Carbonic Anhydrase Activators: X-ray Crystallographic and Spectroscopic Investigations for the Interaction of Isozymes I and II with Histamine[†,‡]

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Received April 1, 1997; Revised Manuscript Received June 2, 1997[⊗]

ABSTRACT: The interaction of native and Co(II)-substituted isozymes I and II of carbonic anhydrase (CA) with histamine, a well-known activator, was investigated kinetically, spectroscopically, and X-ray crystallographically. This activator is of the noncompetitive type with 4-nitrophenyl acetate and CO_2 as substrates for both HCA I and HCA II. The electronic spectrum of the adduct of Co(II)−HCA II with histamine is similar to the spectrum of the Co(II)−HCA II−phenol adduct, being only slightly different from that of the uncomplexed enzyme. This is the first spectroscopic evidence that the activator molecule binds within the active site, but not directly to the metal ion. X-ray crystallographic data for the adduct of HCA II with histamine showed that the activator molecule is bound at the entrance of the active site cavity in a position where it may actively participate in shuttling protons between the active site and the bulk solvent. The role of the activators and the reported X-ray crystal structure of the HCA II−histamine adduct has prompted us to reexamine the X-ray structures of the different CA isozymes in order to find a structural basis accounting for their large differences in catalytic rate. A tentative explanation is proposed on the basis of possible pathways of proton transfer, which constitute the rate-limiting step in the catalytic reaction.

Carbonic anhydrase (CA, EC 4.2.1.1), a zinc enzyme widely spread in the bacterial, vegetal, and animal kingdoms (Maren, 1967; Tashian 1992; Supuran, 1994), catalyzes one of the simplest physiological reactions, the reversible interconversion between CO_2 and the bicarbonate ion (Bertini & Luchinat, 1983; Silverman & Lindskog, 1988; Lindskog & Liljas, 1993; Supuran, 1994; Christianson & Fierke, 1996). Although at least eight distinct isozymes are presently known in higher vertebrates (Hewett-Emmett & Tashian, 1996), the physiological function for many of them is still unknown (Supuran, 1994).

The mode of action of CA inhibitors (of the sulfonamide or anion type) has been elucidated by means of spectroscopic and X-ray crystallographic studies, which showed the inhibitor to displace the zinc-bound solvent of the pseudotetrahedral Zn(II) or to add to the coordination sphere as the fifth ligand (Bertini et al., 1982, 1992; Bertini & Luchinat, 1983; Mangani & Håkansson, 1992; Mangani & Liljas, 1993; Liljas et al., 1994; Supuran, 1994; Håkansson & Liljas, 1994; Vidgren et al., 1993; Supuran et al., 1997).

In contrast to inhibitors, activators of CAs were less studied, probably because CA is one of the most efficient enzymes known (Roughton, 1943; Clark & Perrin, 1951). Still, CA II activation by phosphorylation in the presence of protein kinases and cAMP has been reported (Narumi & Miyamoto, 1974) and recently reconfirmed (Igbo et al., 1994). Some anionic activators of CA III have also been reported (Shelton & Chegwidden, 1988; Paranawithana et al., 1990; Rowlett et al., 1991), the effect being explained as due to the proton shuttling capacities of such molecules. On the other hand, one group (Supuran, 1992; Supuran & Puscas, 1994; Supuran et al., 1993, 1996a,b) studied the activation of CA II and CA I by amines and amino acids, proposing a general scheme that should explain the activation mechanisms for isozymes CA I−III (Supuran, 1992; Clare & Supuran, 1994). It was then proven that compounds acting as CA activators participate to the catalytic cycle of this enzyme (Supuran & Balaban, 1994; Supuran & Puscas, 1994; Puscas et al., 1990, 1994).

The generally accepted catalytic mechanism for the physiological reaction involves the nucleophilic attack of zinc-bound hydroxide to CO_2, optimally activated and oriented in the hydrophobic pocket of the CA active site (Silverman & Lindskog, 1988; Lindskog & Liljas, 1994; Christianson & Fierke, 1996). Bicarbonate formed in this way is then replaced by a water molecule, with generation of the catalytically inactive form of the enzyme $EZn^{2+}-OH_2$ (eq 1). In order to regenerate the catalytically active form, a proton-transfer reaction must occur, from the water bound to Zn(II) within the enzyme active site to the external medium. In isozyme CA II, this step (eq 2) was considered to be assisted by the active site residue His 64 (Tu et al., 1989), placed at the entrance of the active site, as well as by external buffer molecules (Tu et al., 1989; Liang et al., 1993). This step is also rate-determining for the whole catalytic cycle (Steiner et al., 1975), and the shuttling effects of His 64 would explain the very high efficiency of CA II as catalyst, with a maximal turnover number of 1.6×10^6 s^{-1} (Liang & Lipscomb, 1987; Silverman & Lindskog, 1988). Indeed, in several crystal structures, the His 64 side chain has been observed disordered over two orientations, one toward the

[†] This research was financed by European Union Grant ERBCIPDCT 940051 and by Consiglio Nazionale delle Ricerche Grant 96.01270.PF37.

[‡] The structure of the HCA II−histamine adduct has been deposited in the Brookhaven Protein Data Bank, accession code 4TST.

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[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1997.

S0006-2960(97)00760-5 CCC: $14.00

inside and the other one toward the outside of the active site cavity, indicating its flexibility and hence supporting its involvement in the proton shuttling (Nair & Christianson, 1991; Smith et al., 1994).

