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Carbonic anhydrase activators: L-Adrenaline plugs the active site entrance of isozyme II, activating better isoforms I, IV, VA, VII, and XIV☆

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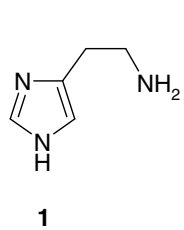
Abstract—The activation of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) with L-adrenaline and histamine has been investigated by kinetic and X-ray crystallographic studies. L-Adrenaline behaves as a potent activator of isozyme CA I (activation constant of 90 nM), being a much weaker activator of isozyme CA II (activation constant of 96 μM). Isoforms CA IV, VA, VII, and XIV were activated by L-adrenaline with K_As in the range of 36–63 μM. The X-ray crystal structure of the CA II–L-adrenaline adduct revealed that the activator plugs the entrance of the active site cavity, obstructing it almost completely.

A multitude of physiologically relevant compounds such as biogenic amines (histamine, serotonin, catecholamines), amino acids, oligopeptides or small proteins act as efficient activators for many of the 15 presently known human carbonic anhydrase (CA, EC 4.2.1.1) isoforms.[1–5] Activation of some of these enzymes 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 to achieve spatial learning and memory therapy.[6] The levels of several CA isozymes were also shown to be diminished in patients affected by Alzheimer's disease or in the older population,[7] supporting thus a possible involvement of brain CA isoforms (such as CA I, II, IV, V, VII, XII, and XIV, all of them present in the CNS)[1–5] in cognitive processes, and their activation as a possible therapeutic intervention.[6]

The binding of CA activators (CAAs) to various isozymes, such as CA I, II, IV, VA, VII, XIII, and XIV, was studied by kinetic and X-ray crystallographic techniques (the last techniques were applied only for the cytosolic isozymes I and II),[5,8–10] which showed the activator to intervene in the rate-determining step of the catalytic cycle, that is, the shuttling of protons between the active site and the reaction medium, a process which in



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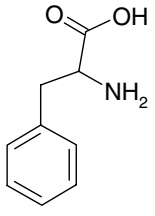

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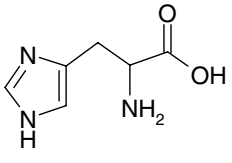

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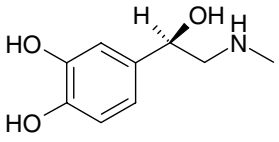

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Keywords: Carbonic anhydrase; L-Adrenaline; Histamine; Enzyme activator; X-ray crystallography.

☆ The X-ray coordinates of the hCA II–adrenaline adduct are available in PDB with the ID 2HKK.

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most CA isoforms is assisted by a histidine residue (His64, CA I numbering) placed in the middle of the active site cavity.[11–13] In the presence of CAAs, there is the possibility of alternative proton transfer pathways, involving a protonatable moiety of the activator bound within the enzyme active site, which explains the enhanced overall catalytic efficiency, reflected in the augmentation of k_{cat}, without any effect on K_M, for all isoforms investigated up to now in detail (i.e., CA I, II, IV, VA, VII, XIII, and XIV).[5,8–10] X-Ray crystallography of adducts of human CA (hCA) II with histamine **1**,[8] L- and D-phenylalanine **2**,[9] and L- and D-histidine **3**,[5c] as well as the adduct of hCA I with L-His **3**,[10a] allowed a better understanding of the CA activation phenomenon at the molecular level, bringing also new insights into problems of ligand recognition by an enzyme active site, since it has been observed that enantiomers such as L-/D-His and L-/D-Phe bind in a very different manner to hCA II, interacting with different amino acid residues from the activator binding site.[5c,8,9] In addition, the interaction between the two best studied isozymes, that is, hCA I and II, with the same activator, L-His, was also very different, with the activator binding deep within the active site cavity in the case of hCA I, and toward the external of the cavity for hCA II.[5c,10a] These studies are helpful for the design of better CAAs or for obtaining compounds with selectivity for an isozyme, and less affinity for another one which is not desirable to be activated (or inhibited).[7c,1]

