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Carbonic anhydrase activators: X-ray crystal structure of the adduct of human isozyme II with L-histidine as a platform for the design of stronger activators

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Abstract—Activation of the carbonic anhydrase (CA, EC 4.2.1.1) isoforms hCA I, II, and IV with L-histidine and some of its derivatives has been investigated by kinetic and X-ray crystallographic methods. L-His was a potent activator of isozymes I and IV (activation constants in the range of 4–33 μM), and a moderate hCA II activator (activation constant of 113 μM). Both carboxy- as well as amino-substituted L-His derivatives, such as the methyl ester or the dipeptide carnosine (β-Ala-His), acted as more efficient activators as compared to L-His. The X-ray crystallographic structure of the hCA II–L-His adduct showed the activator to be anchored at the entrance of the active site cavity, participating in an extended network of hydrogen bonds with the amino acid residues His64, Asn67, and Gln92 and, with three water molecules connecting it to the zinc-bound water. Although the binding site of L-His is similar to that of histamine, the first CA activator for which the X-ray crystal structure has been reported in complex with hCA II (Briganti, F.; Mangani, S.; Orioli, P.; Scozzafava, A.; Vernaglione, G.; Supuran, C. T. *Biochemistry* **1997**, *36*, 10384) there are important differences of binding between the two structurally related activators, since histamine interacts among others with Asn67 and Gln92 (similarly to L-His), but also with Asn62 and not His64, whereas the number of water molecules connecting them to the zinc-bound water is different (two for histamine, three for L-His). Furthermore, the imidazole moieties of the two activators adopt different conformations when bound to the enzyme active site. Since neither the amino- nor carboxy moieties of L-His participate in interactions with amino acid moieties of the active site, they can be derivatized for obtaining more potent activators, with pharmacological applications for the enhancement of synaptic efficacy. This may constitute a novel approach for the treatment of Alzheimer's disease, aging, and other conditions in need of achieving spatial learning and memory therapy.

Activation of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) has recently been shown to constitute a novel therapeutic approach for the enhancement of synaptic efficacy, which may constitute an excellent means for the treatment of Alzheimer's disease, aging, and other conditions in need of achieving spatial learning, and memory therapy.[1,2] In fact, it has previously been shown by this group that 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 CA activators (CAAs) for many of the 16 presently known human CA isozymes.[3–6] 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 of the inhibitor or substrate binding sites, participating thereafter in the rate-determining step of the catalytic cycle, that is, the proton transfer processes between the active site and the environment.[7,8]

In addition to clarifying basic aspects of the CA catalytic mechanism, CAAs might lead to interesting pharmacological applications, although this field is largely unexplored for the moment.[3–6] Thus, recently it has been reported[1] that phenylalanine, a CAA first investigated by our group,[8,9] when administered to experimental animals produces an important pharmacological enhancement of synaptic efficacy, spatial learning, and

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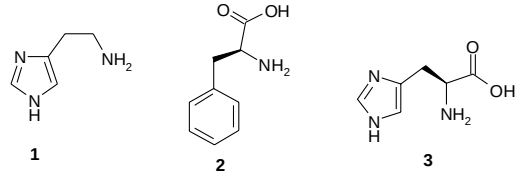
memory, proving that this class of unexplored enzyme modulators may be used for the management of conditions in which learning and memory are impaired. It should also be mentioned that it was previously reported that the levels of several CA isoforms (such as CA I and CA II) are significantly diminished in the brain of patients affected by Alzheimer's disease,[10] a fact strongly supporting the involvement of CAs in cognitive functions.[1,3–6]



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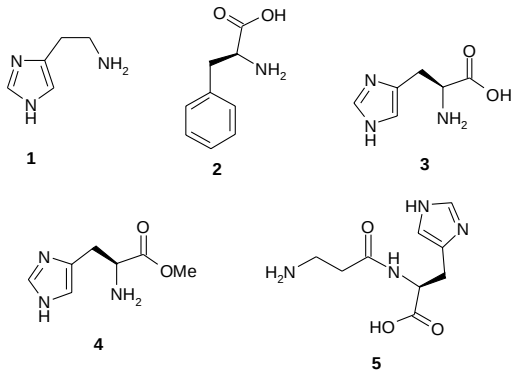
Two X-ray crystallographic structures of adducts of the main isoform, that is, human CA II (hCA II) with activators, are known at this moment: one with histamine,[7] and another one with phenylalanine (a ternary complex in which azide is also bound to the Zn(II) ion of the hCA II active site).[8] Both 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, a 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 the amino acid residue His64 situated in the middle of the active site cavity.[8–10] 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.

