Carbonic Anhydrase Activators. Activation of Isoforms I, II, IV, VA, VII, and XIV with L- and D-Phenylalanine and Crystallographic Analysis of Their Adducts with Isozyme II: Stereospecific Recognition within the Active Site of an Enzyme and Its Consequences for the Drug Design†

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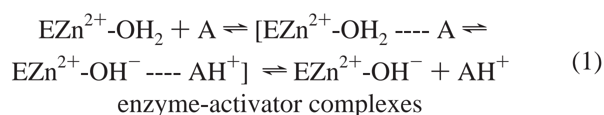
Activation of six human brain carbonic anhydrases (hCAs, EC 4.2.1.1), hCA I, II, IV, VA, VII, and XIV, with L-/D-phenylalanine was investigated kinetically and by X-ray crystallography. L-Phe was a potent activator of isozymes I, II, and XIV (K_As of 13−240 nM), a weaker activator of hCA VA and VII (K_As of 9.8−10.9 μM), and a quite inefficient hCA IV activator (K_A of 52 μM). D-Phe showed good hCA II activatory properties (K_A of 35 nM), being a moderate hCA VA, VII, and XIV (K_As of 4.6−9.7 μM) and a weak hCA I and IV activator (K_As of 63−86 μM). X-ray crystallography of the hCA II−L-Phe/D-Phe adducts showed the activators to be anchored at the entrance of the active site, participating in numerous bonds and hydrophobic interactions with amino acid residues His64, Thr200, Trp5, and Pro201. This is the first study showing different binding modes of stereoisomeric activators within the hCA II active site, with consequences for overall proton transfer processes (rate-determining for the catalytic cycle). It also points out differences of activation efficiency between various isozymes with structurally related activators, exploitable for designing alternative proton transfer pathways. CA activators may lead to the design of pharmacologically useful derivatives 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 spatial learning and memory therapy must be enhanced. As the blood and brain concentrations of L-Phe are quite variable (30−73 μM), activity of some brain CAs may strongly be influenced by the level of activator(s) present in such tissues.

Introduction

In previous work from this laboratory, we have explored the drug design of activators of the metallo-enzyme carbonic anhydrase (CA, EC 4.2.1.1), belonging to various classes of compounds.[1,2] Although activation of this enzyme by amines and amino acids was reported[3] simultaneously with its inhibition by sulfonamides,[4] the two discoveries had completely different consequences for the CA research.[5] While CA inhibitors (CAIs) have been constantly and extensively studied, as well as exploited clinically for the prevention and treatment of several diseases (with at least 10 such derivatives in clinical use),[6,7] CA activation has become—soon after its initial report—a controversial issue.[5] Consequently, little progress has been made in the research of CA activators (CAAs) for a long period. However, in the past decade, by means of electronic spectroscopy, X-ray crystallography, and kinetic measurements, it has been proved that the activator molecule binds within the enzyme active cavity at a site distinct of the inhibitor or substrate binding sites,[5] participating thereafter in the rate-determining step of the catalytic cycle, i.e., the proton transfer processes between the active site and the environment.[5−9] Thus, the CA activator (A in eq 1) interferes directly in the proton transfer step of the catalytic cycle, facilitating such an intramolecular process by means of transient enzyme−activator complex(es).[5] As intramolecular reactions are faster than the intermolecular ones, the result is a significantly enhanced catalytic rate:[5]



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

enzyme-activator complexes

Activation of some members of the α-CA family (one of the genetically unrelated CA gene families presently known, the α-CAs−δ-CAs)[6] was recently shown to constitute a possible therapeutic approach for the enhancement of synaptic efficacy, which may represent a conceptually new treatment for Alzheimer's disease, aging, and other conditions in which it is necessary the achievement of spatial learning and memory therapy.[10,11] Indeed, CAAs might lead to interesting pharmacological applications, although this field is largely unexplored for the moment.[5] A multitude of physiologically relevant compounds such as biogenic amines (histamine, serotonin, catecholamines), amino acids, oligopeptides, or small proteins among others act as efficient CAAs for the human CA isozymes I and II (other isoforms of the 15 presently known in humans have not been investigated in detail up to now).[5]

Sun and Alkon reported[10,11] that phenylalanine, an activator first investigated by our group against isozymes I and II,[12,13] when administered to experimental animals produces a relevant pharmacological enhancement of synaptic efficacy, spatial learning, and memory, proving that this class of unexplored enzyme modulators may be used for the management of conditions in which learning and memory are impaired.[10,11] It should be also mentioned that it was previously discovered that the levels of several CA isozymes (such as CA I and CA II) are significantly diminished in the brain of patients affected by Alzheimer's disease,[14a] as well as in 20-month old rats as compared to young animals,[14b] a fact strongly supporting the

† Both structure coordinates reported in the paper have been deposited in the Brookhaven protein database (PDB ID 2FMZ and 2FMG).

