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Carbonic anhydrase activators: Activation of the human cytosolic isozyme III and membrane-associated isoform IV with amino acids and amines

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ABSTRACT

An activation study of the human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms hCA III (cytosolic) and IV (membrane-associated) with a series of natural and non-natural amino acids and aromatic/heterocyclic amines is reported. hCA III was efficiently activated by D-His, serotonin, pyridyl-alkylamines, and aminoethyl-piperazine/morpholine (K_As of 91 nM–1.12 μM), whereas the best hCA IV activators were 4-aminophenylalanine, serotonin, and 4-(2-aminoethyl)-morpholine (K_As of 79 nM–3.14 μM). Precise steric and electronic requirements are needed to be present in the molecules of effective CA III/IV activators, in order to assure an adequate fit within the enzyme active site for the formation of the enzyme-activator complex, and for efficient proton transfer processes between the active site and the reaction medium. The activation profiles of CA III and IV are distinct from those of all other mammalian CA isoforms investigated so far for their interaction with amino acids and amines.

Among the 16 carbonic anhydrase (CA, EC 4.2.1.1) isoforms described so far in mammals,[1–4] the very slow cytosolic isozyme CA III is the least understood and investigated at this moment. Indeed, CA III is the worst catalyst for CO_2 hydration as compared to other cytosolic, mitochondrial or membrane-associated human CAs,[5,6] its CO_2 hydration catalytic activity being around 0.3% that of the ubiquitous, very rapid catalyst which is CA II.[6] In fact, unlike the other two cytosolic wide-spread isozymes I and II, CA III is mainly present in slow skeletal muscles (10% of the cytosolic protein content), adipocytes (24% of the soluble protein), and liver (8% of the soluble protein), where its primary functions remain largely unknown.[6–8] Recent studies with CA III knockout mice showed CA III to be involved in mitochondrial ATP synthesis,[8] whereas its levels were found to be significantly decreased in mutant mice lacking the gene SULT1E1, indicating a role of CA III in cystic fibrosis liver disease.[9] CA III is also considered as one of the proteins involved in oxidative stress response both in liver[10] and in skeletal muscle,[11] probably acting as a scavenger of reactive oxygen species (ROS) and thus protecting cells from oxidative damage.[12] CA III seems to play an important role (together with E-cadherin) in disruption of the intercellular barrier associated with the down-regulation of E-cadherin in the laryngopharyngeal reflux disease.[13]

These physiologic/pathophysiologic functions of CA III are poorly understood from the mechanistic viewpoint, except for the antioxidant role of this enzyme, which has been shown to be modulated by the *S*-glutathionylation of two cysteine residues (Cys181 and Cys186) present on the surface of the protein (we stress this here again, not within its active site).[14] Indeed, oxidants such as hydrogen peroxide, peroxy radicals or hypochlorous acid were shown to oxidize these two cysteine residues to sulfinic/sulfenic acids (in the absence of glutathione), but when this tripeptide was present in the medium, the S-glutathionylation of the two Cys residues occurred, without damage to the protein.[14] It is thus probable that one of the main in vivo functions of CA III, is that of protecting proteins from irreversible oxidation processes with subsequent cellular damage.[4,14]

A very recent report[15] proves the involvement of CA III in tumor acidification processes (even considering the relatively low catalytic activity of this isoform), similarly with what was demonstrated earlier for the transmembrane, tumor-associated isoform CA IX.[1–3] Indeed, in some hepatic carcinoma cells (SK-Hep1) it has been proven that CA III promoted the invasive ability of these tumors, a process which was hypothesized to be mediated through the focal adhesion kinase (FAK) signaling pathway, which was activated through the intracellular and/or extracellular acidification mediated by the CO_2 hydrase activity of CA III.[15]

Unlike the cytosolic CA I, II, and III, CA IV was the first extracellular isoform to be discovered.[16] Indeed, this isozyme is associated to plasma membranes in lungs, kidneys, ciliary processes within the eye, and several other organs, playing an important function in pH regulation, bicarbonate reabsorption in the kidneys, production of ocular fluid, elimination of CO_2 in the lungs, cerebral blood flow, etc.[1–4] CA IV is tethered to the plasma membrane by means of

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glycosylphosphatidylinositol tails,[17] it has the active site outside the cell, and possesses a high catalytic activity (similar to that of CA II)[1,17] both for the CO_2 hydration and for the bicarbonate dehydration reactions.[1,16,17] Most inhibition and activation studies of CA IV reported up to now have been done with the bovine isoform (bCA IV) usually purified from lungs or kidneys.[18,19] However, we have recently shown[20] that the recombinant human (hCA IV) and bCA IV isoforms are very different in their behavior toward the main class of inhibitors, the sulfonamides, and their bioisosteres, with the human isoform showing frequently less affinity for many such compounds as compared to the corresponding bovine enzyme. Thus, it appeared of interest to investigate the activation of hCA IV as well as hCA III with amino acids and amines. This is in fact the first activation study of both hCA III and hCA IV reported, except for some preliminary data of Chegwidden's group on chicken CA III activation with inorganic phosphates.[21] We have included in our study L-/D-Amino acids and amines **1–18**, which have been investigated earlier for the activation of all other mammalian catalytically active isoforms, that is, hCA I, II, VA, VB, VI, VII, IX, XII, XIII, and XIV by our group.[22–24]

The affinity constant (K_{aff}) of an activator for the corresponding CA isoform has been denominated the activation constant (K_A)[18] in order to obtain a measure of the strength for the interaction between enzyme and activator, similarly with the inhibition constant (K_I) which defines the potency of an inhibitor in the enzyme-inhibitor (E-I) complex.[1–3] By representing the catalytic enhancement as a function of activator concentration, a typical sigmoid curve is obtained, from which the affinity constant (K_A) may be estimated by non-linear least-squares fitting.[24] Detailed kinetic measurements (data not shown) showed that the activators **1–18** investigated here for their interactions with isoforms hCA III and IV do not change the value of the Michaelis–Menten constant (K_M), which is the same in the absence or the presence of activators, similarly with what was observed earlier for the activation of other mammalian CAs.[18,19,22,23] On the contrary, the observed catalytic rate of the enzyme (k_{cat}) is enhanced in the presence of all activators investigated up to now and against all CA isozymes (Table 1), supporting our previous observations[3,18–23] that CA activators (CAAs) do not influence the binding of CO_2 to the CA active site, but intervene in the rate-determining step of the catalysis, that is, the transfer of protons from the active site to the environment.

Data of Table 1 show that from the point of view of catalytic activity for the CO_2 hydration reaction (at 25 °C, in steady-state conditions), there are three types of CA isoforms: (i) the low activity ones, that is, CA I–like, including hCA I (but also hCA VA and hCA XIV among others,[1] data not shown), possessing k_{cat} values in the range of 2.0–3.1 × 105 s^{-1};[1] (ii) the high activity ones (CA II-like), among which hCA II, hCA IX, and hCA IV (k_{cat} values in the range of 1.2–1.4 × 10^6 s^{-1}), and (iii) the very low activity one, including only hCA III, which has the lowest catalytic activity among the mammalian CAs, with a k_{cat} of only 1.3 × 10^4 s^{-1}. Data of Table 1 show that all these investigated CA isoforms are activated by L- and D-His but in a very different manner. Thus, the high activity isoforms hCA II and hCA IV were poorly activated by L- and D-His. Both these activators showed activation constants, in the range of 7.3–10.9 μM for L-His, and 12.3–43.5 μM for D-His, respectively. This is probably due to the fact that the proton transfer processes in hCA II/IV, assisted by the proton shuttle residue His64,[1,18] are already very efficient for assuring a high turnover of the catalytic cycle, and thus quite high concentrations of activator are needed for supplementing these processes. However, it should be noted (Table 1) that even as low concentrations of L-His as 10 μM produce a 3-fold increase in k_{cat}, which is obviously remarkable for such an efficient enzyme. It may also be observed that L-His is a much more efficient hCA II activator (around 4 times) as compared to its stereoisomer D-His. The same is true for hCA IV (an isozyme also possessing His64 as proton shuttle residue),[25] but for this isoform the differences in the activation constants of the two amino acids are much smaller, with L-His being only 1.68-fold, a better activator as compared to D-His. However, the very slow isoform hCA III, is very much activated by D-His (13-fold, see Table 1), whereas the enantiomeric amino acid, L-His, is a much weaker activator of this isoform (only 1.38-fold, at 10 μM activator concentration in the assay system). X-Ray crystal data of the complexes of hCA I and II with L- and D-His showed the two activators to bind in different regions of the enzyme cavity, and also with different orientations, dependent on both the stereoisomer amino acid activator and the particular CA isoform.[18,19,20–22] Such X-ray data (which are not available for the moment for isoforms hCA III and IV) of complexes of activators with various CA isoforms may explain the kinetic data shown in Table 1, as well as the very different activation profile of various isoforms with these and structurally related amino acid and amine activators (see Discussion later in the text).

Data of Table 2 show that all amino acids and amine activators **1–18** investigated here act as CAAa against both hCA III and IV, but their activation profile is characteristic for each of these isoforms, and different of that of the isozymes CA I and II investigated earlier,[22] which have been reported in Table 2 for comparison purposes. Thus, against hCA III, a group of compounds, among which D-His **2** as well as the amines **13–17**, showed effective activation phenomena, with K_As in the range of 91 nM–1.12 μM. It may be observed that only one amino acid acts as a good CA III activator (compound **2**), whereas all other derivatives in this subgroup are heterocyclic amines. The best CA III activator, wit a nanomolar affinity for this isoform, was 4-(2-aminoethyl)-morpholine **17**, followed by the structurally related piperazine **16**. However, other amines included in our study, such as histamine **11**, dopamine **12**, and L-adrenaline **18**, were less effective as CA III activators, with K_As in the range of 33.2–36.9 μM. Among the amino acid investigated here, in addition to L-His, which was an effective CA III activator, other compounds such as D-Phe, L-DOPA, L- and D-Trp showed K_As in the range of 13.5–20.5 μM, whereas the remaining amino acids were even less efficient CAAs for this isozyme (K_As in the range of 28.7–43.2 μM). Several facts should be noted. Thus, important differences can be seen for the activation of CA III (but also that of the cytosolic isozymes I and II or the membrane-associated one CA IV) by compounds which differ only by their enantiomeric form: for example, as mentioned above, L-His is a much less effective CA III activator (32.0 times) as compared to the corresponding D-amino acid. Similar differences are also observed for the coupled L- and D-Phe, L- and D-DOPA, or L-, and D-Trp, respectively. On the other hand, just small modifications in the scaffold of some of these activators (e.g., introduction of a substituent in

Table 1
Activation of hCA isozymes I, II, III, and IV, with L- and D-histidine, at 25°C, for the CO_2 hydration reaction

Isozyme	k_{cat}[a] (s^{-1})	$(k_{cat})_{L\text{-His}}$[b] (s^{-1})	$(k_{cat})_{D\text{-His}}$[b] (s^{-1})	K_A[c] (μM)	
				L-His	D-His
hCA I[d]	2.0 × 10^5	13.4 × 10^5	9.1 × 10^5	0.03	0.09
hCA II[d]	1.4 × 10^6	4.3 × 10^6	2.7 × 10^6	10.9	43.5
hCA III[d]	1.3 × 10^4	1.8 × 10^4	16.9 × 10^4	35.9	1.12
hCA IV[d]	1.2 × 10^6	4.3 × 10^6	3.8 × 10^6	7.3	12.3

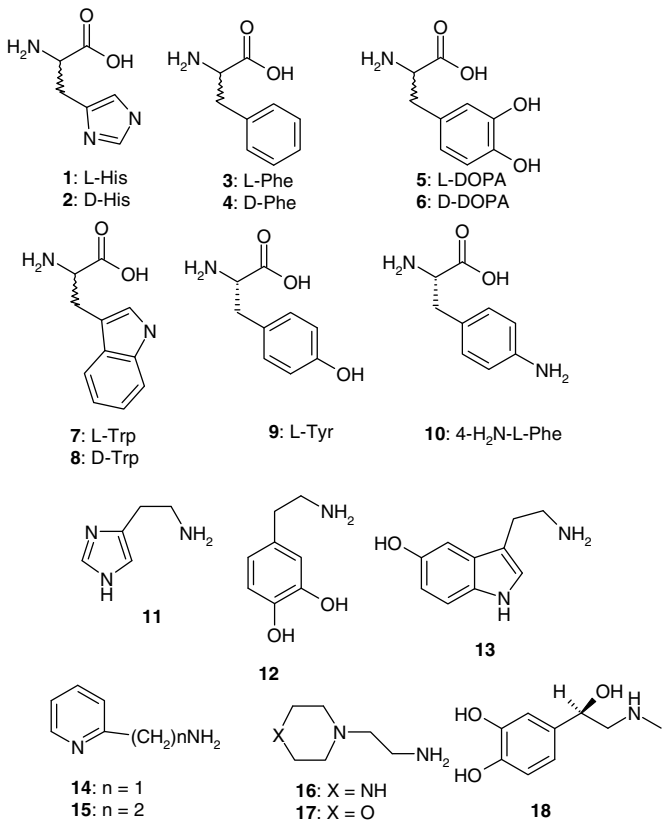
[a] Observed catalytic rate without activator. K_M values in the presence and the absence of activators were the same for the various CA isozymes (data not shown).
[b] Observed catalytic rate in the presence of 10 μM activator.
[c] The activation constant (K_A) for each isozyme was obtained by fitting the observed catalytic enhancements as a function of the activator concentration.[24] Mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[24] Standard errors were in the range of 5–10% of the reported values.
[d] Human recombinant isozymes; [b]Truncated human recombinant isozyme lacking the first 20 amino acid residues, which represent the signal peptide orienting the protein outside the cell.[20]

Table 2
Activation constants of hCA I, hCA II, hCA III (cytosolic isozymes) and hCA IV (membrane-associated isoform), with amino acids and amines **1–18**

No	Compound	K_A (μM)[a]			
		hCA I[b]	hCA II[b]	hCA III[b]	hCA IV[c]
1	L-His	0.03	10.9	35.9	7.30
2	D-His	0.09	43	1.12	12.3
3	L-Phe	0.07	0.013	34.7	36.3
4	D-Phe	86	0.035	15.4	49.3
5	L-DOPA	3.1	11.4	13.5	15.3
6	D-DOPA	4.9	7.8	28.7	34.7
7	L-Trp	44	27	20.5	37.1
8	D-Trp	41	12	19.0	39.6
9	L-Tyr	0.02	0.011	34.1	25.1
10	4-H_2N-L-Phe	0.24	0.15	43.2	0.079
11	Histamine	2.1	125	36.9	25.3
12	Dopamine	13.5	9.2	33.2	30.9
13	Serotonin	45	50	0.78	3.14
14	2-Pyridyl-methylamine	26	34	1.03	5.19
15	2-(2-Aminoethyl)pyridine	13	15	1.10	7.13
16	1-(2-Aminoethyl)-piperazine	7.4	2.3	0.32	24.9
17	4-(2-Aminoethyl)-morpholine	0.14	0.19	0.091	1.30
18	L-Adrenaline	0.09	96	36.4	45.0

Data for hCA I and II activation with these compounds are from Ref. 22.
[a] Mean from three determinations by a stopped-flow, CO_2 hydrase method.[24] Standard errors were in the range of 5–10% of the reported values.
[b] Human recombinant isozymes, stopped-flow CO_2 hydrase assay method.[24]
[c] Human recombinant enzyme lacking the first 20 aminoterminal residues,[20] stopped-flow CO_2 hydrase assay method.[24]

the phenyl moiety of L-Phe **3**, such as the OH moiety in Tyr **9**, the two phenolic moieties of L-DOPA **5**, or the amino one in **10**) again lead to important changes in the CA III activatory properties of the corresponding compounds. Such complex structure activity relationship (SAR) has in fact been observed for the activation of the other CA isozymes with this type of amines and amino acids.[18,19,22,23]



The membrane-associated human isozyme hCA IV was also activated by compounds **1–18** investigated here, but with a diverse profile as compared to that of the cytosolic isoforms hCA I – III shown in Table 2. Thus, the best, nanomolar hCA IV activator was the amino acid derivative 4-aminophenylalanine **10**, which showed an activation constant of 79 nM. It may be observed that this compound is a very weak CA III activator (K_A of 43.2 μM), being on the other hand a stronger CA I and II activator (K_As in the range of 0.15–0.24 μM). Effective hCA IV activation has also been observed with L-His **1**, serotonin **13**, the pyridyl-alkylamines **14** and **15**, as well as amine **17** (K_As in the range of 1.30–7.30 μM, Table 2). The remaining amines and amino acids showed less effective activity for the activation of hCA IV, with K_As in the range of 15.3–49.3 μM. SAR is again (as for the activation of the cytosolic isoforms CA I-III) quite sensitive to small changes in the scaffold of the activator or to its enantiomeric form. Thus, again important differences of activity were observed between enantiomers of the same amino acid (e.g., compare **1** and **2, 3** and **4, 5**, and **6**) or to rather minor changes in the substitution pattern of the scaffold. In this case, again the 3,4-dihydroxyphenyl moiety present in DOPA leads to better CA IV activators as compared to the unsubstituted Phe derivatives. The absence of a COOH moiety (as in histamine **11**) as compared to the amino acids **1** and **2** from which it can be derived leads to a loss of CA IV activatory efficiency. On the other hand it is amazing to see the difference of activity between the structurally related amines **16** and **17**, with the last one being 19.1 times a better CA IV activator as compared to **16**. It is difficult to rationalize these data in the absence of an X-ray crystal structures of CA IV complexed with activators.

In order to rationalize this SAR observed for the activation of hCA III and IV with compounds **1–18**, and also for explaining the quite different activation profiles of various CA isozymes, a comparison of the amino acid sequences of the human isozymes hCA I–IV is presented in Figure 1. The amino acid residues that were previously shown to form the active site cavity in all α-CAs[26] are indicated by a mixture of asterisk, plus sign, and 'z' above the hCA I sequence. Among these active site residues, many amino acids are conserved between these four isozymes, such as among others those involved in the catalytic cycle: (i) the three zinc ligands, His94, 96, and 119; (ii) the 'gate-keeping' residues Thr199 and Glu106, which orient the substrate in the right position to be attacked by the zinc-bound hydroxide ion; and (iii) His64, the proton shuttle residue, which transfers protons from the zinc bound water molecule toward the external medium, leading to the generation of the active form of the enzyme with hydroxide as the fourth zinc ligand but this last amino acid is conserved only or isozymes CA I, II, and IV.[1–4] Indeed, one of the reasons why CA II has such a low catalytic activity (Table 1) as compared to the other investigated isoforms is correlated just with the presence of a Lys residue in position 64 instead of the His present in most other α-CAs. The ε-amino group of Lys64 in CA III is a much worse proton shuttling moiety as compared to the imidazole of His64, at the pH values at which these enzymes work best, that is, in the pH range of 6.5–7.5.[1–4] Another important difference between CA III and the other α-CAs is the bulky amino acid in position 198, which is Phe only in CA III, being Leu in the other three isoforms discussed here. This position (198) is quite important for the fact that the side chain of this amino acid is in close proximity of the zinc ion and of Thr199, one of the amino acid residues critical or catalysis involving α-CAs.[1–4,6c] Thus, the steric impairment provoked by Phe198 and the presence of Lys64, which is a poor proton shuttling moiety, make CA III a weak catalyst for CO_2 hydration.[6c] However, these two amino acids were shown (at least for CA I and II)[18,19,22,23] to be involved in the binding of activators by means of X-ray crystallography, and thus the different activation profiles of these three cytosolic isoforms by

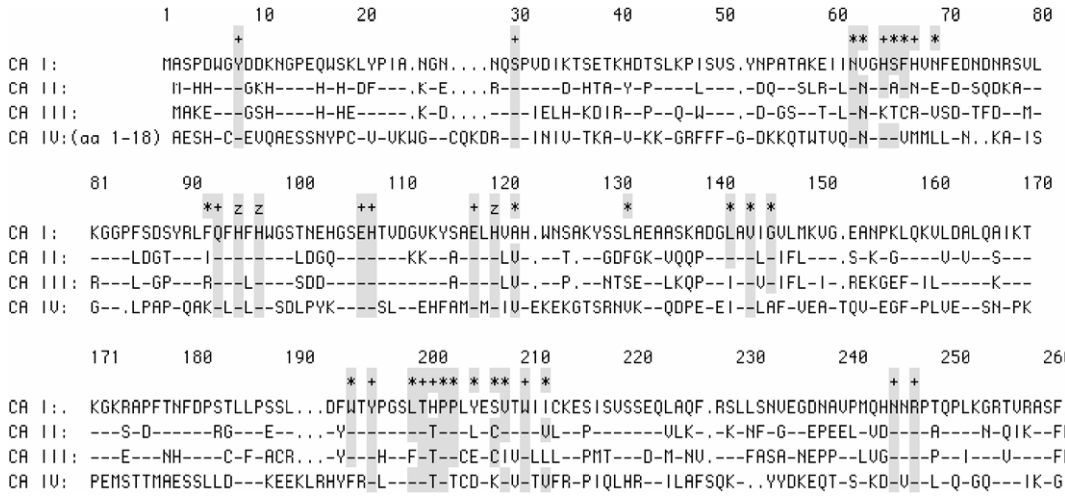


Figure 1. Alignment of the amino acid sequence of human isoforms CA III and CA IV with that of isozymes CA I and II (CA I numbering system used). Thirty-six active site residues previously defined as forming the active site[26] are indicated by a mixture of asterisk, 'plus', and 'z' signs above the CA I sequence. The residues known to participate in a network of hydrogen bonds and being involved in the binding of inhibitors/activators[22–23,27] are indicated by 'plus' and 'z' above the sequence; the latter sign indicates the three zinc-liganded histidine residues (His94, 96, and 119). Conserved amino acids in the three isoforms are indicated by a closed box.

amino acids and amines may be strongly influenced by the different amino acid residues present in these positions of the active site.

There are also several amino acid residues, which are characteristic only to CA IV among the α-CA isozymes discussed here, and they are the four cysteine residues in positions 7, 19, 24, and 203 (Fig. 1, CA I numbering system). Christianson's group[27] showed that they form two S–S bridges, which greatly contribute to the stabilization of the 3D-fold of this isozyme, making it also much more resistant to denaturation by detergents as compared to other α-CAs. It is also possible that they may contribute to the binding of activators, although no X-ray crystal data for adducts of CA IV with such modulators of activity are available for the moment. All these particular amino acid residues different between the α-CAs investigated here may explain the particular activity and activation profiles of these isozymes.

In conclusion, we report here the first activation study of the human CA isoforms III (cytosolic) and IV (membrane-associated), with a series of natural and non-natural amino acids as well as aromatic/heterocyclic amines. hCA III was efficiently activated by D-His, serotonin, pyridyl-alkylamines, and aminoethyl-piperazine/morpholine (K_As of 91–1.12 μM), whereas the best hCA IV activators were 4-amino-phenylalanine, serotonin, and 4-(2-aminoethyl)-morpholine (K_As of 79–3.14 μM). Precise steric and electronic requirements are needed to be present in the molecules of effective CA III/IV activators, in order to assure an adequate fit within the enzyme active site for the formation of the enzyme-activator complex, and for efficient proton transfer processes between the active site and the reaction medium. The activation profile of CA III and IV is distinct from that of all other mammalian CA isoforms investigated so far for their interaction with amino acids and amines.

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

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):[18,19]

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

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[18,19]
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