Bioorganic & Medicinal Chemistry 16 (2008) 8373–8378

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

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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ARTICLE INFO

Article history:
Received 15 July 2008
Revised 8 August 2008
Accepted 20 August 2008
Available online 26 August 2008

Keywords:
Carbonic anhydrase
Isozymes
Activation
L-Tryptophan
D-Tryptophan
X-ray crystallography

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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doi:10.1016/j.bmc.2008.08.043

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