Considering mainly histamine or different di-/tripeptides as lead molecules, many types of potent CAAs have been reported for isozymes CA I, II, and IV,[11–16] but no other X-ray crystallographic studies for this type of pharmacological agents are available in the literature. Here, we report such a study, and more precisely we have resolved the X-ray crystal structure of the adduct of the physiologically most relevant cytosolic isoform hCA II[17] with L-histidine, an activator previously described by our group.[16] The new structure may be used as a platform for the design of new types of activators for many of the physiologically crucial CA isozymes identified up to now in higher vertebrates.

Compounds **1**–**5** investigated as activators of the physiologically relevant isozymes hCA I, II, and IV, that is, histamine, L-Phe, L-His, and its methyl ester, as well as the dipeptide carnosine (β-Ala-L-His) are commercially available from Sigma–Aldrich and were used without further purification.



1 2 3 4 5

Activation data with compounds **1**–**5** against the three above-mentioned human CA isozymes are shown in Table 1. hCA I and II data for **1**–**3** and **5** have previously been reported,[7,8,11–15] whereas the hCA IV data of all the compounds and the L-His-OMe **4** data against all isozymes are presented here for the first time.

It may be observed that all these derivatives act as potent hCA I activators, with activation constants (K_As) in the range of 1.2–4 μM. The best hCA I activators were L-Phe and carnosine, whereas L-His was the least effective one. Thus, derivatization of both the amino- as well as the carboxy moieties of L-His leads to an enhancement of the CA activatory properties, a finding that will be explained later after discussing the X-ray crystal structure of the adduct. Against hCA II, histamine **1**, and L-His **3** act as medium–weak activators, with K_As in the range of 113–125 μM, whereas the methyl ester **4**, the dipeptide **5** or L-Phe **2** were much better activators (K_As in the range of 10.3–84 μM). Similarly, against hCA IV, histamine **1**, and L-His **3** acted as medium potency activators (K_As in the range of 33–37 μM), whereas compounds **2**, **4**, and **5** were more effective CAAs (K_As in the range of 4.6–23 μM).

Thus, L-Phe is a more effective CAA against all the isozymes investigated here, compared to L-His or histamine, whereas derivatization of both the amino- or carboxy moieties of L-His (such as in the ester **4** or the dipeptide **5**) leads to an enhancement of the CA activatory properties against all three investigated isoforms.

Table 1. Activation data of hCA isozymes I, II, and IV with histamine, L-phenylalanine, L-histidine, L-histidine methyl ester, and carnosine

Compound	No.	K_A[a] (μM)		
		hCA I[b]	hCA II[b]	hCA IV[b]
Hst (histamine)	**1**	2	125	37
L-Phe	**2**	1.2	10.3	4.6
L-His	**3**	4	113	33
L-His-OMe	**4**	2.5	84	23
β-Ala-His (carnosine)	**5**	1.3	35	15

[a] Mean from at least three determinations by the esterase method, with 4-nitrophenyl acetate as substrate.[18] Standard error was in the range of 5–10%.

[b] Human recombinant isozymes.[19]

To assess the molecular basis responsible for the activatory properties of L-His **3** toward hCA II, we solved the crystal structure of the complex, which was prepared and crystallized as previously reported for other CA–activator adducts.[7,20] This three-dimensional structure was analyzed by difference Fourier techniques, the crystals being isomorphous to those obtained for the native enzyme[21–23] and refined using the CNS program.[22] The statistics for data collection and refinement are summarized in Table 2.