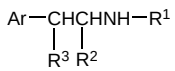
$$EZn^{2+}-OH^- + CO_2 \leftrightarrows EZn^{2+}-HCO_3^- \overset{H_2O}{\leftrightarrow} EZn^{2+}-OH_2 + HCO_3^- \quad (1)$$

$$EZn^{2+}-OH_2 \leftrightarrows EZn^{2+}-HO^- + H^+ \quad (2)$$

In the presence of activators, an enzyme−activator complex may form, in which the activator participates in proton-transfer processes (Supuran, 1992; Supuran & Puscas, 1994). The enhanced catalytic rate might be due to the fact that intramolecular reactions are more rapid than intermolecular ones (Page, 1989). Thus, in the presence of activators (symbolized as "A"), eq 2 becomes

$$EZn^{2+}-OH_2 + A \leftrightarrows [EZn^{2+}-OH_2-A] \leftrightarrows [EZn^{2+}-HO^--AH^+] \leftrightarrows \text{enzyme−activator complexes} \rightarrow EZn^{2+}-HO^- + AH^+ \quad (3)$$

It was shown that, in order to act as a CA activator, a compound needs precise steric and electronic factors to be present in its molecular structure (Puscas et al., 1990; Supuran, 1992; Puscas & Supuran, 1994; Clare & Supuran, 1994; Supuran et al., 1996a,b). Efficient activators are amines with the general formula **1**.



1, Ar = aromatic/heterocyclic group;
$R^1 = R^2 = H$, Me; $R^3 = H$, OH, COOH

All of these derivatives possess a bulky aromatic/heterocyclic moiety, and a primary/secondary amino group in their molecular structure, that may act as a proton acceptor (Supuran & Puscas, 1994). These two structural elements are separated by a two sp^3-hybridized carbon atom chain, possibly substituted as shown above. The kinetics of the hydrase activity of such compounds, which might also possess physiological relevance or pharmacological importance (Supuran & Puscas, 1994; Supuran et al., 1996a), have recently been investigated (Puscas et al., 1994), and a theoretical QSAR study has also been published (Clare & Supuran, 1994). Nevertheless, a detailed spectroscopic or X-ray crystallographic study has never been attempted for CA activators. Crystallographic evidence that the activators bind within the enzyme active site would definitively resolve the long-lasting controversy about the mere existence of CA activators (Van Goor, 1948; Maren, 1967; Supuran & Puscas, 1994).

In this work we report spectroscopic and X-ray crystallographic studies for the interaction of histamine, an efficient amine activator of type **1**, with two native isozymes (HCA I and HCA II) as well as Co(II)-substituted HCA II. Histamine has been investigated for activation of the esterase activity of CA toward 4-nitrophenyl acetate as substrate (isozymes HCA I and HCA II have been used) and spectroscopically toward Co(II)-substituted HCA II. The results of the present investigation induce us also to propose a new hypothesis, based on the reexamination of older X-ray crystallographic data available for different CA isozymes, that may explain the large catalytic differences between the different CAs in catalyzing both the physiological as well as nonphysiological reactions.

EXPERIMENTAL PROCEDURES

Buffers (Tris, Hepes, phosphate), 4-nitrophenyl acetate, histamine, and pyridine-2,6-dicarboxylic acid were from Sigma; acetonitrile was from Acros and was used without further purification. All buffers used in the kinetic measurements were brought to a ionic strength $\mu = 0.1$ by addition of K_2SO_4. Cobalt(II)−HCA II was prepared as described in the literature (Hunt et al., 1977) by removing zinc from the native enzyme in the presence of pyridine-2,6-dicarboxylic acid, followed by dialysis against metal-free Tris−H_2SO_4 buffer and addition of the stoichiometric amount of Co(II) salt.

Human CA I (HCA I) and CA II (HCA II) cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/HCA I and pACA/HCA II as described in the literature (Forsman et al., 1988) (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group (Behravan et al., 1991b), and enzymes were purified by affinity chromatography according to the published method (Khalifah et al., 1977). Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1}\cdot cm^{-1}$ for HCA I and 54 $mM^{-1}\cdot cm^{-1}$ for HCA II, respectively, based on $M_r = 28\,850$ for CA I and 29 300 for CA II, respectively (Henderson et al., 1976; Nyman & Lindskog, 1964).

Initial rates of 4-nitrophenyl acetate hydrolysis were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM-compatible PC (Pocker & Stone, 1967). 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 $M^{-1}\cdot cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.80), as reported in the literature (Pocker & Stone, 1967). Nonenzymic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each activator concentration, and the values reported throughout the paper are the mean of such results. The enzymatic rates (without activator) were obtained as the mean from at least three experiments.

Crystallization trials on the HCA II enzyme, prepared and purified as described above, were performed by the hanging drop method, using a 10 mg/mL HCA II solution in 50 mM Tris·HCl, pH = 7.7−7.8, containing 3 mM NaN_3 and 1 mM $HgCl_2$ or sodium 4-(hydroxymercury)benzoate. Mercuric and organomercury compounds are known to enhance the HCA II crystal quality (Tilander et al., 1965). The drops were obtained by mixing 5 μL of the enzyme solution with 5 μL of precipitant solution. The drop was equilibrated by vapor diffusion against 1 mL of precipitant solution using a 24-well cell-culture plate at 4 °C. The precipitant solution consisted of 2.3−2.5 M $(NH_4)_2SO_4$ in 50 mM Tris·HCl, pH = 7.7−7.8, containing 3 mM NaN_3 and the mercuric compound. Well-shaped parallelepipedons appeared within a week and grew to maximum dimensions of about $0.4 \times 0.4 \times 0.7$ mm in about 20 days. The best single crystals have been obtained with the organomercury compound and

a precipitant solution 2.3 M in $(NH_4)_2SO_4$. HCA II crystals belong to the $P2_1$ space group with typical unit cell parameters of $a = 42.6$ Å, $b = 41.7$ Å, $c = 72.8$ Å, and $\beta = 104.2°$. The HCA II−histamine complex has been prepared by soaking the crystals in a 50 mM Tris·HCl-buffered solution (pH 7.7) containing 2.5 M $(NH_4)_2SO_4$ and 50 mM histamine ($K_a \approx 10^4$ M). The crystals were kept in the soaking solution at 4 °C for 1 week before data collection.