L-Adrenaline (epinephrine) **4**, one of the neurotransmitter catecholamines released by the sympathetic nervous system and adrenal medulla in response to a range of stresses in order to regulate the host physiological functions, is involved in regulation of blood pressure, vasoconstriction, cardiac stimulation, relaxation of the smooth muscles (such as the bronchial ones) as well as in several metabolic processes.[14] As a consequence, **4** has a variety of clinical uses, such as among others for relieving respiratory distress in asthma, in treating hypersensitivity reactions due to various allergens, cardiac arrest, or as a topical hemostatic agent, etc.[14–16] The activating effects of adrenaline **4** on CA II (of bovine origin, bCA II) were first investigated by this group,[17] being shown that the compound is a weaker CAA, as compared to histamine, aromatic/heterocyclic amino acids or other structurally related amines investigated in the same study. However, since adrenaline is such an important endogenous compound, and its concentrations in blood or other tissues seem to be rather high, in the range of 2–5 μM,[18] we decided to investigate in more detail its interaction with various physiologically relevant CA isozymes,[1–5] such as CA I, II, IV, VA, VII, and XIV (all of them present among others in the brain).[1] Here we report kinetic investigations regarding the activation of the above-mentioned isoforms with L-adrenline **4**, as well as an X-ray crystallographic study of the hCA II–**4** adduct. Our work may bring a better understanding of the CA activation processes, potentially useful for the design of pharmacological agents, whereas from the chemical point of view, it reveals a completely new interaction between the activator and the enzyme, which explains at the molecular level the lower efficacy of adrenaline as a CA II activator. Our study may also shed new light in the recognition processes by metalloenzymes of ligands which do not directly interact with the metal ion, phenomena far less investigated up to now, since the majority of ligands interacting with metalloenzymes usually directly coordinate the metal ion(s) from the enzyme active site.

Solution CA activation studies. Activation data with L-adrenaline **4** and histamine **1** (as a standard, since this was the first activator for which the X-ray structure in complex with CA II has been reported[8]) against six physiologically relevant human CA isozymes, that is, hCA I, II, IV, VA, VII, and XIV, for the physiological reaction catalyzed by them, that is, CO_2 hydration to bicarbonate and a proton,[19–23] are shown in Table 1. The histamine CA activation data are available only for isoforms CA I, II, and IV,[9a] for the esterase activity (4-nitrophenyl acetate hydrolysis) of these enzymes. However, as mentioned above, histamine is the only amine derivative for which the X-ray crystal structure in complex with hCA II has been published,[8] and thus, the activation data of various isoforms with this compound are important both because this is a physiologically relevant autacoid,[8] and also for discussing the crystal structure of the adduct of hCA II with adrenaline **4**, reported here, in order to rationalize the differences in activity/binding to the enzyme active site of the two amines, **1** and **4**.

Data of Table 1 show both histamine **1** and L-adrenaline **4** to act as quite potent activators of the slow cytosolic isozyme hCA I, with activation constants in the range of 50–90 nM. On the contrary, both compounds are much weaker CAAs against the other cytosolic, highly abundant isoform, that is, hCA II, which shows a much higher catalytic activity as compared to hCA I (data of Table 1 and Refs.1,11,12), with K_As in the range of 74–96 μM. Thus, there is almost a factor of 1000 between the CA activation of isozyme I (activatable at nanomolar concentrations of CAAs) and II (activatable in the micromolar range), respectively, with the two amines **1** and **4** investigated here. The behavior of the two activators **1** and **4** against the membrane-associated isoform hCA IV is somehow similar to that shown against hCA II, the two compounds being medium potency activators, showing K_As in the range of 31–45 μM. However, important differences of behavior between the two compounds **1** and **4** were observed when studying the activation of the mitochondrial isoform hCA VA, of the cytosolic, brain-specific isoform hCA VII, and the transmembrane isozyme hCA XIV (Table 1). Against all these CAs, histamine **1** behaved as a strong activator, with K_As in the range of 1.3–2.5 μM, whereas L-adrenaline **4** showed much weaker activating properties, with K_As in the range of 36–63 μM. It should be also mentioned that kinetic measurements by a stopped-flow technique, for the physiological reaction catalyzed by these enzymes, showed that the activators lead to a marked augmentation of k_{cat} (Table 1), with no effects on K_M (practically K_M in the presence and the absence of activator was the same; data not shown). These data are in agreement with our other recent kinetic and crys-