The structure refinement allowed us to evidence the spatial arrangement of the activator within the enzyme active site. The unbiased calculated electron density map of the hCA II–L-His adduct showed one molecule of activator (numbered as His300) present within the active site (Fig. 1). The water molecules and amino acid residues involved in the recognition of the activator, together with the Zn(II) ion and its ligands, are also shown in this figure. The overall binding of L-His, at the entrance of the active site cavity of hCA II (similar to the binding of histamine[7] and phenylalanine[8] to this isozyme), is presented in Figure 2A, whereas the key amino acids involved in the recognition of this binding site are shown in Figure 2B.

Inspection of the electron density maps at various stages of the crystallographic refinement, showed features compatible with the presence of the L-His molecule bound to the active site, as clearly illustrated in Figure 3. The binding of **3** to the enzyme did not significantly perturb the enzyme structure, even in close proximity of the ligand. As a matter of fact, the rms deviation, calculated over the entire Cα atoms of hCAII–**3** complex with respect to the unbound enzyme, was of 0.36 Å. Interactions between the protein and Zn(II) ion were entirely preserved in the adduct (Figs. 3 and 4). Thus, the Zn(II) ion remained in its tetrahedral geometry, being coordinated to the imidazolic moieties of His94, His96, and

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

Parameter	Value
X-ray source	Rotating anode
Wavelength (Å)	1.54
Cell parameters	a = 42.70 Å
	b = 41.79 Å
	c = 72.83 Å
	$\alpha = \gamma = 90°$
	β = 104.47°
Space group	$P2_1$
No. of unique reflections	15,867
Completeness (%)[a]	95.0 (90.1)
No. of reflections [$>2\sigma(F_o)$]	14,294
$\langle I/\sigma(I)\rangle$[a]	22.1 (5.1)
Resolution range (Å)	8.0–2.0
R-factor (%)	18.2
R-free (%)[b]	21.8
Rmsd of bonds from ideality (Å)	0.005
Rmsd of angles from ideality (°)	1.34

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

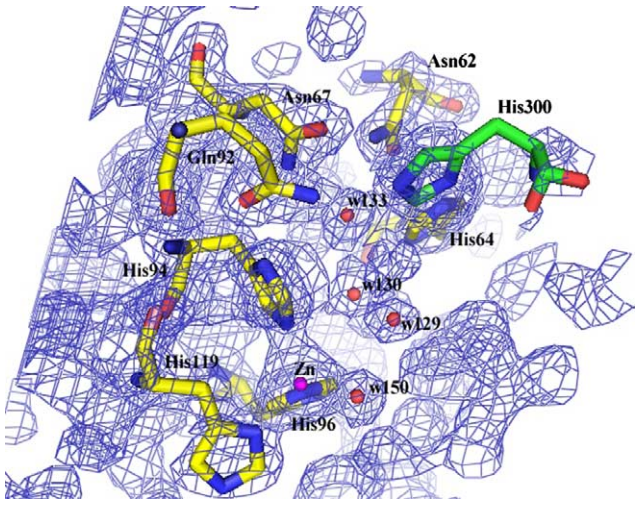


Figure 1. Unbiased calculated electron density map of the hCA II–L-His adduct, showing the activator (His300), water molecules, and amino acid residues involved in the recognition of the activator, together with the Zn(II) ion and its ligands.

His119, as well as to a water molecule (w150, zinc–oxygen distance of 2.23 Å).

As seen in Figures 2–4, L-His binds at the entrance of the hCA II active site, in the activator binding site previously discovered by us,[7,8] participating in three strong hydrogen bonds with Nε of His64 (distance of 3.16 Å), Nδ 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 which 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 ones, the distances between two adjacent oxygen atoms being in the range of 2.83–2.91 Å (Fig. 4). They are probably also involved in the proton transfer processes by which activators facilitate the rate-determining step of the catalytic cycle. It should be mentioned that in hCA II, in the absence of activators, it is the imidazolic moiety of His64 which acts as a proton shuttle between the active site and the environment.[3–8] It must also be noted that similar to histamine,[7] the amino moiety of L-His does not participate in any contacts with the enzyme. The same is true for the carboxy 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, are even enhanced as compared to those of L-His (see Table 1). Indeed, the methyl ester of L-His **4** or the dipeptide **5** showed better hCA II activatory properties, probably because their modified carboxy/amino moieties participate in more favorable contacts with amino acid residues in the neighborhood of the activator binding site, such as Leu198, Pro202, Pro201, and Phe131.