Diffraction data were collected with a Siemens HiStar multiwire area detector using graphite monochromatized Cu Kα X-ray radiation from a Siemens M18X-HF rotating anode X-ray generator operating at 45 kV and 90 mA. All data were collected at room temperature from one crystal sealed in a 0.5 mm Lindemann glass capillary. The crystal dimensions were approximately 0.2 × 0.2 × 0.7 mm. The oscillation method was used with the crystal to detector distance set to 9.5 cm and the detector swing angle fixed at 25°. Frames of 0.1° oscillation about ω were collected with exposure times of 60 s/frame for a total angular rotation range of 100° about ω. A total of 49 849 reflections were measured to a maximum resolution of 1.95 Å. The data were merged to a set of 14 777 independent reflections with the XENGEN software, resulting in a $R_{sym} = 0.089$ ($R_{sym} = \sum_i||F_{hi}| - \langle|F_h|\rangle|/\langle|F_h|\rangle$, where F_{hi} is the modulus of an individual reflection and $\langle|F_h|\rangle$ is the average modulus for this reflection; the summation is over all data).

The CCP4 suite was used for all subsequent calculations (Collaborative Computational Project No. 4, 1994) The program FRODO (Jones, 1978) or TOM (FRODO adaptation for SGI workstations by S. Oatley) was used for model rebuilding and inspection of the $(3F_o - 2F_c)$ and $(F_o - F_c)$ Fourier maps at various stages of the work.

The initial model has been the refined structure of the HCA II native enzyme at 1.54 Å (Håkansson et al., 1992) from which all water molecules, the disordered parts of the structure, and residues 1−10 were removed. This model was subjected to stereochemically restrained least-squares refinement (Konnert, 1976; Konnert & Hendrickson, 1980) of individual atomic positions and temperature factors, as implemented in the CCP4 suite. A low-resolution cutoff of 15 Å was used. No cutoff was applied to amplitudes. A total of 30 cycles of the above refinement lowered the crystallographic *R*-factor ($R = \sum_i||F_{io}| - |F_{ic}||/\sum_i|F_{io}|$, where F_{io} and F_{ic} are the observed and calculated structure factors for each reflection, respectively) from the initial value of 0.32 to 0.25. At this stage the program ARP (Lamzin & Wilson, 1993) was used to model the solvent and to look for the potential activator molecule(s). An additional 10 cycles of ARP refinement lowered the *R* to 0.19 and provided better resolved Fourier maps. At this stage electron density of the shape corresponding to that of one histamine molecule bound at the entrance of the active site cavity was found. A model of the activator molecule was built into the density and the refinement continued with 40 more cycles of conventional restrained refinement which converged to a final *R*-factor of 0.154. The final model contained 2039 protein atoms, one mercury covalently bound to Cys206, 177 water molecules, and one histamine molecule and had very good stereochemistry as reported in Table 1. A difference electron density map with coefficients $(|F_o| - |F_c|)$ and phases calculated from the coordinates of the final model showed the highest peaks, at the 3σ level, close to the zinc ion indicating the anisotropy of its thermal motion. The rms error on atomic positions as estimated from Luzzati and σ_A plots (Luzzati, 1952; Read, 1986) was about 0.15 Å. The coordinates of the HCA II−histamine complex have been deposited with the Brookhaven Protein Data Bank under accession number 4TST (Bernstein et al., 1977).

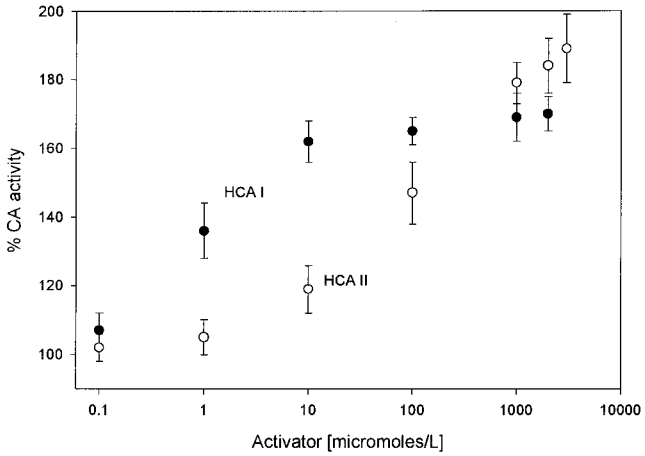


FIGURE 1: Activation of HCA I (0.121 μM) and HCA II (0.083 μM) with histamine, in concentration range of 10^{-7}−5.10^{-3} M, for the hydrolysis of 4-nitrophenyl acetate. CA activity in the absence of activator is taken as 100%. Substrate concentration was 2.5 mM; 10 mM Tris buffer, pH 7.40, at 25 °C and ionic strength of 0.1 (K_2SO_4).

Table 1: Refinement Statistics of the HCA II−Histamine Complex

	target error	HCA II−histamine complex
R_{cryst}		0.154
distances		
bond distance (Å)	0.020	0.018
angle distance (Å)	0.040	0.056
planar 1−4 distance (Å)	0.050	0.080
miscellaneous		
planar groups (Å)	0.020	0.018
chiral centers (Å^3)	0.150	0.174
nonbonded distances		
single torsion (Å)	0.300	0.177
multiple torsion (Å)	0.300	0.183
X−Y−H bonds (Å)	0.300	0.211
torsion angles		
planar (deg)	3.0	6.3
staggered (deg)	15.0	17.9
orthonormal (deg)	20.0	29.5
thermal restraints		
main-chain bond (Å^2)	3.000	2.762
main-chain angle (Å^2)	4.000	3.683
side-chain bond (Å^2)	4.500	5.471
side-chain angle (Å^2)	6.000	8.186

RESULTS

Previous work showed that histamine acts as a strong activator of BCA and HCA II for the CO_2 hydration as well as 4-nitrophenyl acetate hydrolysis reactions (Puscas et al., 1990; Supuran & Puscas, 1994; Clare & Supuran, 1994).

In Figure 1 the HCA I and HCA II activation with histamine for the esterasic activity is presented, with 4-nitrophenyl acetate as substrate, for the two isozymes, in concentration ranges of the activator of 10^{-7}−5×10^{-3} M and in the presence of 10 mM Tris buffer (pH 7.80, 25 °C, and constant ionic strength of 0.1).

