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Carbonic anhydrase activators: Kinetic and X-ray crystallographic study for the interaction of D- and L-tryptophan with the mammalian isoforms I–XIV

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ABSTRACT

An activation study of mammalian carbonic anhydrase (CA, EC 4.2.1.1) isoforms I–XIV with D- and L-tryptophan has been performed both by means of kinetic and X-ray crystallographic techniques. These compounds show a time dependent activity against isozyme CA II, with activation constants of 1.13 μM for L-Trp and 0.37 μM for D-Trp, respectively, after 24 h of incubation between enzyme and activator. The high resolution X-ray crystal structure of the hCA II—D-Trp adduct revealed the activator to bind in a totally unprecedented way to the enzyme active site as compared to histamine, L-/D-Phe, L-/D-His or L-adrenaline. D-Trp is anchored at the edge of the CA II active site entrance, strongly interacting with amino acid residues Asp130, Phe131 and Gly132 as well as with a loop of a second symmetry related protein molecule from the asymmetric unit, by means of hydrogen bonds and several weak van der Waals interactions involving Glu234, Gly235, Glu236 and Glu238. Thus, a second activator binding site (B) within the CA II cavity has been detected, where only D-Trp was shown so far to bind, in addition to the activator binding site A, in which histamine, L-/D-Phe, and L-/D-His are bound. These findings explain the strong affinity of D-Trp for CA II and may be useful for designing novel classes of CA activators by using this compound as lead molecule.

1. Introduction

L-Tryptophan (L-Trp) is an essential amino acid in humans, being a constituent of all structural and catalytic proteins.[1–3] Furthermore, two metabolic pathways, involving a host of enzymes, transform this amino acid to crucial biomolecules. The first one leads to autacoids, such as the neurotransmitter 5-hydroxytryptamine (serotonin), formed from Trp under the action of tryptophan 5-hydroxylase, followed by decarboxylation of the intermediate 5-hydroxy-Trp. A second round of enzymatic transformations leads to the hormone melatonin (by acetylation of serotonin followed by *O*-methylation of the phenolic OH moiety).[1–5] The second metabolic pathway involving L-Trp, the kynurenine one, is even more complicated, and about 95% of this amino acid is metabolized according to it.[5] This pathway leads to the formation of kynurenine derivatives and nicotinamide adenine dinucleotides, being initiated by the enzymes tryptophan pyrrolase (tryptophan 2,3-dioxygenase) and indoleamine 2,3-dioxygenase. Under their action a large number of kynurenine derivatives are formed, among which quinolinic and picolinic acid, 3-hydroxykinurenine, 3-hydroxyanthranilic acid and kynurenic acid.[1–5] Many of these compounds are neuroactive, acting as agonists at *N*-methyL-D-aspartate (NMDA) receptors, kynurenic acid is an antagonist at glutamate and nicotinic receptors, whereas some others possess redox activity, being able to generate free radicals under many physiological and pathological conditions.[1–7] It is thus not at all surprising that L-Trp and its metabolites seem to be implicated in many pathological conditions, such as autoimmune diseases, chronic immune activation, neurodegenerative disorders (such as acquired immunodeficiency syndrome (AIDS)-related dementia, Huntington's disease, Alzheimer's disease, and Parkinson's disease), stroke, epilepsy, multiple sclerosis, amyotrophic lateral sclerosis, as well as in mental failures (such as schizophrenia and depression), or more generally in cognitive processes.[1–8]

Similarly to other amino acid and amine derivatives, [9,10] both L- and D-Trp were shown to be activators of the metalloenzyme carbonic anhydrase, CA (EC 4.2.1.1) by this group.[11] Fifteen CA isoforms are presently known in humans.[9] In earlier work, we have investigated kinetically the activation of the catalytically active mammalian isoforms (h, human; m, murine) hCA I, II, III, IV, VA, VB, VI, VII, IX, XII, mCA XIII and hCA XIV with the two enantiomers of this amino acid,[11–14] showing them to be activators of all of them, but with a varying efficacy. It should be mentioned that activation of some members of the mammalian CA family was recently shown to constitute a possible therapeutic approach[9,10] for the enhancement of synaptic efficacy, which may represent a conceptually new treatment for Alzheimer's disease, aging and other