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involvement of brain CAs in cognitive functions and aging.[10,11] Very recently, several new studies showed[15] by means of redox proteomics that isoforms CA I and CA II are oxidized or nitrosylated in the frontal cortex and hippocampus of Alzheimer patients, or in patients affected by Parkinson's disease[15d] (another neurodegenerative disease), their catalytic activity being significantly decreased. Thus, in the brain of these patients the dysfunctions of CA I and/or CA II activity leads to imbalances of the extra- and intracellular pH, which may trigger aggregation of proteins, contributing thus to progression of the disease.[15] It is probable that agents that may restore to a certain degree the catalytic activity of these particular isozymes (CA I and CA II), or increase that of other CAs present in the brain (isoforms CA IV, CA VA, CA VII and CA XIV have also been found to be present in the human brain)[5−8] of the CAA type, might lead to conceptually novel approaches for the management of Alzheimer's disease. Furthermore, it is known that the level of L-phenylalanine present in the blood and brain in humans is highly variable, being in the range of 30.3−72.7 μM.[15e]

Three X-ray crystallographic structures of adducts of the main human isoform, hCA II, with activators are known at this moment: one with histamine,[16] one with phenylalanine (a ternary complex in which azide is also bound to the Zn(II) ion of the hCA II active site),[13] and another one with L-histidine.[17] All 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 among others) and water molecules, 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, i.e., the proton transfer reaction between the zinc-bound water molecule and the environment (see eq 1), which in many CA isozymes (in the absence of activators) is assisted by the amino acid residue His64 situated in the middle of the active site cavity and, also possessing a pH-dependent conformational mobility, by changing gradually its orientation related to the metal site through a 64° ring-flipping.[16−18] 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.[5,6]

Here we report a detailed activation study (for the CO_2 hydrase activity, i.e., the physiological reaction catalyzed by these enzymes) of all isoforms found in the human brain, i.e., hCA I, II, IV, VA, VII, and XIV, with L- and D-phenylalanine, as well as the X-ray crystal structure of the two adducts with the main physiological isozyme, hCA II. Many of these isozymes (e.g., CA VA, CA VII, or CA XIV) have never been investigated earlier for their interaction with activators, whereas some of them were recently shown to be abundant in the brain where they play important physiological roles, some of which are only now beginning to be understood.[5−8,19] Thus, in addition to bringing novel insights regarding the catalytic/activation mechanisms of several CA isozymes, our study may help the drug design of CAAs with possible pharmacological applications for obtaining anti-Alzheimer's disease agents or derivatives useful in the treatment of CA deficiencies, genetic conditions in which one CA isoform is absent.[20]

Results

Carbonic Anhydrase Activation. No detailed activation studies of other CA isozymes except hCA I and II are available in the literature at this moment. Kinetic data for steady-state conditions and activation constants of the brain hCA isozymes I, II, IV, VA, VII, and XIV with L-phenylalanine and D-phenylalanine, at 25 °C, for the CO_2 hydration reaction are shown in Table 1.

Table 1. Activation[a] of hCA Isozymes I, II, IV, VA, VII, and XIV with L- and D-Phenylalanine, at 25 °C, for the CO_2 Hydration Reaction[3,4,21]

isozyme	k_{cat}[b] (s^{-1})	$(k_{cat})_{L-Phe}$[c] (s^{-1})	$(k_{cat})_{D-Phe}$[c] (s^{-1})	K_A[d] (μM) L-His	K_A[d] (μM) D-His
hCA I[e]	2.0×10^5	19.8×10^5	2.3×10^5	0.071	86
hCA II[e]	1.4×10^6	5.7×10^6	5.2×10^6	0.013	0.035
hCA IV[f]	1.2×10^6	1.6×10^6	1.4×10^6	52	63
hCA VA[g]	2.9×10^5	4.3×10^5	9.7×10^5	9.81	4.63
hCA VII[e]	9.5×10^5	14.6×10^5	15.8×10^5	10.93	9.74
hCA XIV[g]	3.1×10^5	12.5×10^5	6.1×10^5	0.24	7.21

[a] Standard errors were in the range of 5−10% of the reported values. [b] 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). [c] Observed catalytic rate in the presence of 10 μM activator. [d] The activation constant (K_A) for each isozyme was obtained by fitting the observed catalytic enhancements as a function of the activator concentration.[3,14] Mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[28] [e] Human recombinant isozymes. [f] Truncated human recombinant isozyme lacking the first 20 amino acid residues.[26b] [g] Full length, human recombinant isoforms.[24−26]

Table 2. Crystallographic Parameters and Refinement Statistics for the hCA II−D-Phe and hCA II−L-Phe Adducts

parameter	value: CA II/D-Phe complex	value: CA II/L-Phe complex
X-ray source	Enhance Ultra	Enhance Ultra
wavelength (Å)	1.54	1.54
cell parameters	$a = 42.09$ Å	$a = 42.0$ Å
	$b = 41.38$ Å	$b = 41.41$ Å
	$c = 71.10$ Å	$c = 72.20$ Å
	$\alpha = \gamma = 90°$	$\alpha = \gamma = 90°$
	$\beta = 104.40°$	$\beta = 104.40°$
space group	$P2_1$	$P2_1$
no. of unique reflections	16335	16166
completeness (%)[a]	99.0 (98.8)	98.0 (96.2)
multiplicity	3.0(2.0)	3.1(3.0)
no. of reflections [>2$\sigma(F_o)$]	16294	16116
$\langle I/\sigma(I)\rangle$	20.8 (14.5)	14.0 (10.2)
resolution range (Å)	10-2.0	10-2.0
proteins atom	2049	2049
water molecules	175	175
R-merge (%)	4.7 (4.9)	9.0 (10.0)
R-factor (%)	20.0	20.5
R-free (%)[b]	24.0	24.6
rmsd of bonds from ideality (Å)	0.005	0.005
rmsd of angles from ideality (deg)	1.38	1.36

[a] Values in parentheses relate to the highest resolution shell (2.11−2.0). [b] Calculated using 10% of data.

X-ray Crystallography. To rationalize the CA activating properties of these two amino acids, L-Phe and D-Phe, the 2.0 Å resolution X-ray crystal structures of their adducts with the physiologically most relevant isoform, i.e., hCA II, have been resolved. The statistics for data collection and refinement of the two adducts, hCA II−D-Phe and hCA II−L-Phe, are summarized in Table 2, whereas in Table 3, hydrogen bonds and other relevant distances are provided. The details for the binding of the two stereoisomeric activators within the enzyme cavity are shown in Figures 1−3. A comparison between the hCA II−L/D-Phe adducts reported here and the structure of the previously reported[13] ternary complex of hCA II−azide−L-Phe is shown in Figure 4.