From the data of Figure 1 it is clear that histamine is an efficient activator for both isozymes. For CA I, a powerful activation is already observed at 1 μM histamine concentration (around 140% of the control CA activity, observed in the absence of activators). Increasing concentrations of activator lead to enhanced activation until a plateau is reached

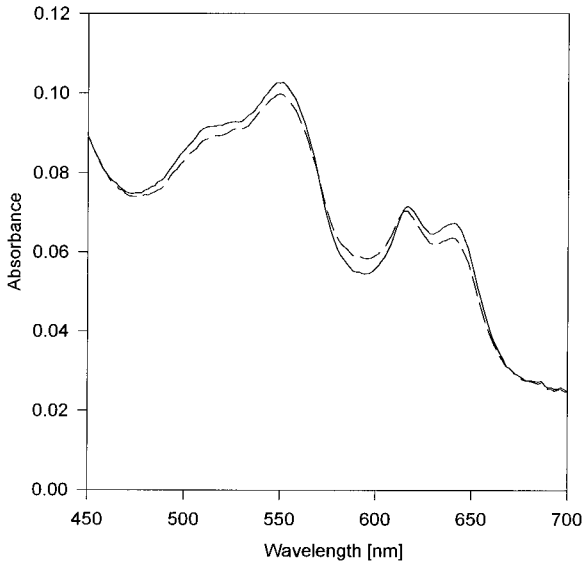


FIGURE 2: Electronic spectrum of Co(II)−HCA II (broken line) and its adduct with histamine (continuous line). Conditions were as follows: enzyme concentration 0.4 mM, in 50 mM Hepes buffer, pH 7.20. Histamine concentration was 3.6 mM.

when the maximal activity is 175% of the initial activity at concentrations larger than 10 μM. For HCA II, histamine starts to significantly activate at concentrations around 10^{-4} M, the final value in this case being 190% of the initial one at concentrations around 5 mM of histamine (Figure 1). Fitting of the observed catalytic enhancements as a function of the histamine concentration allows estimation of the affinity constants of histamine for the two isozymes, $K_a = (5 \pm 0.2) \times 10^5$ M^{-1} for HCA I and $K_a = (8 \pm 0.3) \times 10^3$ M^{-1} for HCA II.

In order to monitor the possible interaction of histamine with the active site of the enzyme, the electronic spectra of pure Co(II)−HCA II and of its adduct with histamine were recorded (Figure 2).

As observed from Figure 2, the spectrum of the adduct of Co(II)−CA with the activator is only slightly different from the spectrum of pure Co(II)−enzyme, suggesting that the activator molecule is not directly bound to the metal ion within the enzyme active site.

The overall three-dimensional structure of the HCA II−histamine complex is close to that of other published HCAs, but the first four residues of the N-terminal part are heavily disordered so as to prevent the building of any satisfactory model for any of them. The least-squares fit of the HCA II−histamine complex backbone with that of HCAII (2CBA) shows that the two structures are identical within experimental error, the resulting rms deviation being only 0.19 Å.

Table 2: Hydrogen Bonds and Contacts of the Histamine Molecule with HCA II

histamine	HCA II residue	distance (Å)
Nϵ2	Nϵ2 Gln 92	2.63
Nϵ2	Nδ2 Asn 62	3.20
Nϵ2	Oδ1 Asn 67	3.47
Nϵ2	Wat 152	2.66
Nδ1	Wat 133	3.12

The histamine molecule is found bound at the entrance of the active site cavity, where is anchored by hydrogen bonds to amino acid side chains and to water molecules. It is noteworthy that such hydrogen bonds are involving only the nitrogen atoms of the imidazole moiety. The terminal aliphatic amino group is not experiencing any contact with the enzyme but is extending from the cavity into the solvent. The electron density corresponding to the histamine molecule as obtained from an omit map is shown in Figure 3 superimposed to the refined atomic model of the complex.

The Nδ1 and Nϵ2 atoms of the histamine imidazole ring are engaged in hydrogen bonds with the side chains of Asn 62 and of Gln 92 and to Wat 133 and Wat 152 as reported in Table 2. The shape of the histamine imidazole ring electron density seems to indicate the presence of some rotational disorder, but no clear alternative conformations are evident. Comparison of the refined temperature factors of the histamine atoms at full occupancy with the average temperature factors of the protein side chains in nearby regions indicates that the molecule has partial occupancy estimatable between 30% and 40%.

Comparison of the refined model of the complex with that of the native enzyme refined at 1.54 Å shows some relevant differences. In native HCA II, as well as in most of its small molecule adducts, the zinc coordination polyhedron is always a quite regular tetrahedron with the three histidine nitrogens and a water/hydroxide molecule as ligands. On the contrary, in the HCA II−histamine complex, the electron density corresponding to the metal-coordinated water/hydroxide molecule has an elongated shape. The distances and angles around zinc are, within experimental error, the same as those of native HCA II, except for that of the nonprotein zinc ligand. Refinement of the water molecule position against this density results in placing it at the unusually long distance of 2.49 Å. Furthermore, the usual electron density corresponding to the so-called *deep water*, which is commonly hosted by the hydrophobic part of the active site cavity, could

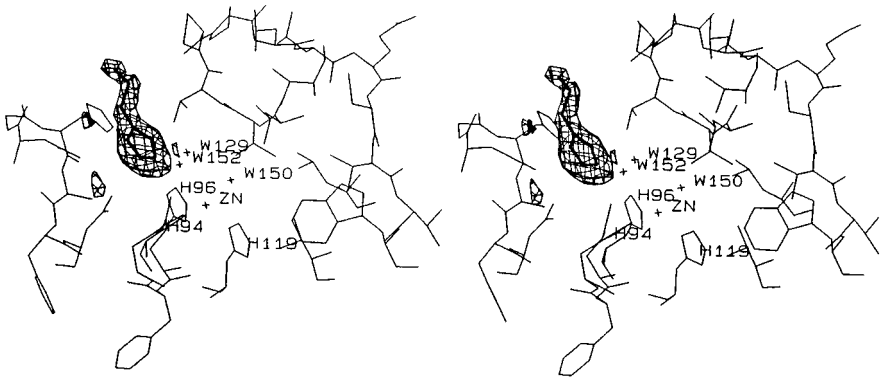


FIGURE 3: Stereoview of the omit map corresponding to the histamine molecule with the refined model of the activator molecule and of some relevant active site residues superimposed. The $|F_o| - |F_c|$ contours were drawn at a 3.0σ level.