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conditions in which it is necessary the achievement of spatial learning and memory therapy.[15,16] Indeed, CA activators (CAAs) might lead to interesting pharmacological applications, although this field is largely unexplored for the moment.[9] A multitude of physiologically relevant compounds such as biogenic amines (histamine, serotonin, catecholamines), amino acids (L- and D-His; L- and D-Phe, etc.), oligopeptides or small proteins among others, act as efficient CAAs for many of the human CA isozymes.[9–14,17–19] However, the physiologically dominant, cytosolic isoforms CA I and II were those better investigated up to now, by means of kinetic, spectroscopic and X-ray crystallographic techniques.[9,10]

Considering the interesting activity of L- and D-Trp as activators of several CA isozymes[9–14,17–19] as well as the interconnections between these amino acids (or their metabolites) and brain function, we decided to investigate in more detail the mechanism of CA activation by these two compounds. Here, we report a detailed kinetic and X-ray crystallographic study for the interaction of L- and D-Trp with the catalytically active mammalian isozymes CA I–XIV.

2. Results and discussion

2.1. CA activation

CA activation data with L- and D-Trp as well as L-adrenaline (LA), an activator studied earlier by means of kinetic and X-ray crystallographic techniques,[13a] against the mammalian CA isoforms I–XIV are shown in Table 1, for the physiological reaction catalyzed by these enzymes, i.e., CO_2 hydration to bicarbonate and protons.[20]

It may be observed that L-Trp acts as a medium potency CAA against most CA isozymes, such as CA I-IV and VI-XIV, with activation constants in the range of 15–57 μM, but it is a quite potent activator of the two mitochondrial isozymes CA VA and CA VB, with K_As in the range of 0.89–1.13 μM. D-Trp is also a medium potency activator of CA I–IV, VI–XII and XIV, with K_As in the range of 12–43 μM, being a better activator of CA VA, VB and CA XIII, with K_As in the range of 0.81–1.35 μM. It is obvious that the CA activating properties of the two enantiomers are quite distinct, with D-Trp for example being a 19.75 times a better CAA as compared to L-Trp against mCA XIII. L-Adrenaline is on the other hand a rather weak activator of isoforms CA II, VA and VII (K_As in the range of 60–96 μM), a medium potency activator of CA III, IV and XIV (K_As in the range of 36–45 μM), and an effective activator of CA I, CA IX and CA XII (K_As in the range of 0.09–0.87 μM). However, an unexpected finding emerged during these investigations, i.e., the fact that D-and L-Trp (but not LA or other amine/amino acid CAAs, data not shown) present a time dependent activating potency against the ubiquitous isoform hCA II, which has been investigated in more detail here due to its physiological importance, widespread distribution in humans, as well as availability (this is the isoform more easily produced in recombinant form and also the most inexpensive one, as compared for example with the mitochondrial or transmembrane CAs).[11–14] Thus, incubation of enzyme and activator for longer periods than the standard one for determination of the activation constant (which is of 15 min)[11–14] led to the observation that K_As tend to diminish time-dependently. After a 24 h incubation this trend was no longer observed even when enzyme and activator were incubated together for additional 24–48 h. However, to our greatest surprise, the activation constants against hCA II of L- and D-Trp aftre 24 h incubation, were much smaller as compared to the same data obtained after the standard, 15 min incubation time. They were of 1.13 μM for L-Trp, and of 0.37 μM for D-Trp, being thus 23.9 times and 32.4 times lower as compared to the same data determined in standard conditions, respectively (Table 1). Thus, it may be considered that both enantiomers of this amino acid act as potent, low micromolar activators of the ubiquitous and physiologically dominant cytosolic isoform CA II. Whether this may have physiologic consequences it is unknown at this moment, but considering the rather high amount of this amino acid in various body tissues, such as, for example, the brain,[1–8] as well as the abundance of CA II (and other isoforms) in many brain tissues,[9,21] we consider that our finding warrants future and detailed investigations.

