance of the hCA II active site cavity (in a region different from the inhibitor binding site), where they are anchored by hydrogen bonds to amino acid side chains (His64, Asn67, Gln92, and Thr200) 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, which in many CA isozymes (in the absence of activators) is assisted by

Keywords: Enzyme mechanism; Carbonic anhydrase; Activator; Amino acid; L-Histidine; X-ray crystallography.

☆ The X-ray coordinates of the hCA I–L-His adduct are available in PDF with the ID 2FW4.

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the amino acid residue His64 situated in the middle of the active site cavity, and also possessing a pH-dependent conformational mobility, changing gradually its orientation related to the metal site through a 64° ring-flipping.[13–16] 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.[4,5] No X-ray crystallographic studies of hCA I with activators have been reported up to now. Here, we report the first X-ray crystal data of the most abundant human CA isoform, hCA I, with L-histidine (L-His) as an activator. The physiological function of this isozyme is largely unknown at this moment,[17] although hCA I is present in very high amounts in red blood cells (there is 10 μM of hCA I in this tissue) and the gastrointestinal tract.[1,17]

Table 1. Crystallographic parameters and refinement statistics for the hCA I–L-His adduct

Parameter	Value
X-ray source	Enhance ultra
Wavelength (Å)	1.54
Cell parameters	$a = 62.14$ Å $b = 70.18$ Å $c = 120.57$ Å $\alpha = \beta = \gamma = 90°$
Space group	$P2_12_12_1$
Molecules/asymmetric unit	2
Total no. of reflections[a]	107,360 (13991)
No. of unique reflections	35,706 (4703)
Completeness (%)	97.4 (90.0)
$\langle I/\sigma(I)\rangle$	7.0 (2.5)
Resolution range (Å)	10.0–2.0
R-merge (%)	16.4 (38.8)
Multiplicity	3.0 (3.0)
Refinement	
No. of reflections [$>2\sigma$(Fo)]	33,884
Water molecules	368
R-factor (%)	19.5
R-free (%)[b]	21.4
Rmsd of bonds from ideality (Å)	0.011
Rmsd of angles from ideality (°)	1.36

[a] Values in parentheses relate to the highest resolution shell (2.10–1.99).
[b] Calculated using 5% of data.

The binding affinity of L-His for hCA I is high, this activator possessing an affinity constant of 30 nM for this isozyme, being on the other hand a weaker hCA II activator, with an affinity constant of around 10 μM[12a] (as determined by a stopped flow technique, for the physiological reaction catalyzed by CAs, CO_2 hydration to bicarbonate).[18] Kinetic measurements lead to the observation that the activation is due to an enhancement of k_{cat}, which for hCA I in the absence of activators (for the physiological reaction, at 25 °C and pH 7.5) is of $2.0 \times 10^5\ s^{-1}$, whereas in the presence of 10 μM L-His, this parameter is of $k_{cat} = 13.4 \times 10^5\ s^{-1}$. In all these experiments, the Michaelis–Menten constant K_m was the same, in the absence or in the presence of activators, the measured values being: $K_m = 25$ mM (in agreement with literature data).[1,2] It may be observed that 10 μM L-His (concentrations much higher than this one, in the range of 60–120 mM, are present in many tissues, including the brain)[19] leads to an enhancement of

