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Carbonic anhydrase activators: Activation of the archaeal β-class (Cab) and γ-class (Cam) carbonic anhydrases with amino acids and amines

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ABSTRACT

Activation of the archaeal β-class (Cab) and γ-class (Cam) carbonic anhydrases (CAs, EC 4.2.1.1) with a series of natural and non-natural amino acids and aromatic/heterocyclic amines has been investigated. Cab, Zn-Cam and Co-Cam showed an activation profile with natural, L- and D-amino acids very different of those of the α-class enzymes CA I, II and III. Most of these compounds showed medium efficacy as archaeal CA activators, except for D-Phe and L-Tyr which were effective Cab activators (K_As of 10.3–10.5 μM), 2-pyridylmethylamine and 1-(2-aminoethyl)-piperazine which effectively activated Zn-Cam (K_As of 10.1–11.4 μM) and serotonin, L-adrenaline and 2-pyridylmethylamine which were the best Co-Cam activators (K_As of 0.97–8.9 μM). We prove here that the activation mechanisms of the α-, β-, and γ-class CAs are similar, although the activation profiles with various compounds differ dramatically between these diverse enzymes.

The reversible hydration of carbon dioxide to bicarbonate (Eq. (1)) is involved in diverse biological functions, being catalyzed by the enzyme carbonic anhydrase (CA, EC 4.2.1.1) which contains an active site metal ion and is present in all three domains of life, *Archaea, Bacteria* and *Eukarya*.[1–9] The metal ion centers of the various CA classes are represented schematically in Figure 1.

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+ \quad (1)$$

Currently, there are five CA classes (α-, β-, γ-, δ-, ζ-) identified, possessing no sequence homology among them, indicative of convergent evolution of this catalytic function. Also, crystal structures so far reported for four of these enzyme classes (α-, β-, γ- and ζ-CAs), reveal no structural homology between these proteins.[9–16] An organism may possess more than one CA classes, each carrying out a distinct physiological or pathological function, and many organisms all over the phylogenetic tree possess more than one CA isoform, with vertebrates for example having 16 α-CA isozymes.[1–9]

The catalytic mechanism of these enzymes is also rather well understood. The metal hydroxide species present in the active site of these enzymes as the fourth ligand (Fig. 1A and B) acts as a strong nucleophile (at physiologic pH) converting the CO_2 molecule loosely bound in a hydrophobic pocket nearby the metal ion to bicarbonate, bis-coordinated to it, in a trigonal bipyramidal geometry.[1–9] This adduct is not very stable and reaction with a water molecule leads to liberation of bicarbonate in solution and generation of an acidic form of the enzyme incorporating a $M^{2+}(OH_2)$ species at the metal center, which is catalytically ineffective for the hydration of CO_2. In order to generate the nucleophilic, $M^{2+}(OH^-)$ species, a proton transfer reaction must occur, which is rate determining for the catalytic cycle in many of these quite rapid enzymes.[1–9] For many α-CAs this step is assisted by a proton shuttle residue, which is His64 in most mammalian isoforms, among which CA II, IV, VI, VII, IX, XII, XIII, XIV, etc. Possessing a flexible orientation, inwards (the *in* conformation) or outwards (the *out* conformation) the zinc ion center, the imidazole moiety of this histidine, with a pK_a of 6.0–7.5 is very appropriate as a proton shuttling residue and crucially important for the CA catalytic cycle.[1–9] The process can be also assisted by endogenous molecules, which bind within the enzyme active site (as proven by X-ray crystallography and other techniques) which have been termed CA activators (CAAs) and which facilitate the proton transfer reactions between the metal ion center and the external medium.[10–14] However, CAAs have only been investigated for the mammalian α-CA isozymes (CA I–XIV), mostly by this group. Several X-ray crystal structures of such adducts, mainly of the cytosolic isoforms CA I and II with amine and amino acid activators were recently reported.[10–14]