Table 1
Activation of mammalian isoforms CA I–XIV with L-Trp, D-Trp and L-adrenaline (LA)

Isoform	K_A (μM)[a]		
	L-Trp	D-Trp	LA
hCA I	44	41	0.09
hCA II	27[b]	12[b]	96[c]
hCA III	20	19	36
hCA IV	37	40	45
hCA VA	1.13	1.24	63
hCA VB	0.89	1.35	nt
hCA VI	15	39	nt
hCA VII	57	39	60
hCA IX	37	43	0.87
hCA XII	26	28	0.41
mCA XIII	16	0.81	nt
hCA XIV	16	18	36

Activator and enzyme were incubated for 15 min prior to assay.[11–14,20]
[a] Mean from three determinations by a stopped-flow, CO_2 hydrase method.[20] Standard errors were in the range of 5–10% of the reported values.
[b] Incubation of enzyme with activator for 24 h lead to a K_A of 1.13 μM for L-Trp and 0.37 μM for D-Trp.
[c] No effect of the incubation period (up to 72 h) between enzyme and activator evidenced.

2.2. X-ray crystallography

Crystals of the hCA II—D-Trp adduct were isomorphous with those of the native protein,[22] allowing for the determination of the crystallographic structure by difference Fourier techniques. The unit cell of the hCA II—D-Trp crystal structure contains four identical protein molecules (denominated A–D, Fig. 1) each of which has the activator bound at the entrance of the active site, where it interacts both with amino acid residues/water molecules from the enzyme active site (molecule A) as well as with amino acid residues/water molecules from one loop of a neighbouring protein (molecule B), as shown in Figure 2. The crystallographic paramaters and refinement statistics are shown 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 r.m.s. deviation value of 0.25 Å was calculated over the entire Cα atoms of hCA II—D-Trp 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 (data not shown). As for other hCA II—activator adducts for which the structure was determined by X-ray crystallography,[11–14] also in the case of the D-Trp complex, the activator molecule binds at the entrance of the cavity (Figs. 2 and 3), interacting with amino acid residues and water molecule which stabilize its binding to the enzyme (Fig. 3). It should be mentioned (Fig 3), that the side chain of His64, an amino acid residue extremely important in the CA catalytic cycle,[9] was observed with both its two characteristic conformations, the "in" and "out" ones in the D-Trp complex, similarly to the hCA II—L-adrenaline adduct reported earlier,[13a] although in other CA—activator adducts investigated earlier (for example the histamine one)[10a] His64 adopted only the out conformation. However, the binding of D-Trp to CA II is very much different as compared to that of all other CAAs investigated up to now by means of X-ray crystallography,[10a,13,14] due to the significant contribute