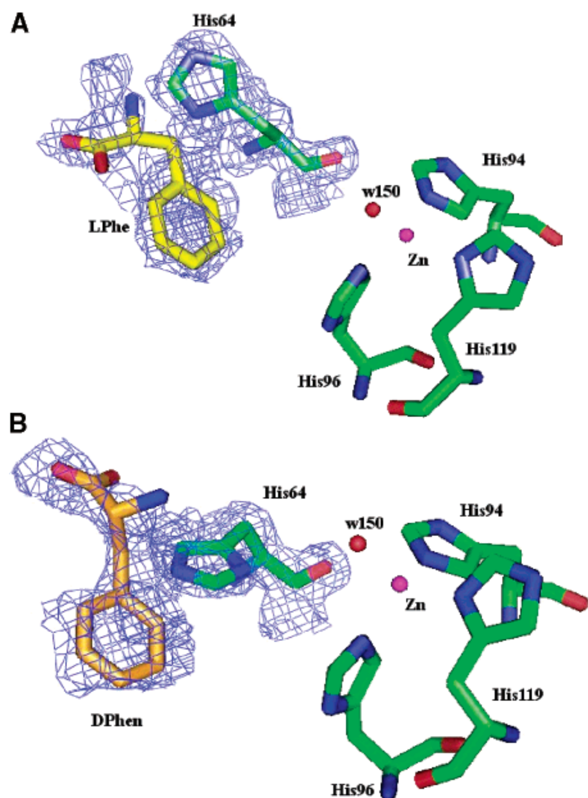

Figure 1. Omit electron density maps of hCA II in complex with L-Phe (A) and D-Phe (B) at 2.0 σ.

Table 3. Hydrogen Bonds and Contacts of the L/D-Phe Molecules in Complex with hCA II

L-Phe atom	D-Phe atom	hCA II residue	distance (Å)
N		Nδ1 His64	3.41
N		Nε1 Trp5	3.21
	N	O Pro201	3.00
	N	Oγ1 Thr200	2.70
	O	Nε1 Trp5	3.00
	O	Nε2 His64	3.60

Discussion

Carbonic Anhydrase Activation. Systematic studies of activation of CA isoforms I and II with amino acids and related compounds, including natural/synthetic amino acids, their esters, *N*-alkyl, *N*-acyl, or pyridinium derivatives, have been reported by this group.[1,2,5,21] Such studies led to the following observations: (i) The most powerful CAAs in these series of compounds were L-histidine, L-proline, L-homoproline, together with aromatic amino acids structurally related to L-phenylalanine (e.g., compounds incorporating substituted phenyl rings, such as 4-halogeno-, 4-hydroxy-, or 4-amino-phenyl moieties among others). In fact, X-ray crystallographic data (for the hCA II−histamine adduct and the hCA II−azide−phenylalanine ternary complex)[12,16] clearly demonstrated that the aryl/hetaryl moieties present in the activator molecule increase the stability of the enzyme−activator complexes. (ii) Derivatization of the amino or carboxylic groups generally diminished the activator efficiency, mainly due to a decrease in the charge on the most electronegative atom of the molecule (via induced electronic effects), as it was later rationalized by means of QSAR calculations.[12] However, some N-derivatized or carboxy-derivatized amino acid derivatives showed very good CA activatory properties against isozymes hCA I and II.[1,2,5,21] (iii) A strong correlation has been observed between the pK_a value of the activator molecule and its potency, with compounds possessing a pK_a in the range 6.0−8.0, for at least one deprotonatable moiety, leading to the best CA I and II activating properties.[5] All these data clearly showed that a CAA must possess specific steric and electronic requirements for good activity: first, it must fit within the restricted active site cavity of the enzyme, but should interact favorably with amino acid residues present in the activator binding pocket, and second, it should possess a moiety able to participate in proton transfer processes, better if with a pK_a in the range of 6.0−8.0 units.

It must be noted that no D-amino acids have been investigated up to now for CA activation effects, and rarely other isoforms than the red blood cell cytosolic ones hCA I and II have been included in such studies. Here we present activation data with L-Phe and D-Phe against six physiologically relevant human brain CA isozymes, i.e., hCA I, II, IV, VA, VII, and XIV (Table 1), for the physiological reaction catalyzed by them, i.e., CO_2 hydration to bicarbonate and a proton. CA activation studies with this amino acid are important, since the blood and brain concentrations of L-Phe are very variable in humans, with a range of 30−72.7 μM.[15e] Among the CA isoforms investigated in the present study, some are cytosolic (hCA I, II, and VII), others are extracellular (CA IV and CA XIV), and CA VA is mitochondrial.[6] Two of the cytosolic forms, hCA I and II, are ubiquitous in the human body and quite abundant in the brain, whereas isoform VII is mainly restricted to the brain.[6] hCA IV and hCA XIV have an extracellular active site, being membrane-associated (CA IV) or transmembrane (CA XIV) isoforms, quite abundant in the brain (and other tissues too)[6,7] where they seem to play important physiological roles in pH homeostasis.[19−21] Finally, CA VA is a mitochondrial isozyme involved in several biosynthetic processes such as gluconeogenesis, ureagenesis, and lipogenesis among others.[22,23] Inhibition of many of these CAs by means of sulfonamides or sulfamates was recently shown to lead to novel approaches for the development of pharmacological agents useful for the treatment of glaucoma, obesity, or cancer among others.[23−26]