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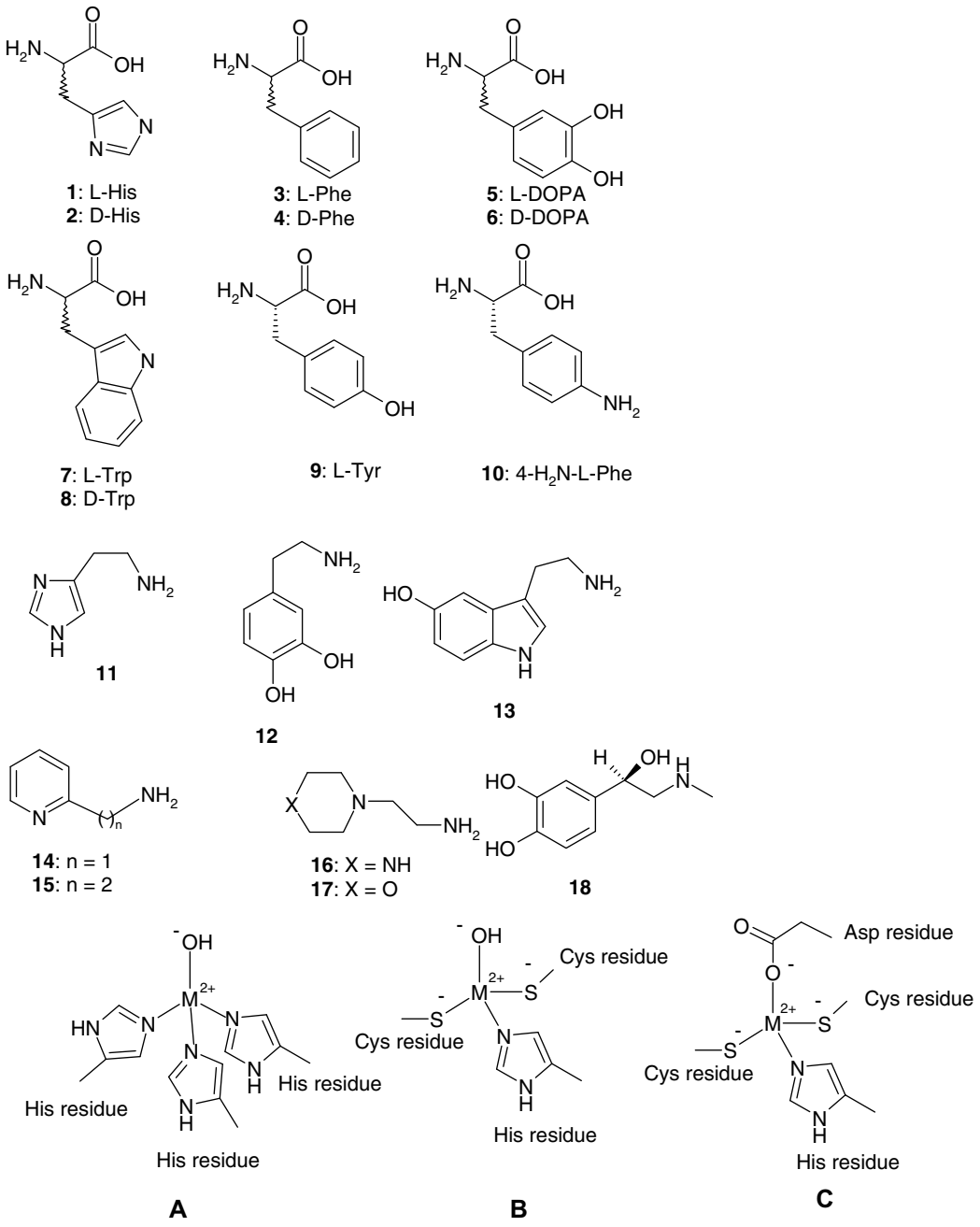

Figure 1. Metal ion coordination sphere in the five classes of CAs: (A) α-CAs and δ-CAs [the three histidine residues from the same polypeptide chain, M = Zn(II)], and γ-class CAs [the histidine residues from different, adjacent polypeptide chains of the homotrimer protein, M = Zn, Fe(II), or Co(II)]; (B) β-CAs with "open active site", that is, a water molecule/hydroxide ion the fourth metal ion ligand, M = Zn(II), and ζ-CAs, M = Cd(II) or Zn(II); (C) β-CAs with "closed active site", that is, the fourth zinc ligand is an aspartate residues which is replaced by a water molecule during the catalytic cycle leading to a **B** type active site.

Ferry's group[15] investigated the proton transfer processes in the catalytic cycle of some β-, and γ-CAs, suggesting that like for the enzymes belonging to the α-class, they are assisted by active site residues, which may be His for the β-CAs, and Glu for the prototypical γ-CA from *Methanosarcina thermophila*, Cam. Thus, either the imidazole belonging to His residues or the carboxylates of Glu active site residues may act similarly to His64 from mammalian CAs, in shuttling protons from the metal center to the environment, facilitating thus the catalytic turnover. In order to better understand the catalytic mechanism of CAs belonging to the the β- and γ-classes, it is of crucial importance to test whether these enzymes, similarly to the α-CAs, can be activated by compounds which can shuttle protons between the active site and the environment. Since no activation studies of these enzymes have been performed so far, here we report the first such study of the archaeal enzymes belonging to the β- and γ-classes, Cab (from *Methanobacterium thermoautotrophicum*) and Cam (from *Methanosarcina thermophila*) with a series of amine and amino acids investigated earlier for the activation of the α-class CA isoforms of mammalian origin (CA I–XIV).[10–14] We have included in our study the 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.[10–14] As both Cab and Cam are prototypical examples of CAs belonging to the β- and γ-classes, respectively, we think that our study may shed some light on the activation phenomena of β- and γ-CAs,

which are widespread enzymes mainly in *Bacteria*, *Archaea* but also in microscopic and macroscopic *Eukarya* such as fungi, plants, etc.[2,3]