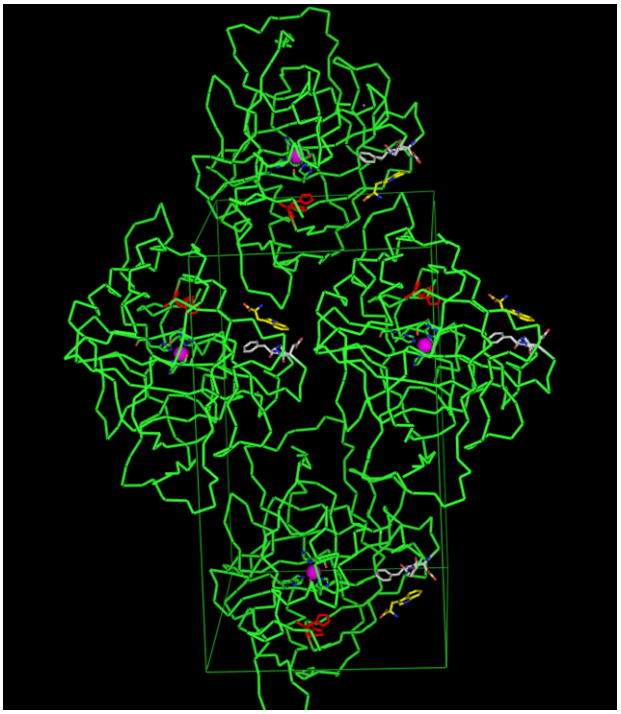

Figure 1. Crystal packing of the hCA II – D-Trp adduct. Four equivalent symmetry related enzyme molecules are present in the cell. The activator (in yellow) is positioned at the edge of the entrance to the enzyme active site and interacts with a loop of a second, neighboring CA molecule. In red are shown the proton shuttle residue His64 and in CPK colors Asp130, Phe131, and Gly132, residues with which the activator interacts. The four adducts (molecules A–D) present in the asymmetric unit are crystallographically identical.

Table 2
Crystallographic parameters and refinement statistics for the hCA II—D-Trp adduct

Parameter	Value
Crystal parameter	
Space group	$P2_1$
Cell parameters	a = 42.0 Å
	b = 41.3 Å
	c = 72.0 Å
	β = 104.4°
Data collection statistics (20.0–1.75 Å)	
No. of total reflections	99060
No. of unique reflections	24480
Completeness (%)[a]	99.3 (99.0)
$F2$/sig($F2$)	15.1 (2.9)
R-sym (%)	13.2 (30.3)
Refinement statistics (20.0–1.75 Å)	
R-factor (%)	19.0
R-free (%)[b]	22.0
R.m.s. of bonds from ideality (Å)	0.014
R.m.s. of angles from ideality (°)	1.58

[a] Values in parenthesis relate to the highest resolution shell (2.0–1.75 Å).
[b] Calculated using 5% of data.

that the loop of a second protein molecule (molecule B, Figs. 2 and 3B; Table 3) gives to the stabilization of the enzyme-activator adduct, in addition to the active site amino acid residues/water molecules belonging to molecule A (i.e., the one in which D-Trp is bound within the active site). Thus, the amino group of the activator molecule participates to a strong hydrogen bond (of 2.75 Å) with the carboxylate of Glu236 belonging to molecule B, whereas the carboxylate moiety of D-Trp takes part in three hydrogen bonds, with three water molecules, two belonging to the protein molecule B (water 44 and 109) and one belonging to molecule A (water 113, Fig. 3B). Thee three hydrogen bond are also quite strong ones, of 2.50–2.90 Å, and significantly contribute to the stabilization of the enzyme-activator adduct. Active site amino acids 130–132 (belonging thus to molecule A), which are known[23] to be important for the binding of CA inhibitors and activators to the enzyme active site, also play a crucial role for the anchoring of D-Trp to hCA II (Figs. 1 and 3 and Table 3). Thus, these amino acids participate to strong (<4 Å) hydrophobic interactions with various moieties of the activator molecule, as shown schematically for some of them in Fig. 3. Indeed, the distance between the amide nitrogen of Gly132 and an aromatic carbon atom of the phenyl ring of D-Trp is of 3.32 Å, whereas exactly the same distance has been measured between the CH_2 carbon atom of the activator and the oxygen carbonyl atom of Asp130 (Fig. 3B and Table 3). On the other hand, several other van der Waals interactions (of 3.64–4.68 Å) have been evidenced between the activator molecule and amino acid residues 234, 235, 236 and 238 belonging to protein molecule B (Table 3). Overall, these multiple polar and hydrophobic interactions strongly stabilize the CA II—D-Trp adduct, explaining thus the submicromolar activation constant measured after prolonged