Data of Table 1 show that L- and D-Phe activate all investigated isoforms for the CO_2 hydration, but in a very different manner. It should also be noted that there are two types of CA isoforms from the catalytic viewpoint: the low activity ones (CA I -like), including hCA I, hCA VA, and hCA XIV (k_{cat} values in the range of 2.0−3.1 $\times$ 10^5 s^{-1}), and the high activity ones (CA II-like), among which are hCA II, hCA IV, and hCA VII (k_{cat} values in the range of 0.95−1.4 $\times$ 10^6 s^{-1}, Table 1). It must be stressed that the activators had no influence on the K_M values (data not shown), as the Michaelis−Menten constants were identical with or without activator, but a very strong influence has been observed on k_{cat} (Table 1). This parameter is generally greatly enhanced in the presence of activators, proving that it is just the rate-determining step, i.e., the proton transfer between the active site and the reaction medium, which is favored by the activators, as already proved in our first CA activation study on histamine.[16]

Thus, L-Phe was a very efficient activator of isoforms hCA I, II, and XIV, with activation constants in the range of 13−240 nM. Isozyme hCA II showed the highest affinity for this compound (K_A of 13 nM), which is indeed an interesting observation, since this isoform is one of the best catalysts known in nature[5−9] and also a very abundant enzyme in the brain.[27] It may also be observed that, at a concentration of 10 μM of L-Phe (much below the one present in the blood),[15e] the enhancement of k_{cat} is of about 4-fold (Table 1). This represents a significant activation of the catalytic cycle for an enzyme already highly

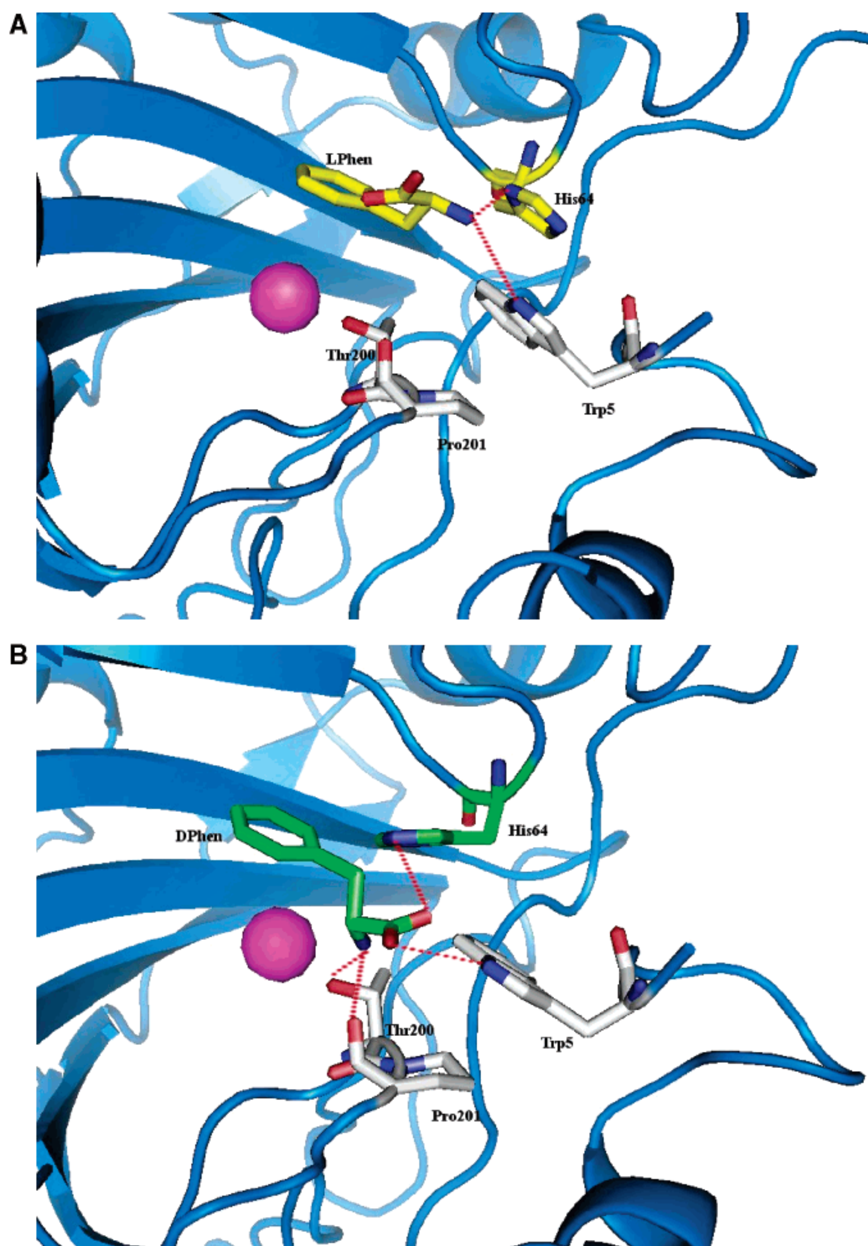

Figure 2. The hCA II–L-Phe (A) and hCA II–D-Phe (B) adducts, with amino acid residues participating in recognition of the activator molecule (reported in yellow for L-Phe, and green for D-Phe) evidenced. The active site Zn(II) ion (magenta sphere) and amino acid residues (His64, Thr200, Trp5, and Pro201) forming hydrogen bonds or in van der Waals contacts with the activators are also shown. Distances are presented in Table 3.

efficient and probably has important physiological consequences. In the case of the cytosolic isozyme hCA I, the enhancement of k_{cat} is even greater, being of about 10-fold (Table 1) with L-Phe. The mitochondrial isoform hCA VA as well as the brain-specific cytosolic isoform hCA VII were moderately activated by L-Phe, with activation constants in the range of 9.81–10.93 μM, whereas the membrane-associated isozyme hCA IV was the least activated one by this amino acid, with a K_A of 52 μM. Thus, there is a factor of 4000 between the most activatable isozyme with L-Phe (hCA II) and the one least prone to be activated (hCA IV). This finding probably indicates that the binding of an activator within a CA isozyme active site is governed by many factors, some of which not well understood at this time.