Table 1. Activation of hCA isozymes I, II, IV, VA, VII, and XIV with histamine (Hsn) **1** and L-adrenaline **4**, at 25°C, for the CO_2 hydration reaction[19]

Isozyme	k_{cat}[a] (s^{-1})	$(k_{cat})_{Hsn}$[b] (s^{-1})	$(k_{cat})_{Adrenaline}$[b] (s^{-1})	K_A[c] (μM)	
				Hsn **1**	Adrenaline **4**
hCA I[d]	2.0×10^5	12.7×10^5	10.4×10^5	0.05	0.09
hCA II[d]	1.4×10^6	2.0×10^6	1.7×10^6	74	96
hCA IV[e]	1.2×10^6	3.5×10^6	2.9×10^6	31	45
hCA VA[f]	2.9×10^5	8.6×10^5	3.9×10^5	2.5	63
hCA VII[f]	9.5×10^5	15.2×10^5	10.4×10^5	1.3	60
hCA XIV[f]	3.1×10^5	10.0×10^5	4.5×10^5	1.9	36

[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 enhancements as a function of the activator concentration.[5c,10] Mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[19] 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.[20]
[f] Full length, human recombinant isoforms.[21–23]

tallographic studies, in which we investigated activation of the same CA isozymes with amino acids, such as L-/D-Phe and L-/D-His.[5c,9,10a] Thus, the first question that arises is why are the two amines **1** and **4** so different in their behavior as CAAs towards the various isozymes investigated here?

For replying to this question, the CA activation mechanism must be considered.[3,4] Thus, it has been show[3–5,8–10] that in order for a compound to act as an efficient CAA, at least two conditions should be satisfied: (i) a steric factor, allowing the compound to bind within the enzyme active site in a favorable orientation for shuttling protons between the active site and the environment; (ii) an electronic factor, connected with the appropriate pK_a value of the protonatable moiety present in the activator molecule, which must be able to engage in hydrogen bonds with other amino acid residues and water molecules, in order to release the proton from the zinc-bound water molecule toward the external of the active site cavity, through a relay of hydrogen bonds.[3–5,8–10,12] We have shown earlier that the best CAAs possess protonatable moieties in their molecule with a pK_a in the range of 6.5–8.0.[3–5,8–10] Although literature data regarding the pK_a of the various protonatable moieties present in L-adrenaline **4** are rather discordant,[24] a recent study assumes that these values for the four acidic groups in this compound are of 8.60 (presumably for the methyl-ammonium moiety), 8.65 (one of the phenolic OH groups), 9.67 (the second phenolic OH), and 11.34 (the alcoholic OH moiety), respectively.[25] Thus, in contrast to histamine **1**, which has the imidazolic moiety with a pK_a of around 6.5–7.0,[8] all protonatable groups of L-adrenaline **4** have acidities with pK_as >8.60, which make them less effective as proton shuttle residues and as a consequence as CAAs (it should be mentioned that the pK_a of the imidazolic moiety of His64, the natural proton shuttling moiety of many CA isoforms, is also around 7.0).[8,11,12] This may explain why L-adrenaline is a less effective CAA as compared to histamine **1**, against all investigated isozymes studied here. Thus, clearly the second factor mentioned above, the electronic one, is not satisfied for **4** in order to act as a potent CAA. However, more insights may be obtained by studying the three-dimensional structure of the hCA II–L-adrenaline adduct, which will be discussed in the next section.

Crystallographic studies. Crystals of the hCA II–**4** adduct were isomorphous with those of the native protein,[26,27] allowing for the determination of the crystallographic structure by difference Fourier techniques. The model was refined using the REFMAC5 program[28] to crystallographic *R*-factor and *R*-free values of 0.186 and 0.230, respectively. The overall quality of the model was excellent, with 100% of the non-glycine residues located in the allowed regions of the Ramachandran plot. The statistics of data collection and refinement are summarized in Table 2.