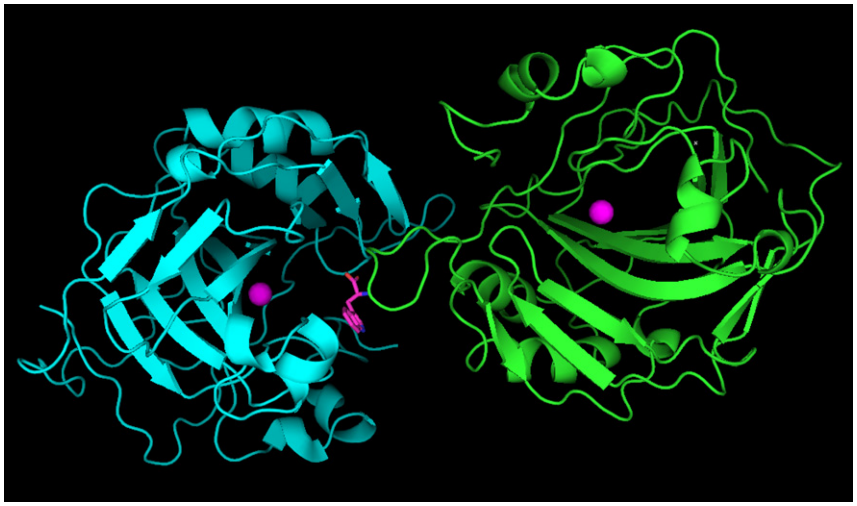

Figure 2. Interaction between two symmetry-related enzyme molecules, showing the activator molecule (magenta) in one of them bound at the entrance of the active site cavity (molecule A, enzyme backbone scaffold as blue ribbon), also interacting with the loop of the symmetric enzyme molecule (B), represented with the backbone as green ribbon. The catalytic Zn(II) ions are shown as violet spheres.