D-Phe showed quite different activating properties against the investigated six CA isozymes as compared to its stereoisomer (Table 1). Thus, this amino acid was a strong activator only for hCA II (K_A of 35 nM), being a medium potency activator for hCA VA, hCA VII, and hCA XIV (K_As in the range of 4.63–9.74 μM) and a weak activator of isoforms hCA I and hCA IV (K_As in the range of 63–86 μM). The tremendous difference of activity between L- and D-Phe against hCA I should be noted, with the L-isomer being 1211 times more efficient an activator as compared to the D-stereoisomer. A similar situation was also observed in the case of hCA XIV, with L-Phe being 30 times a better activator than D-Phe. In the case of other isozymes (hCA II, IV, VA, and VII), the activation constants of the two stereoisomers were of the same order of magnitude.

These data clearly show at least two important facts: (i) There are net differences of activating efficacy of the two stereoisomers, L- and D-Phe, against various brain CAs. Some isoforms, such as CA I, II, IV, and XIV, are better activated by the L-amino acid, whereas others (such as CA VA and CA VII) show a better activation profile with D-phenylalanine. This fact is quite important as it points out that diverse CA isozymes probably possess different activator binding sites on one hand,

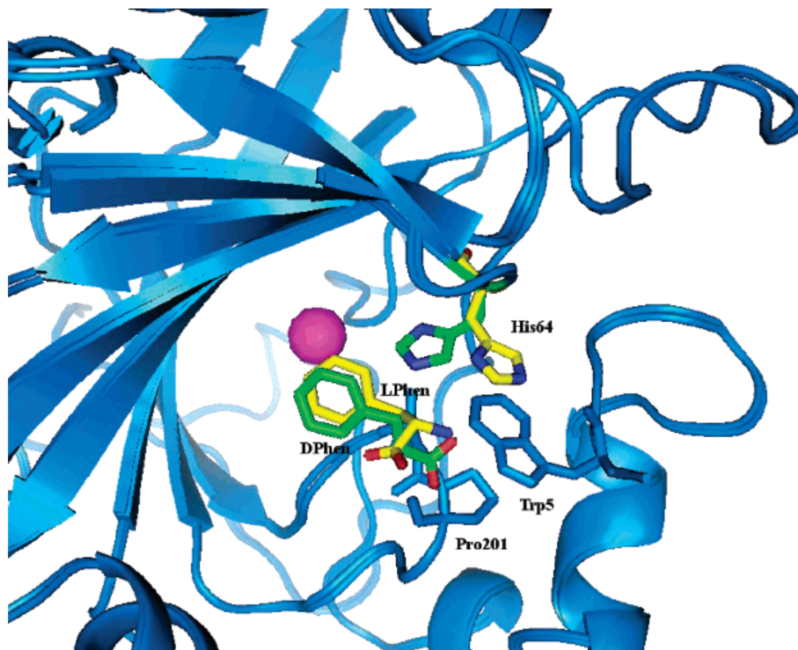

Figure 3. Superposition of the two hCA II adducts with L-Phe (yellow) and D-Phe (green), with the zinc ion (magenta sphere) and amino acid residues present in the activator binding site evidenced. His64 is in the "out" conformation in the L-Phe adduct (yellow), and in the "in" conformation in the D-Phe adduct (green).

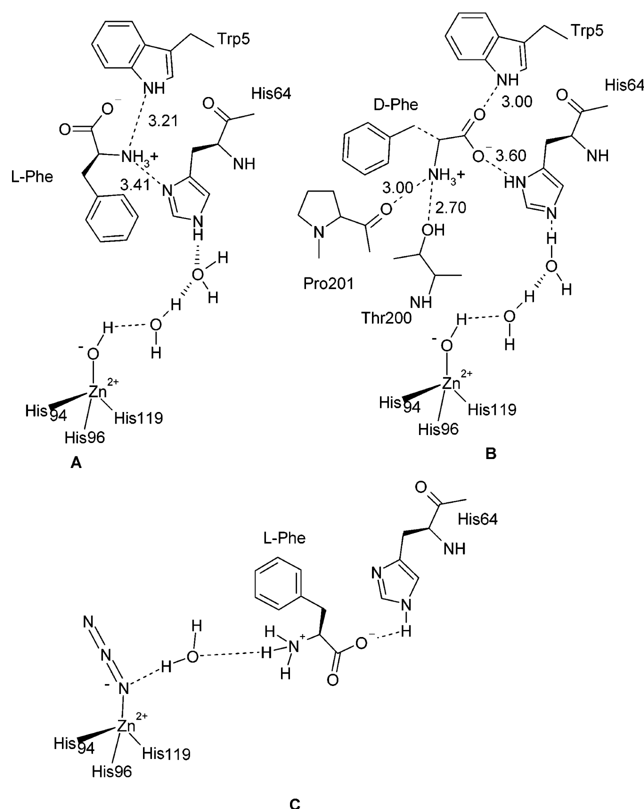

Figure 4. Schematic representation of the active site in the hCA II–L-Phe adduct (A), hCA II–D-Phe adduct (B), and the hCA II–azide–L-Phe ternary complex (C). Figures represent distances (in Å).