Analysis of the three-dimensional structure of the complex revealed that the overall protein structure remained largely unchanged upon binding of the activator. As a matter of fact, an rms deviation value of 0.29 Å was calculated over the entire Cα atoms of hCA II–**4** complex with respect to the unbound enzyme. The analysis of the electron density maps within the enzyme cavity showed features compatible with the presence of one activator molecule bound within the active site. The structure of the activator **4** perfectly fitted to the shape of this electron density (Fig. 1).

As for other hCA II–activator adducts for which the structure was determined by X-ray crystallography,[5,8,9] also in the case of the L-adrenaline complex, the activator molecule binds at the entrance of the cavity (Fig. 2), interacting with amino acid residues and water molecules which stabilize its binding to the enzyme (Fig. 3). It should be stressed (Fig. 2) that the side chain of His64, an amino acid residue extremely important in the CA catalytic cycle,[10–12] was observed with both its two characteristic conformations, the 'in' and 'out' ones in the L-adrenaline adduct reported here, although in other CA–activator adducts investi-

Table 2. Crystallographic parameters and refinement statistics for the hCA II–L-adrenaline **4** adduct

Parameter	Value
Crystal parameter	
Space group	$P2_1$
Cell parameters	a = 42.10Å
	b = 41.43 Å
	c = 72.20 Å
	β = 104.48Å
Data collection statistics (20.0–1.90 Å)	
No. of total reflections	46684
No. of unique reflections	18677
Completeness (%)[a]	97.4 (90.0)
$\langle I/\sigma(I)\rangle$	13.2 (4.5)
R-merge (%)	5.20 (13.0)
Refinement statistics (20.0–1.90 Å)	
R-factor (%)	18.6
R-free (%)[b]	23.0
Rmsd of bonds from ideality (Å)	0.008
Rmsd of angles from ideality (°)	1.21

[a] Values in parentheses relate to the highest resolution shell (2.10–1.90).
[b] Calculated using 5% of data.

gated earlier (e.g., the histamine one)[8] His64 adopted only the out conformation. Thus, L-adrenaline participates to an extended network of hydrogen bonds involving five water molecules and several amino acid residues, when bound to the hCA II active site (Fig. 3). In particular, the zinc-coordinated water molecule (w114) is hydrogen bonded to the OH moiety of Thr200 through another water molecule (w64) acting as a bridge. In turn, the same threonine OH moiety is connected to one of the phenolic OH groups of the activator molecule by another bridging water molecule, w105, which makes two strong hydrogen bonds, one with the OH moiety of Thr200 and another one

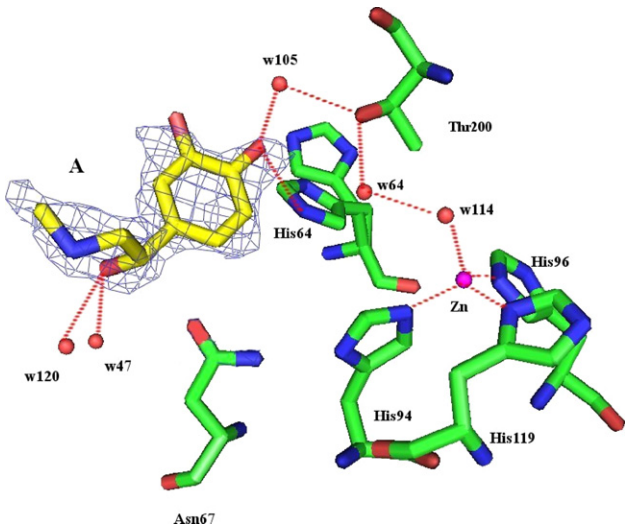

Figure 1. Electron density omit map contoured at 1σ of L-adrenaline (labeled as A) bound to hCA II. The Zn(II) coordination by His94, 96, and 119, as well as residues involved in the catalytic/activation mechanism (such as Thr200, His64, Asn67, and five water molecules) are also evidenced.