Although L-His and histamine 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

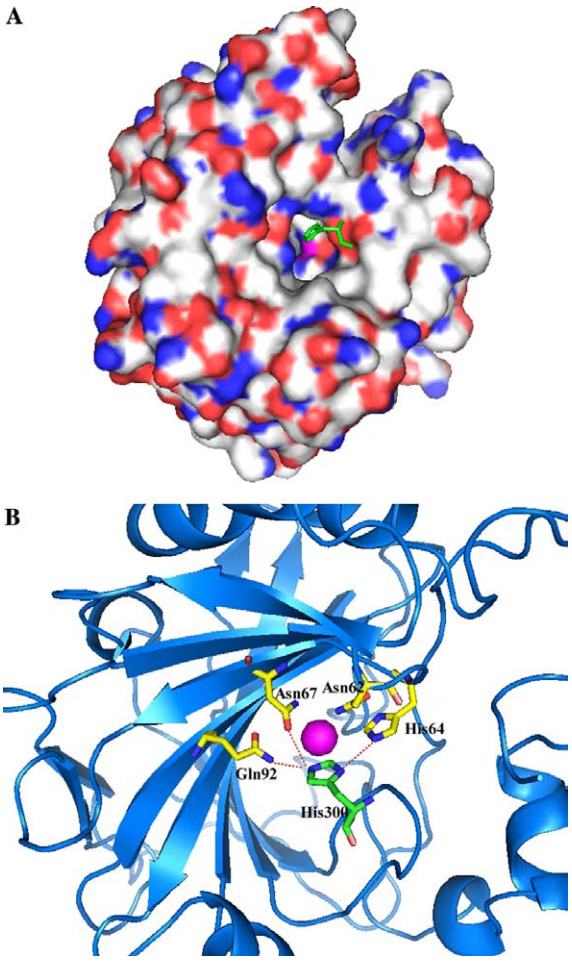


Figure 2. (A) Electrostatic surface potential of hCA II in complex with L-His **3**. Polar atoms are colored in red (negatively charged) and blue (positively charged). The activator molecule is shown in green. (B) Ribbon diagram of the hCA II with L-His bound (in green) and key amino acid residues involved in binding of the activator molecule (Asn62, His64, Asn67, and Gln92, in yellow). The Zn(II) ion is in magenta.

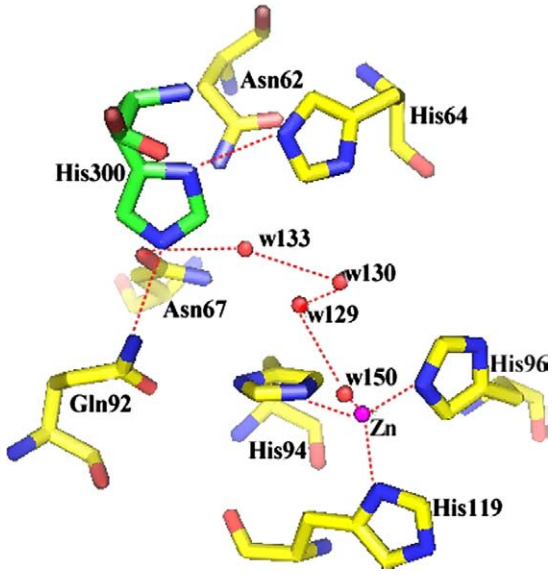


Figure 3. Details of the active site in the hCA II–L-His adduct, showing amino acid residues participating in recognition of the activator molecule, reported in green and numbered as His300. Hydrogen bonds, water molecules (w150, 129, 130, and 133), and the active site Zn(II) ion coordination are also shown.