and on the other hand, such structural differences may lead to the discovery (or the development) of isozyme-selective CAAs. (ii) By selecting different CA isozymes and diverse CAAs possessing different stereochemistry among the many classes of such derivatives already reported,[1,2,5,6,21] it is possible to engineer proton transfer processes between the active site of the enzyme and the reaction medium, enhancing the catalytic efficiency of a particular isoform. Such phenomena are critical for better understanding the catalytic mechanisms of enzymes for which the rate-determining step is a proton transfer reaction (as the CAs) as well as, in this particular case, for the drug design of more efficient and possibly isozyme-specific CAAs, some of which may show biomedical applications for the management of CA deficiencies, Alzheimer's disease, or aging among others.[21]

X-ray Crystallography. To assess the molecular basis responsible for the activating properties of L-Phe and D-Phe toward hCA II and presumably also other isoforms (as the active site residues differing among the various α-CA isozymes are sometimes rather limited),[5-9] we solved the crystal structure of these complexes, which were prepared and crystallized as previously reported for other CA–activator adducts.[13,16,17] These three-dimensional structures were analyzed by difference Fourier techniques, the crystals being isomorphous to those obtained for the native enzyme (see the Experimental Section for details). The structure refinement allowed us to evidence the spatial arrangement of the activators within the enzyme active site (Figure 1). Analysis of the three-dimensional structure of the complexes revealed that the overall protein structure remained largely unchanged upon binding of the activators, except residue His64 which will be discussed shortly. As a matter of fact, an rms deviation value of 0.27–0.29 Å was calculated over the entire Cα atoms of hCA II–L/D-Phe complexes, with respect to the unbound enzyme. From the omit maps of Figure 1, it may be observed that one molecule of L-Phe or D-Phe are bound at the entrance of the active site cavity, similarly to the binding of histamine[16] or histidine[17] to this isozyme. The electron density of the activators bound within the hCA II active site is clearly (and entirely) defined in the neighborhood of the enzyme's natural proton shuttle, His64 (Figure 1). However, it is interesting to note that the electron density of His64 shows this residue to be only in the "out" conformation[18] for the L-Phe adduct, whereas for the D-Phe adduct (Figure 1B), the predominant conformation is the "in" one, with some "out" conformation also observable (which may explain the additional density around His64 shown in Figure 1B).

It may also be observed that the two stereoisomers bind differently to the enzyme (Figures 2−4). Unlike the two previously investigated activators, histamine[16] or histidine,[17] in the hCA II−L-Phe and hCA II−D-Phe complexes, the pattern of hydrogen bonds and hydrophobic contacts with amino acid residues present within the activator binding site are completely different. Thus, in the hCA II−L-Phe adduct, the activator molecule is anchored by its amino group by means of two hydrogen bonds with the indole nitrogen of Trp5 (of 3.21 Å), and with one of the imidazolic nitrogen atoms of His64, of 3.41 Å (Figures 2A and 4A and Table 3). In addition, van der Waals contact (<4.5 Å) is formed with Thr200 and Pro201 (Figure 2A). In fact, some of these amino acid residues are part of the activator binding pocket, and they were shown to be involved in the binding of the other activators (histamine[16] and histidine[17]) investigated earlier by means of X-ray crystallography. It is interesting to note that the carboxyl moiety of L-Phe is not involved in any hydrogen bond with amino acid residues or water molecules present in the hCA II active site. For the hCA II−D-Phe adduct, the same four amino acid residues interacted with the activator but in a completely different manner as compared to the hCA II−L-Phe adduct. Thus, the amino group of the activator participates in two hydrogen bonds with the carbonyl oxygen of Pro201 and the OH moiety of Thr200 (of 3.00 and 2.70 Å, respectively), whereas the carboxylate moiety of D-Phe makes a hydrogen bond with the indole NH of Trp5 (of 3.00 Å) and a good contact with a nitrogen atom of His64. We consider this last contact at the boundary between a hydrogen bond and a van der Waals interaction, due to its length of 3.60 Å. Although the same amino acid residues are involved in the interactions with both activators, it should be noted that the conformation of L- and D-Phe when bound to the active site are quite different, with the amino group of L-Phe bridging by means of two hydrogen bonds His64 and Trp5, and the carboxylate group of D-Phe interacting with these two amino acid residues, whereas its amino group is available to the formation of two other hydrogen bonds with Thr200 and Pro201 (Figures 2 and 4).

As mentioned earlier, the rate-limiting step in the CA catalytic process, the proton transfer reaction between the zinc-bound water molecule and the environment, is assisted by the amino acid residue His64 situated in the middle of the active site cavity. His64 shows a pH-dependent conformational mobility, as shown in the work of Christianson's group,[18a] changing gradually its orientation related to the metal site through a 64° ring-flipping.[15−18] This proton transfer reaction leads to the formation of the catalytically active nucleophilic species of the enzyme, with hydroxide coordinated to the zinc ion.[3,4] In most X-ray crystallographic structures reported up to now this amino acid residue appeared disordered, due to this flexibility critical for the catalytic cycle.[15−18] However, in all structures of hCA II with activators reported up to now,[13,16,17] except the L-Phe one reported here, the side chain of His64 appears well defined and oriented toward the inside of the cavity (the so-called "in" conformation)[18a] which is probably the conformation involved in the uptake of protons that will be shuttled toward the external part of the active site. As seen from Figures 1A and 3, for the L-Phe−hCA II adduct the conformation of His64 is entirely the "out" one, whereas as seen from Figures 1B and 3, in the D-Phe−hCA II adduct the predominant conformation of His64 is the "in" one (with some electronic density assignable to the "out" conformation also observed, Figure 1B). A relay of two water molecules (w129 and w130) also connects the zinc-bound water/hydroxide anion to the imidazole nitrogen of His64 in both hCA II adducts with activators reported here (Figure 4A,B).