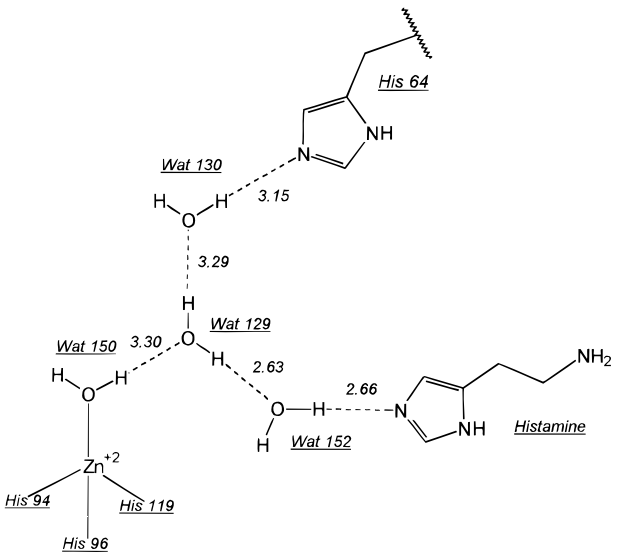


FIGURE 4: Scheme of the hydrogen-bonding pathways linking the zinc-bound Wat 150 to the histamine molecule and to His 64.

not be located in the present structure. The above two observations indicate that there is partial occupancy of the water coordination site by an azide molecule which is present in the crystallization solution in slight stoichiometric excess with respect to the enzyme. Azide is a known HCA inhibitor, and crystal structures of its complex with the enzyme have been reported (Jönsson et al., 1993; Nair & Christianson, 1993) where it has been found bound to the zinc ion replacing the native water molecule and displacing the *deep water* from its location.

The binding of histamine to HCA II displaces at least three water molecules from the active site cavity (as compared with the 2CBA structure), and this is accompanied by a substantial rearrangement of the water structure in the cavity. A further difference with respect to the native structure is found about the orientation of the His 64 side chain. While this residue has almost always been found disordered both in the native and in many HCA II complexes (Nair & Christianson, 1991; Håkansson et al., 1992), in the present structure the side chain of His 64 appears well defined and oriented toward the inside of the cavity pointing to the metal site. The His 64 imidazole ring is involved in a hydrogen bond with a nearby water molecule and makes short contacts with the histamine imidazole moiety.

The above differences may be related to the binding of the histamine molecule to the enzyme. Inspection of the refined model of the HCA II−histamine complex reveals the presence of a hydrogen bond pathway linking the zinc-bound water molecule to histamine through Wat 129 and Wat 152 as shown in Figure 4. A second alternative pathway exists through Wat 129 and Wat 130 reaching His 64 (Figure 4).

This is similar to the pathway existing in the native enzyme between the zinc-bound water and His 64, which has been considered to be the normal proton release pathway (Christianson & Fierke, 1996).

DISCUSSION

Previous work on CA activators showed differences between the two isozymes, in their behavior toward amine and amino acid activators, for the hydrase activity of the enzyme (Supuran & Puscas, 1994; Supuran & Balaban, 1993; Supuran et al., 1996a,b). One of the strongest activators described up to now is histamine (Supuran & Puscas, 1994). Thus, for HCA I, an important activating effect (around 140%) is already seen at micromolar concentrations of histamine, the maximal value which is measurable being around 175% of the initial rate, at concentrations of histamine larger than 10^{-5} M. The value for the affinity constant of histamine for HCA I is about 5×10^5 M^{-1}, which represents a quite large number, comparable to that of the strong CA inhibitors, i.e., aromatic sulfonamides.

Toward HCA II, histamine shows a more pronounced activation effect, corresponding to a doubling of the initial rate. However, its affinity constant decreases about 2 orders of magnitude, K_a being around 8×10^3 M^{-1}.

The high values of the affinity constants and the sigmoidal shape of the curves (enzymatic activity *vs* activator concentrations) are strongly indicative of the existence of a specific interaction between histamine and a single binding site located on the enzyme. The location of this until now unknown binding site can be at this point tentatively assigned.

First of all, it has been reported recently (Supuran & Puscas, 1994; Puscas et al., 1994) that activators of type **1**, including histamine, are noncompetitive with the substrate CO_2, for BCA as well as HCA II. As for the corresponding hydrase activity, also in the case of 4-nitrophenyl acetate hydrolysis, it was recently proven that activators of the amine type bind noncompetitively to isozymes HCA I and HCA II, in agreement with the scheme proposed for explaining their mechanism of action (eq 3) (Supuran & Puscas, 1994; Coltau et al., 1994).

The binding site of histamine does not overlap therefore with that of the substrates of the enzyme.

From the electronic spectra of the adduct of Co(II)-substituted HCA II with histamine, reported here (Figure 2), it can be seen that slight differences appear between the spectra of the enzyme−activator adduct, as compared to the spectrum of pure Co(II)−HCA II, at the same pH. This spectrum, on the other hand, is not similar with those of any known anionic or sulfonamide CA inhibitor adduct (Bertini et al., 1978, 1982, 1992; Bertini & Luchinat, 1983). The conclusion is that the binding site of histamine is not located on the Zn(II) ion. The observed spectrum is instead reminescent of that of the adduct of Co(II)−HCA II with phenol, the only reported competitive inhibitor with CO_2 as substrate of this isozyme (Simonsson et al., 1982). This inhibitor has been shown to bind in the hydrophobic pocket of the enzyme, without displacing the metal-bound solvent molecule. This peculiar mode of binding has been recently confirmed after the X-ray structure of the adduct had been reported (Nair et al., 1994). Phenol does not coordinate to zinc but binds the zinc-bound solvent through a 2.6 Å hydrogen bond, and a second, poorly oriented hydrogen bond has also been detected between the phenolic hydroxyl and the NH of Thr 199 (of 3.2 Å) (Nair et al., 1994).