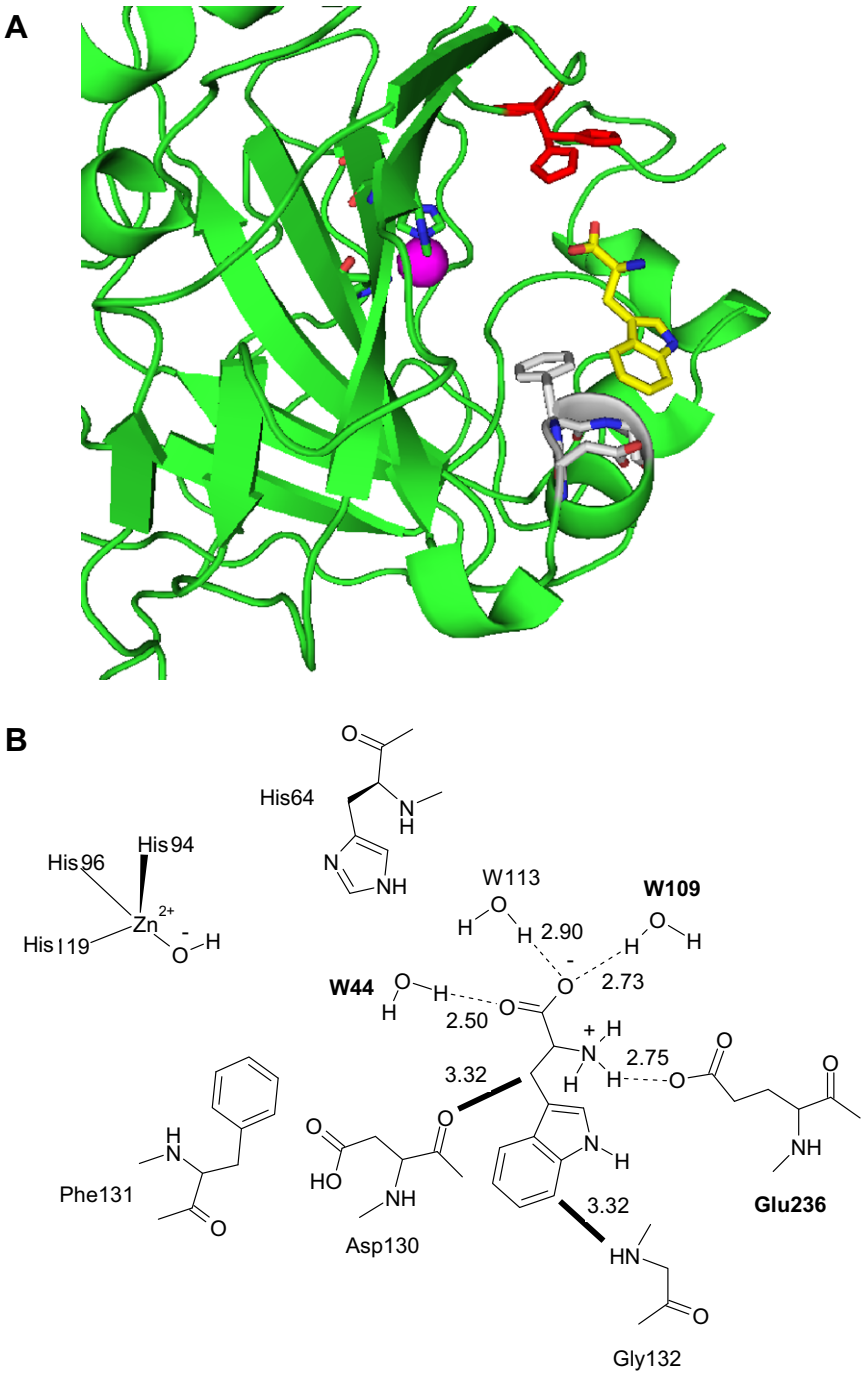

Figure 3. (A) Amino acid residues from the CA active site (enzyme molecule A) and the loop (enzyme molecule B), with which D-Trp (yellow) interacts when in adduct with hCA II (ribbon diagram, in green). The metal ion (violet sphere, its ligands, His94, 96 and 119, in green), proton shuttle residue (His64, in red, in both its conformations, *in* and *out*) as well as Asp130, Phe131, and Gly132 shown as stick models, in CPK colors. (B) Detailed schematic representations of the interactions between D-Trp and the residues/water molecules involved in its binding. In bold characters amino acid residues/water molecules belonging to the symmetry related enzyme molecule. Dashed lines represent hydrogen bonds and bold lines strong van der Waals interactions. Figures represent distances (in Å).

(24 h) incubation times between enzyme and activator. It should be stressed here that although D-Trp makes more interactions with the protein molecule B, its binding to the entrance of the active site of the protein molecules A is on our opinion, mainly responsible for the activating effects of this compound, since the activator is placed in a favorable position to participate in the rate-determining step of catalytic turnover, i.e., shuttling protons between the active site and the environment. The interactions observed between this activator and the loop of the protein molecule B may be a due to the packing of the CA molecules in the crystal and favored by the external binding site (see discussion later in the text) of this compound as compared to the CAAs investigated earlier.[9–14] It must be also mentioned as an argument in favour of this interpretation that the conformation of the loop interacting with D-Trp is the same in the hCA II—D-Trp adduct as well as in the uncomplexed enzyme (data not shown). Furthermore, there are no conformational changes in CAs when complexed to activators or inhibitors, as shown in this and many other crystallographic studies from our and other groups.[9–14,21,22]

Indeed, the most interesting feature of this adduct regards the activator binding site per se. As mentioned above, all CAAs investigated up to now in some detail by means of X-ray crystal-

Table 3
Interactions between D-Trp and amino acid residues involved in its binding belonging to the CA II active site in which the activator is found (protein molecule A) and the symmetry related enzyme loop (protein molecule B) interacting with molecule A