with the *para*-OH (phenolic) moiety of L-adrenaline, of 2.66 and 2.90 Å, respectively (all the distances of these hydrogen bonds are shown in Fig. 3). The same phenolic OH moiety of the activator also participates in another hydrogen bond, of 3.10 Å, with one of the imidazolic nitrogens of His64 in its 'in' conformation (when in the 'out' conformation, this hydrogen bond is not formed). The alcoholic OH moiety of the activator makes two strong hydrogen bonds (of 2.82 and 2.83 Å, respectively) with two other water molecules from the active site (w120 and w47), and a weaker one (we are at the limits of distances accepted as hydrogen bonds, i.e., 3.39 Å) with the carboxamide oxygen atom of Asn67, an amino acid residue involved in the binding of other CAAs studied by means of crystallography, such as histamine, L-/D-histidine, and L-/D-phenylalanine.[5,8,10] It is interesting to note that the second phenolic OH moiety (in *meta* to the aliphatic chain of the catecholamine) as well as the methylamino group of L-adrenaline, do not participate in any polar interaction with water molecules or amino acid residues from the active site. On the other hand, the methylamino group extends towards the hydrophobic half of the CA II active site, the amino acid residue most close to it being Phe131. In fact one carbon atom of the phenyl ring of this residue is at about 3.70 Å from the methyl group of the activator. This particularly interesting binding mode has never been evidenced earlier for any other CAA. Actually, L-adrenaline adopts an extended conformation when complexed to the CA II active site, practically plugging the entrance to the cavity, in contrast to activators such as histamine or L-/D-histidine, L-/D-phenylalanine, which penetrate to a larger extent within the active site, and adopt completely different orientations, that is, more or less parallel to His64, the natural proton shuttle residue of the CA II active site. This is particularly clear from data shown in Fig. 4 where the hCA II–histamine **1** and hCA II–L-adrenaline **4** adducts were superposed. It may be observed that both activators are bound towards the entrance of the active site cavity. However, histamine adopts a conformation which is almost parallel with His64 (in its out conformation, that is, the conformation believed to shuttle the protons toward the outside of the active site, transferring them to the buffer and contributing thus to generation of the nucleophilic species of the enzyme, with hydroxide coordinated to the zinc ion)[10–12], whereas L-adrenaline adopts an extended conformation, practically perpendicular on that of histamine, so that the activator molecule plugs the entrance to the active site. In this orientation, the activator rather obstructs the entrance to the active site and must be much less effective in acting as a proton shuttle, as compared to the imidazolic moiety of histamine in the hCA II–histamine adduct. Thus, the first condition mentioned earlier for obtaining potent CAAs, the steric one, is also largely unfulfilled in the hCA II–L-adrenaline adduct, since as shown above, the binding of this activator is achieved in a rather unfavorable manner as compared to that of other CAAs investigated earlier, such as histamine, L-/D-His or L-/D-Phe.[5,8–10]