The close resemblance between the electronic spectra of the histamine adduct of Co(II)−CA II and that of phenol strongly suggests that the activator should bind to the enzyme in a somehow similar manner to phenol, i.e., without displacing the zinc-bound solvent molecule. In this way, it would be able to participate in efficient proton-shuttling processes between the active site and the medium.

Indeed, the crystal structure of the complex shows that histamine binds at the entrance of the active site cavity leaving it unaltered, but establishing new hydrogen-bonding

pathways linking the zinc-bound water/hydroxide to the bulk solution. The interactions of the amine with the protein are limited to residues Asn 62, Asn 67, and Gln 92 and do not appear to be specific for this kind of molecule. Indeed, preliminary X-ray investigations on other activators show that they bind in the same position of histamine (unpublished results from our laboratory). The discriminating factor between the classical HCA inhibitors which bind to the metal replacing the zinc-bound water/hydroxide and this class of molecules "stopped" at the entrance of the active site cavity may be due to their limited Lewis base character, making them unable to replace the strong OH^- zinc ligand.

The hydrogen bond pathway linking the zinc-bound water (Wat 150) to the histamine molecule reported in Figure 4 provides an alternative route for proton release besides the His 64 shuttling. The mere availability for the proton of more than one pathway to leave the active site appears to be a satisfactory explanation for the activatory property of histamine toward HCA II. The crystal structure of the complex shows that the histamine molecule is held in the active site by few interactions only involving the imidazole moiety. This is consistent with the partial occupancy found from the crystallographic refinement for the histamine molecule and with the measured affinity constant which is in the millimolar range. The entropic contribution to the histamine free energy of binding to HCA II provided by the release of water molecules appears to dominate the complex formation although the ability of histamine to simultaneously make two hydrogen bonds surely provides further stabilization for the complex. The magnitude of the interaction seems to be such as to favor the activatory effect. Indeed, the ability of histamine to easily leave the active site cavity and act as a second proton shuttle seems to perfectly fit the activation mechanism as emerging from the structural findings.

Although important advances were registered in the last period regarding the molecular mechanisms of catalysis/inhibition of CA isozymes, one of the puzzling and unresolved problems in CA research is the understanding of the large catalytic differences between the diverse isozymes. Indeed, among them can be found a perfectly evolved catalyst, CA II [one of the most rapid enzymes known (Silverman & Lindskog, 1988)], a 10 times less efficient one, CA I (Lindskog et al., 1991), and a very inefficient isozyme, CA III, possessing 0.3% of the HCA II CO_2 hydration activity (Engberg et al., 1985; Paranawithana et al., 1991).

As mentioned in the introductory section, the very high efficiency of isozyme CA II in catalyzing CO_2 hydration was explained as being due to the proton-shuttling capacity of active site residue His 64, possessing a pK_a around 7 (Campbell et al., 1975; Tu et al., 1989). This residue is situated at the entrance of the active site (Eriksson et al., 1988) and has a large mobility (Smith et al., 1994). Although CA I has the same residue in position 64, it is currently considered to be unable to shuttle protons efficiently, as its pK_a is much lower, around 4.5 (Campbell et al., 1975; Lindskog et al., 1991). But CA I has other histidine residues within its active site, such as His 67, His 200, or His 243, possibly able to participate in proton-transfer processes (Lindskog et al., 1991; Behravan et al., 1990, 1991a). Thus, for example, much research of Lindskog's group was directed toward changing amino acid residues in HCA II with the corresponding residues of HCA I (by site-directed mutagenesis), in order to transform the high-activity isozyme II into the low-activity one, HCA I (Lindskog et al., 1991; Behravan et al., 1990, 1991a,b; Engstrand et al., 1992; Liang et al., 1993). Although the mutated isozymes obtained in this way had smaller catalytic efficiency than the wild-type HCA II (around 70−80% of the activity of native HCA II in some cases), they did not approach the 10 times lower CO_2 hydration activity of HCA I (as compared to wild-type HCA II) (Lindskog et al., 1991; Lindskog & Liljas, 1994; Liljas et al., 1994).

FIGURE 5: Schematic view of the HCA II structure. The Zn(II) ion and its three ligands, as well as the His cluster, are shown. The cluster is situated at the entrance of the cavity and consists of the following residues: His 64, two conformations; His 4, two conformations, His 3; His 10; His 15; and His 17. The figure was generated from the X-ray coordinates of HCA II from Brookhaven Protein Data Bank (file code 2CBA).

On the basis of the present results on the mechanism of action of CA activators, and by analyzing the X-ray crystallographic data of known CA isozymes, we suggest here a possible explanation for the large difference in the catalytic efficiency of diverse CAs. The high efficiency of the most active isozyme, HCA II, is due to a unique feature of its active site: the presence of a histidine cluster, consisting of the following residues: His 64, two conformations; His 4, two conformations, His 3; His 10; His 15; and His 17 (Figure 5). This cluster extends from the interior of the active site (His 64) to its entrance (His 4 and His 3) into the surface of the protein (in the proximity of the active site entrance), and we postulate that it constitutes a very appropriate "channel" for efficiently transferring protons from the active site to the reaction medium (Figure 5). As seen from Figure 6, in the low-activity isozyme CA I, such a cluster does not exist. Moreover, the pathways for the proton transfer are somehow bifurcated and divergent as the four histidines present within the active site His 64, His 67, His 200, and His 243 [except, of course, for the three Zn(II) ligands, which in both isozymes are His 94, His 96, and His 119] are placed at bifurcating positions. As seen from Figure 6, these four histidines in HCA I (His 64, His 67, His 200, and His 243) are rather buried in the active site so that, probably, the proton transfer cannot be as efficient as the one assisted by the histidine cluster present in HCA II.