Figure 3 shows the superposition of the two hCA II adducts with activators reported here. It may be observed that although binding strictly in the same region of the active site, the orientation of the two aromatic rings and of the amino and carboxy moieties of the activators is rather different. Thus, the phenyl rings point in both cases toward the inner part of the cavity, but they cannot be superposed. The same may be noticed regarding the carboxylate and amino moieties, which point toward different parts of the active site, as discussed above. These data clearly show that the sterospecific recognition within the active site of hCA II is responsible for the activation efficacy of diverse compounds and may also lead to novel directions in the drug design of more efficient and also isozyme-specific activators. It should again be stressed the very different conformation of the His64 side chain in the two adducts, which can easily be observed in the superposition presented in Figure 3.

The present kinetic and crystallographic data show on one hand that the activator has a dramatic, dose-dependent effect on augmenting k_{cat} of the enzyme, with no effect on K_M (Table 1). On the other hand, the binding of the activators at the entrance of the active site cavity, in close vicinity of the natural proton shuttlle of most CAs (i.e., His64), leads to a drastic rearrangement of the hydrogen bond network connecting the zinc-bound water to amino acid residues relevant for catalysis (Thr199, Thr200, His64, etc). Thus, it is plausible that the activator molecule per se may engage in supplementary proton shuttling processes, since both the amino or carboxylate moieties of these amino acids when bound to the active site of the enzyme may be appropriate for such processes (i.e., their pK_a values may be different of those of the free amino acid in solution). Another possibility is that the binding of the activator enhances the efficacy of His64 as a proton shuttle as compared to its mobility in the wild type (nonactivated) enzyme. In fact, the present structures clearly show the conformation of this critical amino acid residue to vary considerably in the L-Phe and D-Phe complexes. It should be also noted that, in our view, the process of CA activation is a dynamic one, with the activator probably binding to the activator binding site, enhancing the proton shuttling processes (by itself, or by ensuring an optimal conformation to His64), dissociating then from the active site, with the return of His64 in the "in" conformation appropriate for the uptake of protons from the zinc-bound water, and the binding of another molecule of activator. This process is then repeated many times during the catalytic turnover, with the overall result of an enhaced catalytic activity of the enzyme.

In a previous communication,[13] serendipitously, we have discovered the first ternary complex of hCA II with an inhibitor (azide) and an activator (L-Phe). In fact we were interested to resolve the X-ray crystal structure of the hCA II−L-Phe adduct (reported here for the first time), but our precipitant solution for obtaining hCA II crystals also contained azide (in order to avoid fungi colonization of the protein solution), which is a CA inhibitor.[6] Thus, we obtained the structure of the ternary adduct shown schamatically in Figure 4C,[13] in which the electron density part of the phenyl ring was poorly defined. We decided to compare the structure of the presently reported hCA II−L/D-Phe complexes with that of the hCA II−azide−L-Phe ternary complex (Figure 4). As seen from data of Figure 4, the binding of the activator is completely different for the two adducts. In the binary complex (Figure 4A), L-Phe interacts with the amino acid residues mentioned earlier (His64, Trp5, Thr200,

and Pro201), and a relay of two water molecules connects the zinc-bound water/hydroxide to the side chain of His64. For the ternary complex (Figure 4C), the zinc-bound water was replaced by the azide anion, which by means of only one water molecule connects the nitrogen atom coordinated to Zn(II) to the amino moiety of the activator. Furthermore, the carboxylate moiety of L-Phe is hydrogen bonded to the imidazole NH group of His64, and the phenyl ring is oriented toward the cavity opening (unlike the case of the binary adduct in which it is oriented toward the inside of the cavity). Thus, important differences are observed between these two complexes of hCA II with the same activator molecule, in the absence or the presence of anion inhibitors.

Conclusions

Activation of six human brain CAs, i.e., hCA I, II, IV, VA, VII, and XIV, with L-/D-phenylalanine was investigated kinetically and by X-ray crystallography. L-Phe was a potent activator of isozymes I, II, and XIV (K_As of 13−240 nM), a weaker activator of hCA VA and VII (K_As of 9.8−10.9 μM), and a quite inefficient hCA IV activator (K_A of 52 μM). D-Phe showed good hCA II activatory properties (K_A of 35 nM), being a moderate hCA VA, VII, and XIV (K_As of 4.6−9.7 μM) and a weak hCA I and IV activator (K_As of 63−86 μM). X-ray crystallography of the hCA II−L-Phe/D-Phe adducts at 2.0 Å resolution showed the activators to be anchored at the entrance of active site, participating in numerous hydrogen bonds and hydrophobic interactions with amino acid residues His64, Trp5, Thr200, and Pro201. This is the first study showing different binding modes of stereoisomeric activators within the hCA II active site, with consequences for overall proton transfer processes (rate-determining for the catalytic cycle). It also points out differences of activation efficiency between various isozymes with structurally related activators, exploitable for designing alternative proton transfer pathways. CA activators may lead to the drug design of pharmacologically useful derivatives 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 spatial learning and memory therapy must be enhanced.