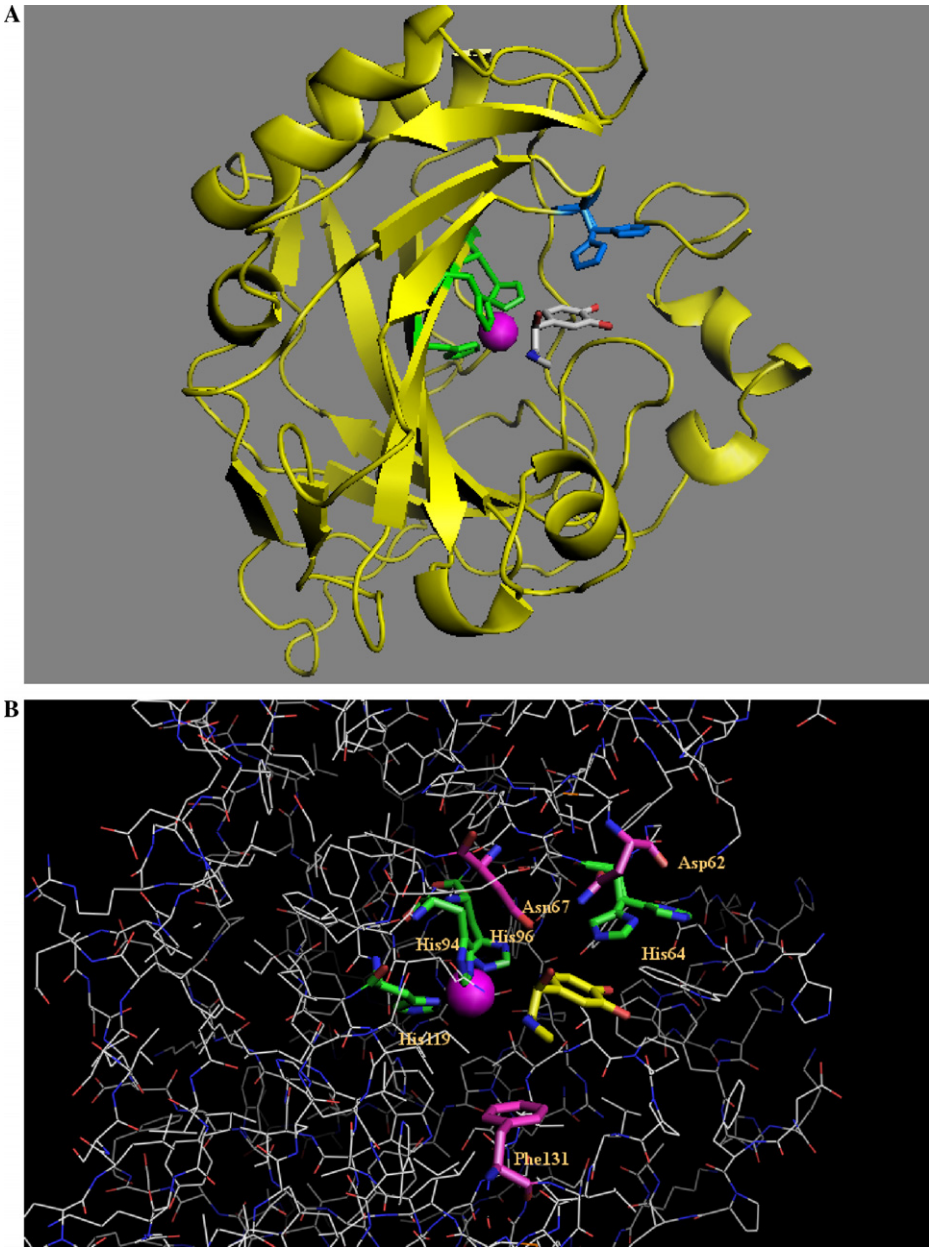

Figure 2. (A) Binding of L-adrenaline (CPK colors) at the entrance of the hCA II active site. The zinc ion (violet sphere), its three histidine ligands (His94, 96, and 119, in green), and the proton shuttle residue His64 (with its two conformation, 'in' and 'out', in blue) are also evidenced. (B) L-adrenaline (in yellow) plugs the entrance to the hCA II active site, obstructing it entirely, from the hydrophobic part (where Phe131 lies) toward the hydrophilic half, where residues His64, Asn67, and Asp62 are situated.

Conclusions. The activation of CA with L-adrenaline and histamine has been investigated by kinetic and X-ray crystallographic studies. L-Adrenaline behaved as a potent activator of isozyme CA I (activation constant of 90 nM), being a much weaker activator of isozyme CA II (activation constant of 96 μM). The isozymes IV, VA, VII, and XIV were activated by L-adrenaline with K_As in the range of 36–63 μM. Histamine was a better CA activator against all investigated isozymes, with an affinity of 50 nM against CA I, 74 μM against CA II, 31 μM against CA IV, 2.5 μM against CA VA, 1.3 μM against CA VII and 1.9 μM against CA XIV, respectively. The enhancement of the catalytic activity was due to an augmentation of k_{cat}, with no effects on K_M, against all investigated isozymes, with both activators. The X-ray crystal structure of the CA II–L-adrenaline adduct revealed the reason why this compound is a weaker activator as compared to histamine and related biogenic amines/amino acids. Thus, in contrast to other activators investigated earlier, L-adrenaline plugs the entrance of the active site cavity, obstructing it almost completely. In this conformation, it is unable to facilitate the shuttling of protons between the active site and the environment, also because the pK_as of its protonatable moieties are in the range of 8.6–11.34. On the contrary, histamine bound to the enzyme active site adopts a conformation that allows its imidazolic moiety (with a pK_a around 7) to easily participate in proton