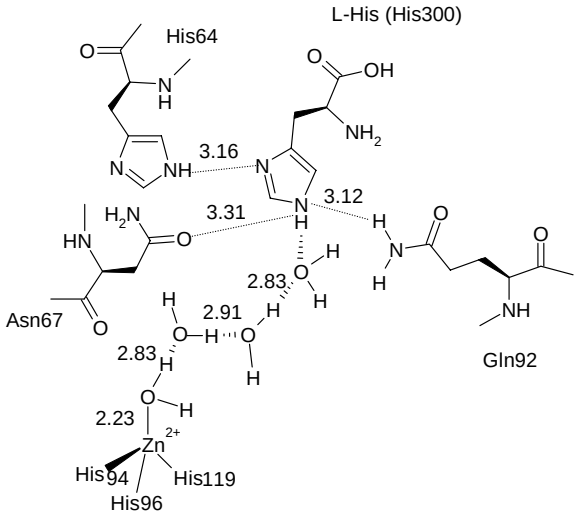


Figure 4. Schematic representation for the binding of L-His (numbered as His300) to the hCA II active site. The Zn(II) ligands and hydrogen bonds connecting the Zn(II) ion and the activator through a network of four water molecules are shown, as well as the three hydrogen bonds (dotted lines) between the activator molecule and amino acid residues His64, Asn67, and Gln92 (figures represent distances in Å).

site (Fig. 5). A superposition of the two structures is shown in Figure 5. Thus, as shown earlier by our group,[7] histamine participates in three strong hydrogen bonds when complexed to the hCA II active site, one with Gln92 (similarly with L-His, see above), another one with Asn62 (whereas L-His makes the second hydrogen bond with His64), and the third one with Asn67 (similarly to L-His). Indeed, in the present structure, the distance between Nδ of L-His and Nδ of Asn62 is of 4.07 Å (the corresponding distance in the hCA II–histamine adduct is of 3.20 Å),[7] which results in the impossibility of hydrogen bond formation. In addition, histamine makes a weaker hydrogen bond with Oδ of Asn67 (of 3.47 Å), interaction which is more favorable in the hCA II–histidine adduct investigated here (distance Oδ of Asn67 to Nε of Asn67 of 3.31 Å). Furthermore, the orientation of the imidazole moieties of the two activators when bound to the hCA II active site is rather different (Fig. 5). Indeed, although the aliphatic chains of the two compounds are almost entirely superposable, the two imidazole rings make an angle of around 45°, which in the case of L-His orients the ring in a favorable conformation for participating in the above-mentioned hydrogen bond with His64. For histamine, which is placed in a somehow more vertical conformation as compared to L-His, the imidazole ring is too far from His64 for making this favorable hydrogen bond (Fig. 5). Another major difference between the two structures concerns the relay of water molecules connecting the activator molecules to the zinc-bound water. Thus, in the hCA II–histamine adduct there are

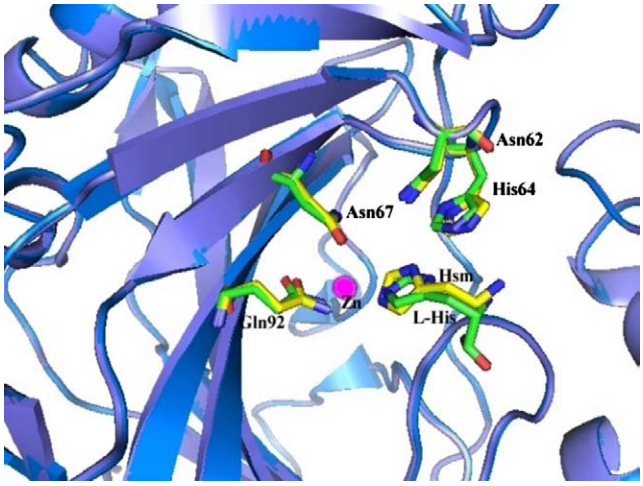


Figure 5. Superposition of the hCA II active sites complexed with L-His (green) and histamine (yellow), showing also the amino acid residues involved in the binding of the two activators.

two such water molecules, whereas in the hCA II–L-His adduct, three water molecules are present in this relay (Fig. 4). It is thus clear that a complete reorganization of the hydrogen bond network occurs after the binding of activators, which may be different for diverse structural classes of CAAs. This is, in fact, the first example in which such a difference is being identified.