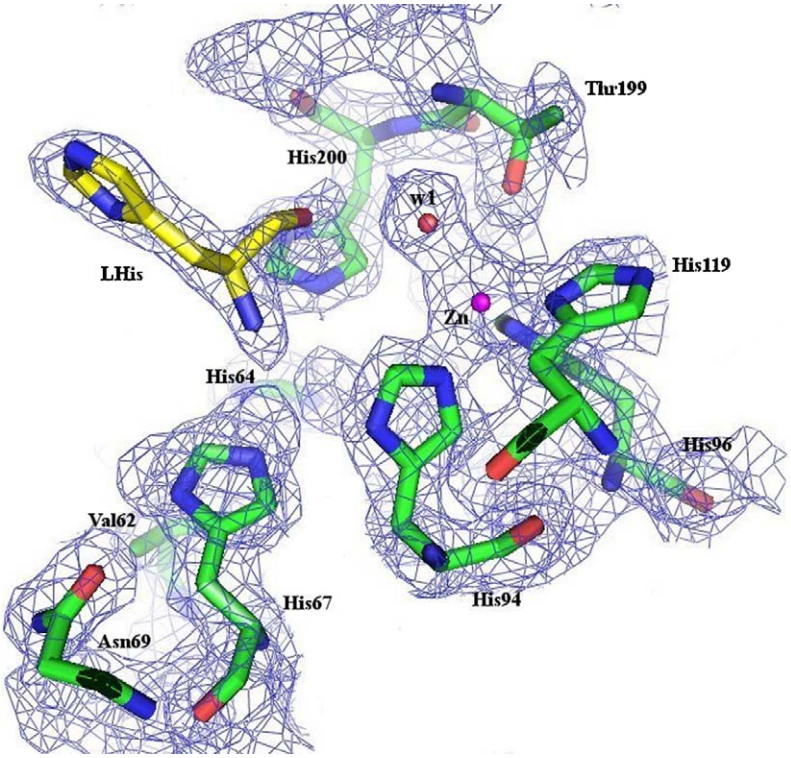

Figure 1. Electron density omit map contoured at 1σ of L-His (in yellow) binding site to hCA I. The Zn(II) coordination by His94, 96, and 119, as well as residues involved in the catalytic/activation mechanism (such as Thr199, His64, His67, Asn69, and Val162) are also evidenced.

670% of the hCA I catalytic activity. Such a phenomenon surely translates in important physiological consequences, probably correlated with pH homeostasis or ion transport processes in which hCA I is known to be involved.[1]

Although the sequence similarity between isozymes hCA I and II is quite high,[1,2] at least two critical amino acid residues from the active site cavity are different, that is, those at position 200 (His in hCA I and Thr in hCA II), and 67 (His in hCA I again and Asn in hCA II).[11] Indeed, the two bulky histidines (His200 and His67) lead to a highly restricted active site cavity for hCA I as compared to hCA II, which may explain both the lower catalytic activity of isoform I, as well as the fact that it is usually 100 times less prone than hCA II to be inhibited by sulfonamides.[7,11] As His200 is close to the Zn(II) ion, it is also probable that this residue may influence the binding of inhibitors, substrates or activators to the enzyme cavity. In fact it is well established that the corresponding residue in hCA II (i.e., Thr200) hydrogen bonds with inhibitors of the sulfamate/sulfamide type, as demonstrated by means of X-ray crystallography earlier.[17b] On the other hand, His67 is situated in the middle of the active site cavity, near His64 (conserved in both isoforms hCA I and II), a residue playing a critical role in catalysis, as mentioned above.[11] Another amino acid residue conserved in the two isozymes is Thr199, whose OH moiety hydrogen bonds the zinc-bound solvent molecule in all CAs investigated up to now by means of X-ray crystallography.[1–3,11] Thus, we expect a quite diverse pattern of interaction between the hCA I active site cavity and the activators, as compared to the corresponding adducts with isozyme hCA II.

In order to understand the binding of L-His to hCA I, the X-ray crystal structure of the adduct has been solved at 2 Å resolution.[20–24] Two independent molecules, related by a translation operator, were present in the asymmetric unit of the hCA I adduct with L-His (data not shown). The overall structure of the adduct is very close to that of the native enzyme, with no inhibitors/activators bound to it (Table 1).[20] On the other hand, it is important to stress that L-His binds very similarly to both molecules A and B of enzyme in the asymmetric unit of the adduct. Thus, for the following discussion, only molecule A will be taken into consideration, as the binding mode of the activator as well as relevant distances between various groups of the activator and the enzyme are very similar between molecules A and B (data not shown). Inspection of the electron density maps showed only one molecule of L-His bound within the active site of hCA I, both for molecules A and B of the asymmetric unit (Fig. 1).