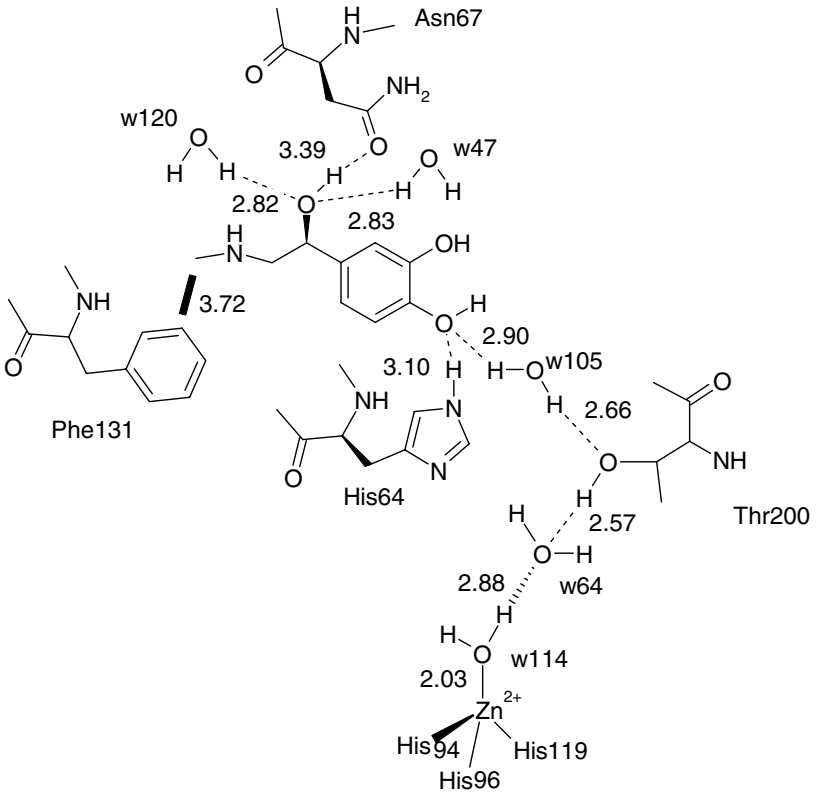

Figure 3. Schematic representation for the binding of L-adrenaline to the hCA II active site. The Zn(II) ligands, hydrogen bonds connecting the Zn(II) ion and the activator molecule with other amino acid residues/water molecules through a network of hydrogen bonds, stabilizing the enzyme–activator complex, are also evidenced (dotted lines, figures represent distances in Å). His64 is shown only in the 'in' conformation, the only one making a hydrogen bond with the activator molecule. The 'out' conformation of His64 does not interact with the activator. The methylamino group of **4** does not participate in any polar interaction, being rather close to the phenyl ring of Phe131 (bold line, figure represents distance in Å).

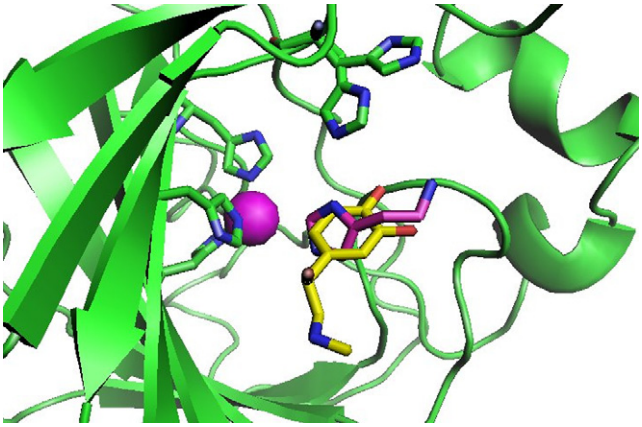

Figure 4. Superposition of the hCA II–histamine **1** adduct (PDB code 4TST,[8] the activator is shown in magenta) and the hCA II–L-adrenaline **4** adduct (PDB code 2HKK, the activator molecule in yellow). The Zn(II) ion (violet central sphere) and its three protein ligands (His94, 96, and 119, green) together with the proton shuttle residue His64 (in its two conformations, in and out) are also shown, being completely superposable in the two structures. His64 is present only in the out conformation in the histamine adduct,[8] and in both the in and out conformations in the L-adrenaline adduct.