The affinity constant (K_{aff}) of an activator for the corresponding CA isoform has been denominated the activation constant (K_A)[10] 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.[16] Detailed kinetic measurements (data not shown) showed that the activators **1–18** investigated here for their interactions with Cab as well as Zn(II)-Cam and Co(II)-Cam, 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 observed earlier for the activation of other mammalian CAs.[10–14] 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 enzymes (Table 1), supporting our previous observations[1,10–14] 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. Thus, it is apparent from the above data that the activation mechanisms of the α-, β-, and γ-CAs are similar, involving the formation of enzyme-activator (E-A) complexes in which the proton shuttling is favored by the proton accepting moieties present in the activator structure, which lead to an overall increase of the catalytic turnover.

Data of Table 1 show that L-Tyr (at 10 μM concentration), which is an effective CAA for hCA I and II, and a less effective one for Cab and Zn-/Co-Cam, enhances k_{cat} values for all these enzymes, whereas K_M remains unchanged. It may be observed that L-Tyr is a nanomolar activator for the α-class enzymes (hCA I and II, with K_As in the range of 11–20 nM)[11–14] and a micromolar one for the archaeal ones (K_As in the range of 10.5–53 μM, see discussion later in the text).

Data of Table 2 show that all amino acids and amines **1–18** investigated here act as CAAs against both the α-class enzymes hCA I, II and III (investigated earlier[10–14] and included here for comparison reasons) as well as the archaeal, β- and γ-CAs Cab and Cam. Since the metal ion requirements for Cam in Nature may be variable [the enzyme functions equally as well with Zn(II), Co(II) and Fe(II)][17] we used both the zinc(II) and cobalt(II) derivatives of this enzyme in our experiments, also because we have shown earlier that the inhibition profile of the two metal ion derivatives with sulfonamides and anions are rather different.[18] Indeed, the following structure activity relationship (SAR) can be observed for the activation of these CAs with compounds **1–18**:

(i) Cab was moderately activated by the following compounds: D-Phe, L- and D-DOPA, L-trp, L-Tyr, 2-pyridylmethylamine **14** as well as the structurally related heterocyclic amines **16–18** which showed activation constants in the range of 10.3–18.7 μM, whereas the remaining compounds (**1–3, 8, 10–13** and **15**) showed much less effective activating properties, with activation constants in the range of 40–89 μM. Several issues must be discussed here. Thus, unlike for the α-CAs hCA I–III investigated earlier,[10–14] no submicromolar or nanomolar CAAs were evidenced so far for Cab, with the best activators (D-Phe and L-Tyr) having a K_A of 10.3–10.5 μM. Thus, important differences in the affinity of this class of CAAs for the active site of α- and β-/γ-CAs (see later in the text) are already obvious. This may be due to the very different architecture of the corresponding active sites, with those of the β-/γ-CAs being generally more shallow as compared to those of the α-class enzymes. Some amino acid enantiomers were observed to possess very different activating properties against Cab, such as for example L- and D-Phe, with the last enantiomer being 6.8 times more effective as a Cab activator compared to L-Phe. This type of behavior was on the other hand observed also for the activation of α-CAs with such derivatives, and explained by means of X-ray crystallography, being documented a very different binding mode for enantiomers of the same amino acid within the active site cavity of hCA II.[10e,11e] Furthermore, small structural changes in the scaffold of these activators (or example an extra CH_2 moiety between amines **14** and **15**, or the loss of a COOH moiety in dopamine **12** as compared to L-/D-DOPA **5** and **6**) again led to dramatic changes of Cab activating properties of these derivatives. This type of SAR has also been documented for the activation of various α-CAs, such as for example hCA I-III (Table 1).[10–14]

(ii) Zn-Cam was even less activated with derivatives **1–18** compared to Cab. Indeed, only two compounds, 2-pyridylmethylamine **14** and 1-(2-aminoethyl)-piperazine **16** showed activation constants in the low micromolar arnge (K_As of 10.1–11.4 μM) with the remaining derivatives behaving as weak CAAs for this enzyme, with K_As in the range of 24–72 μM. SAR features discussed above for Cab are also valid for this enzyme, but they are more flat, as the variation of the activating properties was not as high for many of the various amino acid enantiomers or structurally related amines.

(iii) Co-Cam showed on the other hand a very different behavior towards activators 1–18 as compared to the zinc derivative Zn-Cam (Table 2). