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Carbonic anhydrase activators: Activation of isozyme XIII with amino acids and amines

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Abstract—The first activation study of isoform XIII of carbonic anhydrase (CA, EC 4.2.1.1) is reported. A series of amino acids and amines incorporating protonatable moieties of the primary/heterocyclic amine type were included in the study. As for CA I and II, CA XIII activators enhance k_{cat} and show no effect on K_M, for the physiologic reaction catalyzed by this isoform. Excellent CA XIII activating properties were shown by D-amino acids (His, Phe, DOPA, and Trp), serotonin, and 4-(2-aminoethyl)-morpholine, whereas the corresponding L-amino acids, dopamine, histamine, and 1-(2-aminoethyl)-piperazine, were weaker activators.

A multitude of physiologically relevant compounds such as amino acids, oligopeptides or small proteins, as well as biogenic amines (histamine, serotonin, and catecholamines among others), efficiently activate the catalytic activity of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), of which 16 isoforms were presently detected in mammals.[1–3] Activation of some CA isozymes, such as the cytosolic, ubiquitous isoforms CA I and II,[2,3] was shown to constitute a possible therapy for the enhancement of synaptic efficacy, which may represent a conceptually new approach in the treatment of Alzheimer's disease, aging, and some other disease conditions characterized with an eventual loss of memory functions.[4,5] Unlike CA inhibitors, widely used clinically for the treatment or prevention of a multitude of diseases,[6,7] CA activators (CAAs) have been much less investigated.[1–3] Only recently, by means of electronic spectroscopy, X-ray crystallography, and kinetic measurements, it has been proved that CAAs bind within the enzyme active cavity (in the case of the physiologically most important isoform, human CA II, hCA II) at a site distinct of the inhibitor or substrate binding-sites,[8,9] participating thereafter in the rate-determining step of the catalytic cycle, a proton transfer reaction between the active site and the environment.[1–3,8,9]

Two X-ray crystallographic structures of adducts of the main human isoform, hCA II, with activators are known at this moment: one with histamine,[8] and another one with L-histidine.[9] The first crystal structure of hCA I in complex with an activator, that is, L-His, has been reported very recently.[10] All of them showed the activator molecule to be bound in a distinct region from the inhibitor binding site, at the entrance of the active site cavity in the case of hCA II, being anchored by hydrogen bonds to amino acid residues (His64, Asn67, and Gln92) and water molecules, and also leading to a complete reorganization of the hydrogen bond network within the active site cavity (Fig. 1A).[8,9] For hCA I, the activator binding site is situated deeper within the cavity, in the neighborhood of residues His200, His67, and His64 (Fig. 1B).[10] Positioned in this way, the activator facilitates the rate-limiting step of CA catalysis, that is, the proton transfer reaction between the zinc-bound water molecule and the environment, which in many CA isozymes (in the absence of activators) is assisted by the amino acid residue His64 situated in the middle of the cavity, and also possessing a pH-dependent

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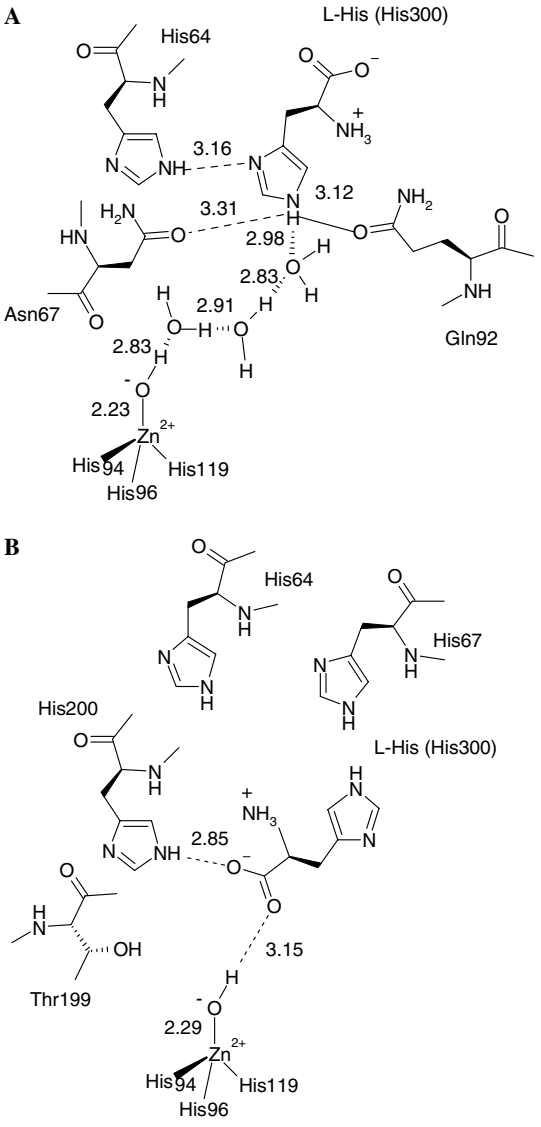
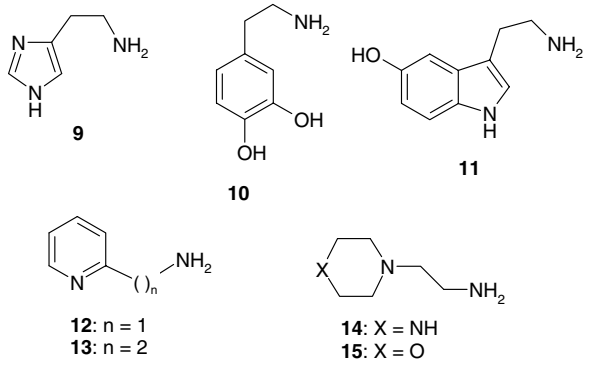


Figure 1. Schematic representation for the binding of L-His (numbered as His300) to the hCA II[9] (A) and hCA I,[10] (B) active sites. The Zn(II) ligands and hydrogen bonds connecting the Zn(II) ion and the activator molecule through a network of water molecules are shown, as well as the hydrogen bonds (dotted lines) between the activator and amino acid residues involved in their binding (figures represent distances in Å).

conformational mobility, changing gradually its orientation related to the metal site through a 64° ring-flipping.[11–13] 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.[1–3]

Few other isozymes except the above-mentioned CA I and II have been investigated earlier for their activation behavior.[1–3] CA XIII, one of the last isozymes to be cloned and characterized,[14] has a cytosolic localization as CA I and II, showing lower catalytic activity (similar to that of the mitochondrial isozyme V), with k_{cat}/K_M of 4.3×10^7 M^{-1} s^{-1} and k_{cat} of 8.3×10^4 s^{-1}.[14] Its distribution is unique when compared to the other cytosolic CA isozymes (mainly CA I and II).[14] The most important differences between CA XIII and II (the most widely spread and physiologically/pharmacologically relevant cytosolic isoform)[1] were observed in the human testis and uterus, organs in which pH and ion balance must be tightly regulated to ensure normal fertilization.[14] CA XIII was found to be expressed in all stages of developing human sperm cells, whereas CA II was confined to the mature sperm cells as shown earlier by some of us.[4,15] In the female genital tract, the endometrial and oviductal epithelium produce an alkaline environment for maintaining the sperm motility,[4] CA XIII being the key factor contributing to the appropriate bicarbonate concentration in the cervical and endometrial mucus needed for normal fertilization processes.[14,15] Being such a widely expressed isozyme, CA XIII could compensate other CAs, and thus, needs to be considered when CA-deficient animal models are tested in phenotypic analyses, or when inhibitors are clinically used in the treatment and prevention of diverse disorders.[1] All these findings suggest that CA XIII plays a major physiological role, and perturbation of its function could potentially lead to significant abnormalities.[14] In previous work, we investigated the inhibition of CA XIII with sulfonamides, sulfamates, and inorganic anions.[16,17] Here, we present the first CA XIII activation study with a series of amino acids and amines, some of which are present in relevant concentrations in many tissues.[18]

L-/D-Amino acids **1**–**8** and amines **9**–**15** investigated as CA XIII activators were commercially available from Sigma–Aldrich (Milan, Italy) and were used without further purification.



Kinetic experiments[18] for the physiological reaction (carbon dioxide hydration to bicarbonate and a proton) (Table 1) showed that as for hCA I and II,[1–3] activators of the amino acid or amine type enhance k_{cat} of the enzyme, with no effect on K_M. Indeed, as observed from data of Table 1, L- or D-His (compounds **1** and **2**) at a concentration of 10 μM produced a notable enhancement of k_{cat} for all three investigated cytosolic isoforms, that is, hCA I, hCA II, and mCA XIII. Thus, for hCA I, this parameter for the pure enzyme is of 2.0×10^5 s^{-1},[10] whereas in the presence of activators **1** and **2** at a concentration of 10 μM, it becomes of $9.1–13.4 \times 10^5$ s^{-1}. For hCA II, the enhancement of k_{cat} from the value of

Table 1. Kinetic parameters for the activation of CA isozymes I, II, and XIII with L- and D-histidine, at 25 °C, for the CO_2 hydration reaction

Isozyme	k_{cat}[c] (s^{-1})	$(k_{cat})_{L\text{-His}}$[d] (s^{-1})	$(k_{cat})_{D\text{-His}}$[d] (s^{-1})	K_A[e] (μM)	
				L-His	D-His
hCA I[a]	2.0×10^5	13.4×10^5	9.1×10^5	0.03	0.09
hCA II[a]	1.4×10^6	4.3×10^6	2.7×10^6	10.9	43.5
mCA XIII[b]	0.83×10^5	3.3×10^5	3.7×10^5	0.13	0.09

[a] Human recombinant isozymes.
[b] Murine recombinant isozyme.[14]
[c] 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).
[d] Observed catalytic rate in the presence of 10 μM activator.
[e] The activation constant (K_A) for each isozyme was obtained as described earlier, and represents the mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[18] Standard errors were in the range of 5–10% of the reported values.

the pure enzyme (1.4×10^6 s^{-1}) is in the range of 2.7–4.3×10^6 s^{-1}, whereas for mCA XIII, this enhancement is of 3.3–3.7×10^5 s^{-1}, from the initial value of 0.83×10^5 s^{-1} for the pure enzyme (Table 1).

mCA XIII activation constants[1–3,18] for a series of structurally related amino acids and amines of types **1**–**15** are shown in Table 2. The activation constants for the ubiquitous isozymes hCA I and hCA II are also provided for comparison. These compounds were shown earlier to act as activators of isozymes hCA I and II.[1–3] All of them possess protonatable moieties of the primary amino or heterocyclic amine type (or both of them), being thus able in principle to participate in proton transfer processes leading to the generation of the nucleophilic species of the enzyme, with hydroxide coordinated to the active site zinc ion. It should be noted that the amines included in our study possess aminoethyl or aminomethyl moieties, in addition to aromatic/heterocyclic groups, the last of which usually incorporate nitrogen atoms that can be protonated at pH values in the physiological range. Similarly, with the inhibition constant K_I (for the enzyme inhibitors), the activation constant K_A measures the affinity of the activator for the enzyme. The lower this parameter is, most strong is the activator against the corresponding isoform.[1–3]

Data of Table 2 show that amines and amino acids investigated here, of type **1**–**15**, show good activating properties against mCA XIII, with activation constants in the range of 0.013–54 μM. The following SAR can be observed for this small series of CA XIII activators: (i) a number of derivatives, such as L-DOPA, dopamine as well as the heterocyclic amines **13** and **14**, behave as weak mCA XIII activators, with K_A-s in the range of 27–54 μM; (ii) more potent mCA XIII activators are the following derivatives: L-Phe, L-Trp, histamine, and 2-pyridyl-methylamine **12**, which possess K_A-s in the range of 1.02–16 μM. Basically, both these compounds as well as the weaker activators mentioned earlier possess rather similar structural features. It is thus clear that quite precise steric and electronic requirements govern the interaction between CA XIII and its activators, a situation similar to that observed for the better investigated isoforms CA I and II;[1–3] (iii) very good mCA XIII activating properties have been detected for compounds **1, 2, 4, 6, 8, 11**, and **15**, which showed K_A-s in the range of 13–810 nM. Among these activators, there are physiologically relevant compounds, such as L-His or serotonin, some of which are present in different tissues in high enough concentrations to produce relevant CA XIII activating effects.[19,20] Also other CAAs investigated here for their interaction with CA XIII, such as L-Phe, show blood and brain concentrations in the range of

Table 2. Activation constants of hCA I, hCA II, and mCA XIII with amino acids and amines **1**–**15**

No.	Compound	K_A[a] (μM)		
		hCA I	hCA II	mCA XIII
1	L-His	0.03	10.9	0.13
2	D-His	0.09	43	0.090
3	L-Phe	0.07	0.013	1.02
4	D-Phe	86	0.035	0.051
5	L-DOPA	3.1	11.4	43
6	D-DOPA	4.9	7.8	0.73
7	L-Trp	44	27	16
8	D-Trp	41	12	0.81
9	Histamine	2.1	125	4.6
10	Dopamine	13.5	9.2	27
11	Serotonin	45	50	0.51
12	2-Pyridyl-methylamine	26	34	3.8
13	2-(2-Aminoethyl)pyridine	13	15	46
14	1-(2-Aminoethyl)-piperazine	7.4	2.3	54
15	4-(2-Aminoethyl)-morpholine	0.14	0.19	0.013

[a] Mean from three determinations by a stopped-flow, CO_2 hydrase method.[18] Standard errors were in the range of 5–10% of the reported values.

30–73 μM,[20] suggesting that they may strongly activate this isoform, and thus lead to physiological responses. It must be noted that both amino acids (such as L-/D-His, D-Phe, D-DOPA or D-Trp) as well as amines (such as serotonin or 4-(2-aminoethyl)-morpholine **15**) may act as very potent CA XIII activators; (iv) the steric requirements for CA XIII activators are very strict, since important differences of activating efficiency are observed for various amino acid stereoisomers, such as the pairs L-/D-His, L-/D-Phe, L-/D-DOPA, and L-/D-Trp. Unexpectedly, the non-natural stereoisomer was always a much better CA XIII activator, as compared to the corresponding L-enantiomer. Thus, for Phe and Trp, the D-enantiomer is around 20 times more effective as a CA XIII activator as compared to the corresponding L-enantiomer. For DOPA, the effect is much higher, with D-DOPA being 58.9 times more effective as an activator as compared to L-DOPA, whereas for the pair L-/D-His, the difference is rather small (a factor of 1.44 times differentiating L- and D-His for their CA XIII activating properties). Another very interesting observation is that the structurally quite similar derivatives **14** and **15** (differing by an NH group in **14** replaced by an oxygen atom in **15**) show very different CA XIII activating properties, with the morpholine derivative **15** being 4153 times more efficient as a CAA as compared to the piperazine **14**. It is difficult to rationalize these results, as the X-ray crystal structure of this enzyme is unknown for the moment; (v) the activation profile of compounds **1**–**15** toward mCA XIII is completely different of their activation profiles versus the other two cytosolic (ubiquitous) isozymes CA I and II investigated earlier[1–3] (Table 2). Indeed, most L-amino acids investigated here were better hCA I and II activators as compared to the corresponding D-amino acid (see, for example, His, Phe for both isoforms, DOPA for CA I, etc.), whereas for CA XIII, just the D-enantiomers were better activators as compared to the corresponding L-enantiomer. There were also important differences of activation efficacy for the three cytosolic isoforms in this small series of investigated compounds. Thus, the best CA XIII activator was **15**, which behaved as a quite efficient CA I and II activator too (but an order of magnitude less efficient against CA I and II as compared to its activity for CA XIII). The best CA I activator was L-His, which is a medium potency CA II activator, and an efficient CA XIII activator. The best CA II activator on the other hand was L-Phe, which is also a rather good CA I and XIII activator. The most CA XIII-specific activator was serotonin, which is a submicromolar CA XIII activator (K_A of 0.51 μM), being at the same time an 88 times less efficient CA I and a 98 times less efficient CA II activator. These data prompt us to make the assumption that it will be possible to design CA XIII-selective activators.

In conclusion, we report here the first CA XIII activator study. A series of amino acids and amines incorporating protonatable moieties of the primary/heterocyclic amine type were included in the study. As for CA I and II, CA XIII activators enhance k_{cat} and show no effect on K_M for the physiologic reaction catalyzed by this isoform. Excellent CA XIII activating properties were shown by D-amino acids (His, Phe, DOPA, and Trp), serotonin, and 4-(2-aminoethyl)-morpholine, whereas the corresponding L-amino acids, dopamine, histamine, and 1-(2-aminoethyl)-piperazine were weaker activators.

Acknowledgments

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An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM HEPES (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s. The CO_2 concentra-

tions ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition 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 activator (10 mM) were prepared in distilled-deionized water and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Activator and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–A complex. The activation constant (K_A), defined similarly with the inhibition constant K_I,[6] may be obtained by considering the classical Michaelis–Menten equation (Eq. 1), which has been fitted by non-linear least squares by using PRISM 3:

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

where $[A]_f$ is the free concentration of activator. Working at substrate concentrations considerably lower than K_M ($[S] \ll K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by Eq. 2:[9]

$$v = v_0 \cdot K_A/\{K_A + ([A]_t - 0.5\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)_2 - 4[A]_t \cdot [E]_t)_{1/2}\}\} \quad (2)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[9]

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