In this context it is enlightening that histamine, which is able to enhance the catalytic efficiency of the enzyme, does really bind in this region of the active site of HCA II. The activator molecule is situated in such a position at the entrance of the active site as to participate to supplementary proton-transfer reactions (the rate-determining step in ca-

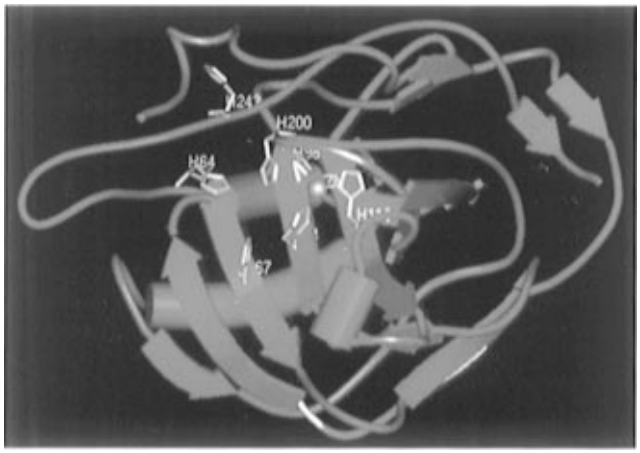


FIGURE 6: Schematic view of the HCA I structure. The Zn(II) ion (central sphere) and its three histidine ligands (His 94, 96, and 119; red), as well as active site residues His 64, His 67, His 200, and His 243, are shown. The figure was generated from the X-ray coordinates of HCA I from Brookhaven Protein Data Bank (file code 1huh).

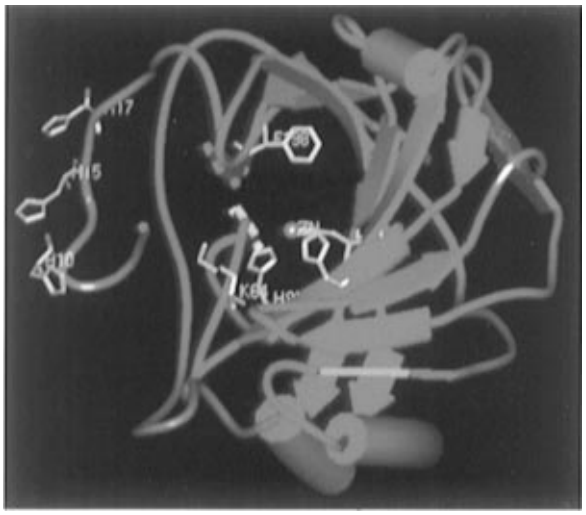


FIGURE 7: Schematic view of the BCA III structure, with the Zn-(II) ion and its three histidine ligands, His 94, 96, and 119. The active site residues Lys 64 and Phe 198 and the histidine semicluster His 10, 15, and 17, are also shown. The figure was generated by using the program MSViewer, from the X-ray coordinates of Eriksson and Liljas, provided by the authors.

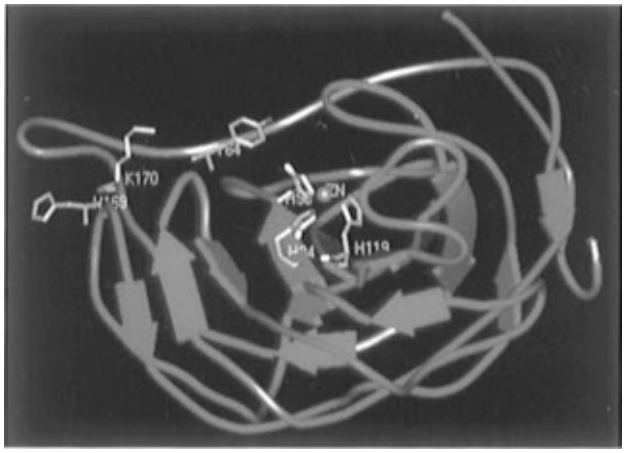


FIGURE 8: Schematic view of the murine CA V structure, with the Zn(II) ion and its three histidine ligands (His 94, 96, and 119) as well as residues Tyr 64, His 169, and Lys 170 shown. The figure was generated by using the program MSViewer, from the X-ray coordinates of Christianson et al., provided by the authors.

talysis). For instance, the effect of histamine as activator on HCA I is more marked at low concentrations of activator (see Figure 1) as compared to the similar effect on HCA II, in which "natural activators"−the histidine cluster−are present in the molecule of the enzyme.

The presence of the histidine cluster in HCA II can also explain many data regarding the behavior of isozyme III. Its three-dimensional structure (the bovine enzyme, BCA III) has been recently reported at 2.0 Å resolution (Eriksson & Liljas, 1993). Although the structure is highly similar to that of HCA II, as mentioned before, CA III has a CO_2 hydration activity of about 0.3% that of HCA II (Engberg et al., 1985). It does not possess a His in position 64, but a Lys residue, whereas position 198 is occupied by a Phe, possessing a very bulky side chain; furthermore, the water bound to Zn(II) has a pK_a around 5.5 (Kararli & Silverman, 1984). These particularities should explain the low catalytic activity of this isozyme, as well as its insensitivity to sulfonamide inhibitors (Koester et al., 1977; Sanyal et al., 1982, Pullan & Noltmann, 1984). Still, efforts of Silverman's group to transform the high-activity isozyme CA II into the very inefficient catalyst which is CA III, again by introducing isozyme III-characteristic amino acid residues into isozyme II, by site-directed mutagenesis (such as Phe 198, Lys 64, etc.), only led to slightly less active enzymes as compared to wild-type CA II (Paranawithana et al., 1991; Jewell et al., 1991). In our opinion this is due to the fact that even if a small portion of the histidine cluster of CA II is modified (for instance, by changing His 64 by Lys), the mutant cluster is still able to efficiently shuttle protons, the overall catalytic activity being influenced in a minimal way. It is much more interesting to consider the structure of the wild-type CA III, in which a histidine semicluster is still present (Figure 7), consisting of residues His 10, His 15, and His 17. This semicluster is placed on the surface of the enzyme, not far away from the entrance within the active site cavity, somehow similarly to the corresponding part of the cluster in HCA II. But, in contrast to HCA II, the other half of the cluster is missing, as no other histidine residues (or other residues able to shuttle protons efficiently in the pK_a range of 6−8) are present within the active site of CA III. As shown by the research of Silverman's group, Lys 64 only acts as a very inefficient proton-transferring group (Silverman & Lindskog, 1988; Jewell et al., 1991; Paranawithana et al., 1991). Moreover, the bulky phenyl moiety of Phe 198 disturbs the binding of the substrate and inhibitors in the vicinity of the catalytically critical Zn(II) ion.