The activator binding site of hCA I is rather different of that of hCA II (Figs. 2 and 3), case in which the same activator, L-His, was shown to bind at the entrance of the cavity, being anchored by several strong hydrogen bonds to His64, Asn67, and Gln92 (Fig. 3A).[12a] In the case of the hCA I–L-His adduct, it may be observed that the activator is bound much deeper within the enzyme

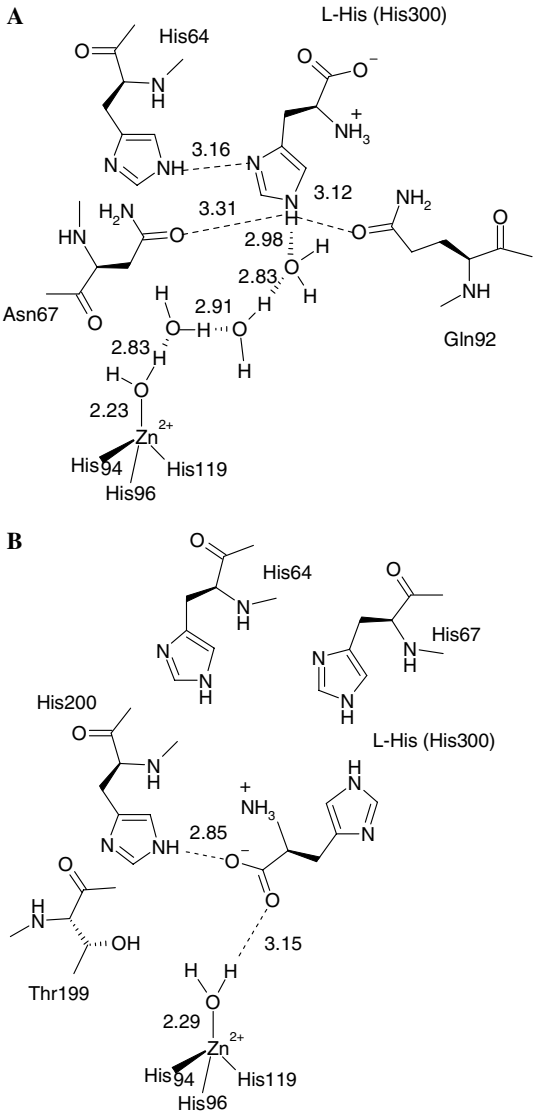

Figure 2. Schematic representation for the binding of L-His (numbered as His300) to the hCA II (A) and hCA I (B) active sites. The Zn(II) ligands and hydrogen bonds connecting the Zn(II) ion and the activator molecules through a network of water molecules are shown, as well as the hydrogen bonds (dotted lines) between the activator molecules and amino acid residues involved in their binding (figures represent distances in Å).

active site, bridging by means of two hydrogen bonds (of 3.15 and 2.85 Å, respectively) the zinc-bound hydroxide ion and the Nε imidazole atom of His200 (an amino acid residue characteristic for this isozyme, as in hCA II there is a Thr in position 200, Figs. 2 and 3).[12a] Furthermore, the orientation of the activator molecule is quite different in the two adducts, as it is the carboxylate moiety of L-His participating in the main interactions with the hCA I active site (in fact the COO^- points toward the zinc ion, Fig. 2B), whereas the imidazole moiety of L-His points toward the Zn(II) ion in the hCA II adduct, and also participates in a network of four hydrogen bonds with three amino acid residues and a water molecule bound within the cavity (Fig. 2A). In addition, L-His is in van der Waals contacts with Thr199, His64, His67, and His200 in the