D-Trp atom	hCA II residue (molecule A)	hCA II residue (molecule B)	Distance (Å)
N		O Glu236	2.75
O		O Glu236	3.64
NE1		N Gly235	4.02
N		O Gly 235	4.22
NE1		Oε1 Glu234	4.68
O		N Glu238	3.86
CZ3	N Gly132		3.32
CH2	Oδ2 Asp130		3.32
OXT	W113		2.90
O		W44	2.50
OXT		W109	2.73

lography [11–14] were found bound more or less in the same region of the CA II active site, except for L-adrenaline[13a] which was observed with an unusual, extended conformation, plugging the active site of the enzyme (which also explained its lower activating efficacy against CA II, as compared to other CAAs). A superposition of the hCA II—D-Trp and hCA II—L-adrenaline (LA) adducts shown in Figure 4A, reveals both the unusual binding of LA, as well as the fact that D-Trp binds in a completely different mode, lying towards an even more external part of the active site as compared to LA. The differences of binding between D-Trp and all other CAAs of the amine (histamine) or amino acid type (L- and D-Phe, L- and D-His) for which the crystal structures were reported in complex with hCA II,[10a,12,13] reported in Figure 4B, clearly show these significant differences. Indeed, all these amine/amino acid activators bind at the entrance of the cavity, in the activator binding site[10a] which will be from now on denominated the *CA activator binding site A*. However, D-Trp binds to a second, previously unknown activator binding site, for which we propose the name *CA activator binding site B*. This new binding site is situated more externally as compared to the A site, at the interface between two protein molecules in the asymmetric unit, with the activator thus participating to interactions both with active site residues belonging to a first enzyme molecule, as well as with amino acid residues/water molecules belonging to one loop of a second protein molecule. This may explain the enhanced stability of this enzyme-activator complex (259-fold as compared to the hCA II—LA adduct) and might be also used for the rationale drug design of novel classes of such agents. Indeed, up to now all reported CAAs[17,18] were designed considering histamine as lead molecule, since this was the first activator for which the X-ray crystal structure in complex with hCA II has been reported.[10a]

3. Conclusions

A detailed activation study of mammalian CA isoforms I–XIV with D- and L-tryptophan has been performed both by means of kinetic and X-ray crystallographic techniques. These compounds showed a time dependent activity against isozyme CA II, with activation constants of 1.13 μM for L-Trp and 0.37 μM for D-Trp, respectively, after 24 h of incubation between enzyme and activator. The high resolution X-ray crystal structure of the hCA II—D-Trp adduct revealed the activator to bind in a totally unprecedented way to the enzyme active site as compared to histamine, L-/D-Phe, L-/D-His or L-adrenaline. D-Trp is anchored at the edge of the CA II active site entrance, strongly interacting with amino acid residues Asp130, Phe131 and Gly132 of the enzyme active site, as well as with a loop of a second symmetry related protein molecule from the asymmetric unit by means of hydrogen bonds and van der Waals interactions involving Glu234, Gly235, Glu236 and Glu238. Thus, a second activator binding site (B) within the CA II cavity has been detected, where only D-Trp was shown so far to bind, in addition to the activator binding site A, in which histamine, L-/D-Phe, and L-/D-His are bound. These findings explain the strong affinity of D-Trp for CA II and may be useful for designing novel classes of CA activators by using this compound as lead molecule.

4. Experimental

4.1. Materials

L- and D-Trp as well as LA were from Sigma–Aldrich (Milan, Italy), whereas, CAs were recombinant enzymes prepared as reported earlier by our group.[10a,11–14]