Finally, the only other isozymes for which the three-dimensional structure has been reported are CA V (Boriack-Sjodin et al., 1995) and CA IV (Stams et al., 1996). The first isozyme, present in the mitochondria, has the particularity of being most active at high pH [optimal activity at pH 8.5 (Heck et al., 1994)]. Similarly to CA III, it does not possess a His at position 64, but a tyrosine residue (Figure 8). Although it has been proven that Tyr 64 or Tyr 131 is not the major proton shuttle at the pH values at which the enzyme functions (Heck et al., 1996), we showed in Figure 8 some residues at the entrance of the active site (Lys 170, His 169) which might be able to participate in proton-transfer processes, although with an efficiency difficult to estimate. One should also take into account that the surface of this mitochondrial protein is very rich in arginine residues, making it relatively basic, in contrast to all other CA isozymes. Such basic residues might have a critical role in

these proton-transfer processes. Although the structure of CA IV has been recently reported (Stams et al., 1996), the file is not yet available in the Brookhaven Protein Data Bank, and this isozyme has not been analyzed for the eventual presence of a histidine cluster.

SUMMARY AND CONCLUSIONS

Amine-type activators possessing the general formula **1** have been shown to directly bind within the CA II active site, without interacting with the metal center. In fact, we propose this binding site of activators as the third important binding site in CAs, after the metal center (which is the binding site for inorganic anions and sulfonamide inhibitors) and the hydrophobic pocket [the binding site of the substrate-(s) and of phenol, the unique CO_2-competitive inhibitor]. Histamine binds in the hydrophilic region located at the entrance of the HCA II active site, between residues His 64, Gln 92, Asn 62, and Asn 67. Moreover, His 64, which possesses a high flexibility and at least two conformations in all reported structures, appears with only one conformation in its adduct with histamine, the "out" conformation not being evidenced, presumably due to the binding of the activator molecule. The mechanism of action for activators suggests a facilitation of the rate-determining proton-transfer reaction between the active site and the reaction medium. In isozyme CA II, the most active one, a histidine cluster extending from within the middle of the active site itself to the surface surrounding the entrance in it and consisting of residues His 64, His 4, His 3, His 10, His 15, and His 17, is postulated to play an important role in such processes. This explains why different attempts of transforming this rapid isozyme (by means of site-directed mutagenesis) into the more inefficient CA I or the highly inefficient CA III failed (from the point of view of the catalytic activity of the obtained mutants, which were more or less similar to CA II but not to CA I or CA III). CA I possesses only four histidine residues able to participate in proton-transfer processes (His 64, His 67, His 200, and His 243), with lower efficiency, explaining thus the 10 times lower catalytic power of this isozyme. Activators of these two isozymes, of the amine type, as those described in the present paper, bind at the entrance of the active site, actively facilitating such proton-transfer processes.

In addition to isozymes I and II, the other isozymes for which X-ray crystallographic structures were reported, such as CA III or CA V, possess particularities that explain their lower catalytic power: CA III does not possess a His residue in position 64, but a lysine, less efficient as a proton shuttle group; its water bound to the Zn(II) ion has a pK_a of 5.5, and a bulky phenylalanine residue (Phe 198) disturbs the binding of substrate or inhibitor molecules within the active site. Although this isozyme possesses a histidine semicluster on its surface, in the vicinity of the entrance to the active site, this is useless for the catalytic power of the enzyme, due to the above-mentioned structural features. CA V, on the other hand, a mitochondrial enzyme, functions at high pH values (around 8.5−9.0), characteristic of the environment within these cellular organelles, and as a consequence the water bound to Zn(II) has a pK_a value of around 8.5, and the proton-transfer processes presumably involve tyrosine and not histidine residues.

Predictions can be made in light of the above data on how to transform a low-activity CA into a high-type one and *vice versa*. Thus, in order to transform the catalytically efficient CA II into the low-activity form CA I, as already mentioned, it is not enough to change amino acids at positions 67 and 200 (as already done by Lindskog group); instead, the whole histidine cluster should be mutated in order to avoid the high efficiency of proton transfer that it promotes. The same is true for the eventual attempts of transforming CA II into a CA III-type enzyme: in addition to changing His 64 for Lys 64 and the introduction of the bulky residue Phe 198, such an enzyme should not have to contain the first part of the cluster, consisting in addition of His 64, His 3, and His 4.

But activators of these widely spread enzymes presumably possess important physiological functions too. In addition to histamine, serotonin, dopamine, and other catecholamines are important autacoids, present in concentrations high enough to elicit important CA activatory effects in many tissues (Supuran & Puscas, 1994). In fact, many of them have been recently investigated by this group for their interaction with different CA isozymes [for a review see Supuran and Puscas (1994)]. More than that, CA activation has also been reported with amino acids and oligopeptides, some of which are important hormones (Supuran & Puscas, 1994; Puscas et al., 1994). Although hypotheses have been made (Puscas & Supuran, 1994) regarding the role of such activators in intracellular signal-transducing systems, more detailed studies are needed in order to understand the role played by CA activators *in vivo*, in physiologic as well as pathologic conditions, now that the mechanism of action at the molecular level of CA activators has been probed.

ACKNOWLEDGMENT

We are indebted to Dr. Sven Lindskog (Umea University, Sweden) for the gift of the two plasmids producing HCA I and II and to Drs. Anders Liljas (Lund University, Sweden) and David W. Christianson (University of Pennsylvania) for X-ray coordinates of different CA isozymes and their adducts with inhibitors. We also express our gratitude to Prof. G. Zanotti (Padova University, Italy) and to the "Centro Biopolimeri" of Padova University for the access to X-ray diffraction facilities and help in data collection and reduction.

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BI970760V