shuttling, similarly with residue His64, the natural proton shuttle amino acid in the CA II active site. These findings explain thus that both the steric requirements (orientation in which the activator binds within the active site) and electronic factors (pK_a of the proton shuttle moiety) are important for a compound to act as an effective CA activator, and may shed new light in the recognition processes by metalloenzymes of ligands which do not directly interact with the metal ion.

Crystallography. The hCA II–**4** complex was co-crystallized at 4 °C by the hanging drop vapor diffusion method. Drops containing 5 μl of 20 mg/ml hCA II in 50 mM Tris–HCl buffer, pH 7.8, were mixed with 5 μl of precipitant buffer (2.4 M $(NH_4)_2SO_4$ in 50 mM Tris–HCl, pH 7.8, and 1 mM sodium 4-(hydroxymercury)benzoate) with added 5 mM L-adrenaline **4** and equilibrated over a reservoir of 1 ml of precipitant buffer. Diffraction data were collected under cryogenic conditions (100 K) on a CCD Detector KM4 CCD/Sapphire using CuKα radiation (1.5418 Å). The unit cell dimensions were determined to be: $a = 42.10$ Å, $b = 41.43$ Å, $c = 72.20$ Å and $\alpha = \gamma = 90°$, $\beta = 104.48°$ in the space group $P2_1$. Data were processed with MOSFLM[28] and scaled with CCP4 suite.[29] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2[26] as starting model. The refinement was carried out with the program REFMAC5;[27] model building and map inspections were performing using the COOT program.[30] The final model of the hCA II–**4** complex had an *R*-factor of 18.6% and *R*-free 23.0% in the resolution range 20.0–1.90 Å, with a

rms deviation from standard geometry of 0.008 Å in bond lengths and 1.21° in angles. The correctness of stereochemistry was checked using PROCHECK.[31] Coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank (Accession Code 2HKK).

Acknowledgments

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An Applied Photophysics stopped-flow instrument has been used for assaying the CA, catalyzed CO_2 hydration activity. 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,[1,5] may be obtained by considering the classical Michaelis–Menten equation (equation 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 the following equation:[1,5]

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

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

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26. The hCA II-**4** complex was co-crystallized at 4 °C by the hanging drop vapor diffusion method. Drops containing 5 μl of 20 mg/ml hCA II in 50 mM Tris–HCl, buffer, pH 7.8, were mixed with 5 μl of precipitant buffer (2.4 M $(NH_4)_2SO_4$ in 50 mM Tris–HCl, pH 7.8, and 1 mM sodium 4-(hydroxymercury)benzoate) with added 5 mM L-adrenaline **4** and equilibrated over a reservoir of 1 ml of precipitant buffer. Diffraction data were collected under cryogenic conditions (100 K) on a CCD Detector KM4 CCD/Sapphire using CuKα radiation (1.5418 Å). The unit cell dimensions were determined to be: $a = 42.10$ Å, $b = 41.43$ Å, $c = 72.20$ Å, and $\alpha = \gamma = 90°$, $\beta = 104.48°$ in the space group $P2_1$. Data were processed with MOSFLM[29] and scaled with CCP4 suite.[30] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2[27] as starting model. The refinement was carried out with the program REFMAC5;[28] model building and map inspections were performed using the COOT program.[31] The final model of the hCA II–**4** complex had an *R*-factor of 18.6% and *R*-free 23.0% in the resolution range 20.0–1.90 Å, with an rms deviation from standard geometry of 0.008 Å in bond lengths and 1.21° in angles. The correctness of stereochemistry was checked using PROCHECK.[32] Coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank (Accession Code 2HKK).
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