Experimental Section

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

Expression and Purification of the Recombinant Human Carbonic Anhydrase Isozymes I, II, IV, VA, VII, and XIV. The expression vector pMA-5-8 containing the hCA I or hCA II genes (at the Sma I and BamH I restriction cleavage sites) were a gift from Dr. Sven Lindskog. The corresponding plasmid was transformed into *Escherichia coli* BL21 codon plusDE3(RIL) host cells by following the standard molecular biology protocol.[6] The transformed cells were grown in LB medium, supplemented with 100 μg of ampicillin/mL, 50 μg/mL of chloramphenicol, and 60 μg/mL of ZnSO4 at 37 °C until A_{600} was 0.6. The expression of hCA I/hCA II was induced by addition of 400 μM IPTG and 400 μM $ZnSO_4$. The cells were incubated further at 25 °C overnight and then centrifuged at 5000*g* for 15 min. The pellet was washed in 20 mM Tris-HCl, pH 8.7, and resuspended in the same buffer containing 0.5 mM EDTA. A working concentration of 1 mM PMSF in 2-propanol was added prior to sonication. The cells were sonicated for a total time of 10 min in a Branson bath sonifier utilizing 40% duty cycle in an ice cold bath. The sonicated extract was centrifuged at 15000 rpm for 30 min, and the supernatant (crude extract) was collected for further purification. The enzyme activity of the recombinant hCA I or II was determined by spectrophotometric measurements by the stopped-flow method, with CO_2 as substrate.[28] The protein concentration was determined according to the Bradford method, utilizing BSA as the standard protein. The recombinant form of human carbonic anhydrase II was purified from the crude extract using *p*-aminomethyl benzenesulfonamide-agarose column.[29] The purified enzymes were subjected to the SDS−PAGE analysis to confirm the homogeneity of the enzyme.

For obtaining isoforms hCA IV, VA, VII, and XIV, the GST fusion protein approach has been used, as described earlier by our group.[24−26] The various constructs reported earlier by this group[24−26] were transfected into *E. coli* strain BL21 for production of the GST−hCA fusion protein. Following induction of the protein expression by adding 1 mM IPTG, the bacteria were harvested and sonicated in PBS. The cell homogenate was incubated at room temperature for 15 min and homogenized twice with a Polytron (Brinkmann) for 30 s each at 4 °C. Centrifugation at 30000*g* for 30 min afforded the supernatant containing the soluble proteins. The obtained supernatant was then applied to a prepacked Glutathione Sepharose 4B column. The column was extensively washed with buffer, and then the GST−hCA VB fusion protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris-HCl pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin.[24−26] The advantages of this method are that the hCA isoform is purified easily and the procedure is quite simple. The obtained hCA was then further purified by sulfonamide affinity chromatography,[29] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[28]

CA Activation Assay. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[28] 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 and 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 inhibitor 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 solutions of activator (1 mM) were 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 to allow for the formation of the E−A complex. The activation constant (K_A), defined similarly with the inhibition constant K_I,[24−26] may be obtained by considering the classical Michaelis−Menten equation (eq 2), which has been fitted by nonlinear least squares by using PRISM 3:

$$v = v_{max}/\{1 + K_M/[S]\ (1 + [A]_f/K_A)\} \qquad (2)$$

where $[A]_f$ is the free concentration of activator.

Working at substrate concentrations considerably lower than K_M ($[S] \ll K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by eq 3:[16]

$$v = v_0 \cdot K_A/\{K_A + ([A]_t - 0.5\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)^2 - 4[A]_t \cdot [E]_t)^{1/2}\}\} \qquad (3)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[16]

X-ray Crystallography. CA II−D/L-Phe complexes were cocrystallized at 4° C by the hanging drop vapor diffusion method. Drops containing 5 μL of 10−20 mg/mL CA II in 50 mM Tris-HCl buffer, pH 7.7−7.8, were mixed with 5 μL of precipitant buffer (2.4−2.5 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl, pH 7.7−7.8, and 1 mM sodium 4-(hydroxymercury)benzoate) with added 50 mM d/L-phenylalanine and equilibrated over a reservoir of 1 mL of precipitant buffer. Crystals were transferred into a cryoprotectant solution (20% ethylene glycol) and mounted in nylon loop and exposed to a cold (100° K) nitrogen stream. Diffraction data were collected on a CCD detector KM4 CCD/Sapphire using Cu Kα radiation (1.5418 Å). The unit cell dimensions were determined to be $a = 42.0$ Å, $b = 41.41$ Å, $c = 72.20$ Å and $\alpha = \gamma = 90°$, $\beta = 104.40°$ for the complex CA II/L-Phen and $a = 41.09$ Å, $b = 42.38$ Å, $c = 72.10$ Å and $\alpha = \gamma = 90°$, $\beta = 104.40°$ for the complex CA II/D-Phen, in the space group $P2_1$. Data were processed with MOSFLM[30] and CCP4 suite.[31] The structure was analyzed by difference Fourier technique, using the PDB file 1AVN as starting model for refinement. Electron density maps ($2F_o - F_c$) and ($F_o - F_c$) were calculated with CNS program[32] and displayed using the graphic program O.[33] The final model of the complex CA II/L-Phen had an *R*-factor of 20.5%, *R*-free 24.6% in the resolution range 10−2.0 Å with an rms deviation from standard geometry of 0.005 Å in bond lengths and 1.36° in bond angles. The final model of the complex CA II−D-Phen had an *R*-factor of 20.0%, *R*-free 24.0% in the resolution range 10−2.0 Å with an rms deviation from standard geometry of 0.005 Å in bond lengths and 1.38° in bond angles. Crystallographic parameters and refinement statistics are summarized in Table 2. Both structure coordinates have been deposited in the Brookhaven Protein Databank (PDB ID 2FMZ and 2FMG).

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