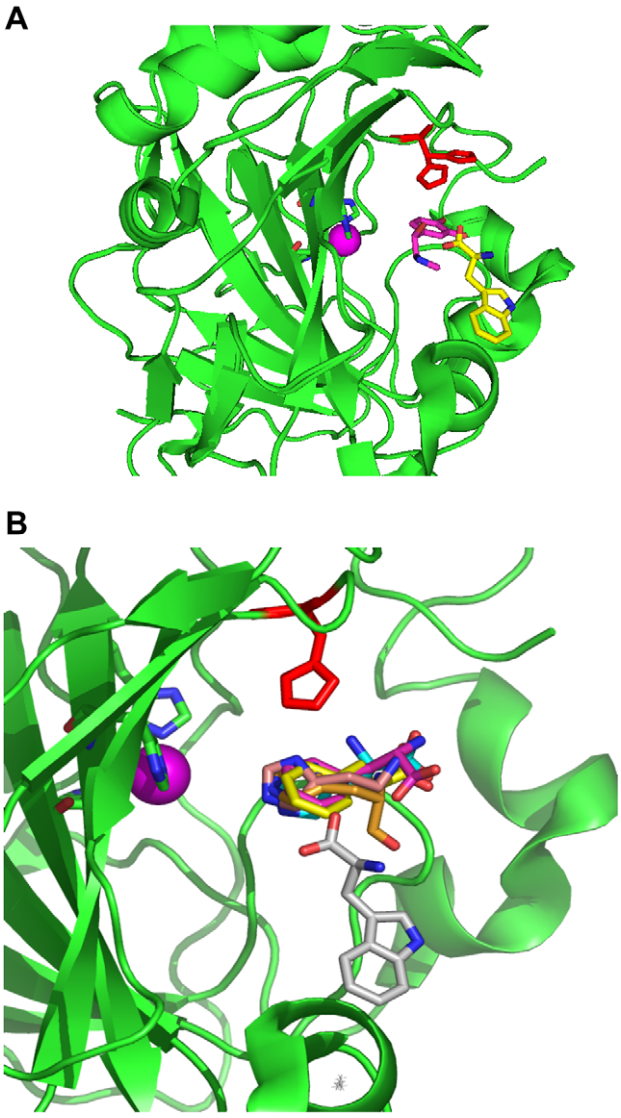

Figure 4. Superposition of the hCA II—D-Trp (yellow in A, gray in B) adduct: (A) with the hCA II—L-adrenaline (magenta) adduct, PDB code 2HKK); and (B) with the hCA II adducts of the following CAAs: L-His (gold, PDB code 2ABE), D-His(sky blue, PDB code 2EZ7), L-Phe (magenta, PDB code 2FMG), D-Phe (yellow, PDB code 2FMZ) and histamine (pink, PDB code 1AVN). The protein backbone is shown as ribbon (in green), with the zinc ion (violet sphere), its three histidine ligands (His94, 96 and 119, in green) and proton shuttle residues (His64) evidenced in red (only one conformation).

4.2. CA enzyme assay

An applied photophysics stopped-flow instrument was used for assaying the CA catalysed CO_2 hydration activity.[20] Phenol red (at a concentration of 0.2 mM) was 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 ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s at 25 °C. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator 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 activators (10 mM) were prepared in distilled–deionized water and dilutions up to 0.001 μM were done thereafter with distilled–deionized water. Activator and enzyme solutions were preincubated together for 15 min (standard assay at room temperature, or for prolonged periods of 24–72 h, at 4 °C) 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,[10a,11–14] can be obtained by considering the classical Michaelis–Menten 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 ($[S] << K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by Eq. (2)[11–14]:

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

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[11–14]

4.3. X-ray crystallography

The hCA II—D-Trp complex was crystallized as previously described.[13,14] 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: $a = 42.0$ Å, $b = 42.3$ Å, $c = 72.0$ Å and $\alpha = \gamma = 90°$, $\beta = 104.4°$ in the space group $P2_1$ Data were processed with CrysAlis RED.[24] The structure was analyzed by the difference Fourier technique, using the PDB file 1CA2[22] as starting model. The refinement was carried out with the program REFMAC5;[25] model building and map inspections were performed by using the COOT program.[26] The final model of the hCA II—D-Trp complex had an *R*-factor of 19.0% and *R*-free 22.0% in the resolution range 20.0–1.75 Å, with an r.m.s. deviation from standard geometry of 0.014 Å in bond lengths and 1.58° in angles. The correctness of stereochemistry was finally checked using PROCHECK.[27] Coordinates and structure factors have been deposited with the Protein Data Bank (Accession Code 3BL2). Crystallographic parameters and refinement statistics are summarized in Table 2.

Acknowledgments

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