The activation of other CA isozymes involved in critical physiological processes with the derivatives investigated here is also of great importance, considering the fact that the active site geometry of many such isozymes (for example, the cytosolic CA VII[24] and CA XIII,[25] or the tumor-associated isozymes CA IX[26] and XII[27]) is rather similar to that of CA II, but some of the key amino acid residues involved in the binding of activators (such as L-His or histamine) are different in some of them. Such studies may lead both to novel pharmacological applications of CAAs as well as to a better understanding of the physiological role of some of these CA isoforms.

In conclusion, activation of three CA isoforms, that is, hCA I, II, and IV, with L-histidine and some of its derivatives has been investigated by kinetic and X-ray crystallographic methods. L-His was a potent activator of isozymes I and IV (activation constants in the range of 4–33 μM) and a moderate hCA II activator (activation constant of 113 μM). Both carboxy- as well as amino-substituted L-His derivatives, such as the methyl ester or the dipeptide carnosine (β-Ala-His), acted as more efficient activators as compared to L-His. The X-ray crystallographic structure of the hCA II–L-His adduct showed the activator to be anchored at the entrance of the active site cavity, participating in an extended network of hydrogen bonds with the amino acid residues His64, Asn67, and Gln92, and with three water molecules connecting it to the zinc-bound water. Although the binding site of L-His is similar to that of histamine, the first CA activator for which the X-ray crystal structure has been reported in complex with hCA II, there are important differences of binding between the two structurally related activators, since histamine interacts among others with Asn67 and Gln92 (similarly to L-His), but also with Asn62 and not His64, whereas the number of water molecules connecting them to the zinc-bound water is different (two for histamine, three for L-His). Since neither the amino- nor carboxy moieties of L-His participate in interactions with amino acid moieties of the active site, they can be derivatized for obtaining more potent activators, with pharmacological applications for the enhancement of synaptic efficacy. This may constitute a novel approach for the treatment of Alzheimer's disease, aging, and other conditions in need of achieving spatial learning, and memory therapy.[3]

Acknowledgments

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18. Pocker, Y.; Stone, J. T. *Biochemistry* **1967**, *6*, 668. Typical CA activation measurements were done as described below: the initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC. Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1×10^{-6} M, working at 25 °C. A molar absorption coefficient ε of 18,400 M^{-1} cm^{-1} was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40). The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activator (1 mM) were prepared in distilled–deionized water and dilutions up to 1 nM were done thereafter with the assay buffer. Activator (A) and enzyme (E) 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 constants were obtained by non-linear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier,[11–15] and represent the mean from at least three different determinations. The cloned CA isozymes (hCA I, hCA II, and hCA IV) were obtained as reported earlier by this group.[19]
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20. Crystals of hCA II–L-His complex were obtained by co-crystallization at 4 °C in hanging drops, 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 using a Rigaku RU200 rotating anode, operating at 50 V, 180 mA, in ω scan mode with Cu Kα radiation (1.5418 Å). The unit cell dimensions were determined to be $a = 42.70$ Å, $b = 41.79$ Å, $c = 72.83$ Å and $\alpha = \gamma = 90°$, $\beta = 104.47°$ in the space group $P2_1$. Data were collected using a Mar300 ImagePlate Detector from MarReasearch and processed with the software DENZO.[21] 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, using the PDB file 1AVN as starting model for refinement.[7] Electron density maps ($2F_o - F_c$) and ($F_o - F_c$) were calculated with CNS program[22] and displayed using the graphic program O.[23] The final model had an *R*-factor of 18.2%, *R*-free 21.8%, for 14,294 reflections at $F > 2\sigma(F_o)$ in the resolution range 8–2.0 Å with a rms deviation from standard geometry of 0.005 Å in bond lengths and 1.34° in bond angles. The coordinates and the structure factors have been deposited in the Protein Data Bank: PDB ID 2ABE. Crystallographic parameters and refinement statistics are summarized in Table 2.
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