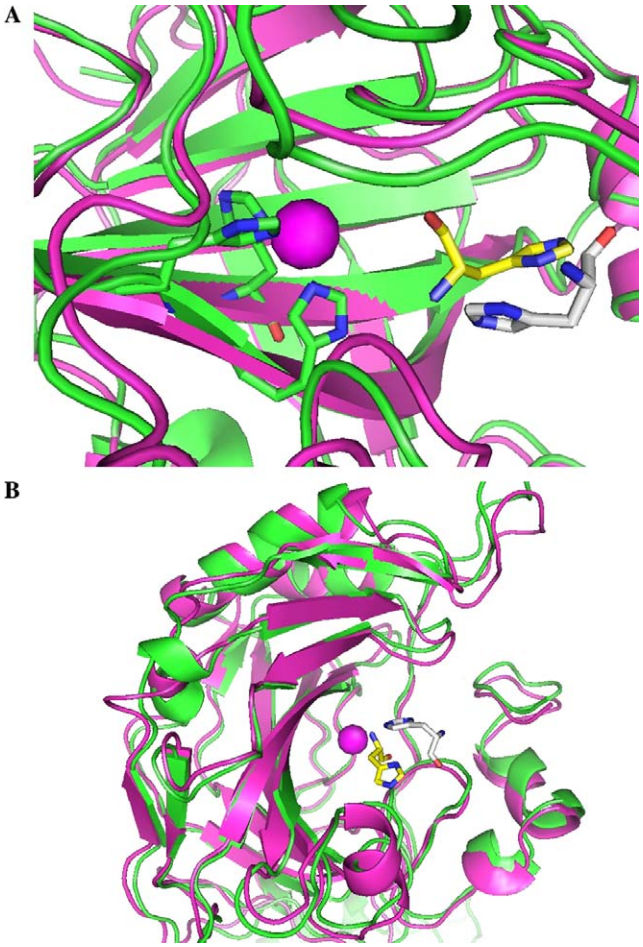

Figure 3. Superposition of the hCA II (magenta)/L-His (CPK) adduct (PDB ID 2ABE)[12] with the hCA I (green)/L-His (yellow) adduct (PDB ID 2FW4). (A) Details of the activator binding site with the Zn(II) ion, its three histidine ligands (His94, 96, and 119), and Thr199 evidenced (the zinc-bound solvent molecule is not shown). (B) Overall protein superposition, clearly showing the deep binding of the activator to hCA I and the external binding to hCA II.

hCA I adduct (Fig. 2B). In addition, the zinc-bound hydroxide is directly hydrogen-bonded to the activator molecule in the hCA I adduct, but is bridged by three different water molecules in the hCA II adduct, respectively (Figs. 2A and B). The important differences in binding of L-His to hCA I and II are also clearly observed in Fig. 3, where the two adducts are superposed. It may be observed that the globular shape and active site architecture of the two CA isozymes are quite similar, but the activator binds at the entrance of the active site cavity for hCA II, and quite deep within this site for hCA I. This may also explain the rather different affinity of this activator for the two isozymes, which is in the nanomolar range for hCA I, and in the micromolar range for hCA II.

In conclusion, we report here the first X-ray crystal structure of the highly abundant cytosolic isoform hCA I with an activator, L-His. The physiological function of this isozyme is not at all understood at this moment, as it is less understood its catalytic mechanism, as compared to that of the physiologically relevant and most studied isoform hCA II. L-His has nanomolar affinity for hCA I (being a very potent activator) and only micromolar affinity for hCA II, and these differences are well explained by comparing the two X-ray crystal structures of these isozymes with L-His, which show the activator to be bound very differently by the two closely related enzymes. This study clearly illustrates that very minor differences in the active site architecture of similar isozymes lead to a completely different pattern of interactions with small molecules acting as activators/inhibitors. It may also lead to the design of better hCA I activators, useful, for example, in patients affected by a genetic syndrome of hCA II deficiency (who possess normal levels of hCA I)[1b] or in increasing synaptic efficiency, since it is well established[10,11] that levels of various CA isozymes, including CA I, are diminished in patients affected by Alzheimer's disease or in the aging population.

Acknowledgments

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An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity. Solutions were purged with nitrogen prior to assay for 15 min in order to remove CO_2 present in them, and preincubation between enzyme and activator was done under N_2. Phenol red (at a concentration of 0.2 mM) has been 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 constant the ionic strength). The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator concentration at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solution of activator (1 mM) was prepared in distilled-deionized water and dilutions up to 1 nM were done thereafter with the same. 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 activation constant (K_A), defined similarly with the inhibition constant K_I,[12] may be obtained by considering the classical Michaelis–Menten equation (Eq. 1), which has been fitted by non-linear least squares by using PRISM 3:

$$v = v_{max}/\{1 + K_m/[S](1 + [A]_f/K_A)\} \quad (1)$$

where $[A]_f$ is the free concentration of activator. Working at substrate concentrations considerably lower than K_M ($[S] \ll K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by Eq. (2):[12]

$$v = v_0 \cdot K_A/\{K_A + ([A]_t - 0.5\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)^2 - 4[A]_t \cdot [E]t)^{1/2}\}\} \quad (2)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.
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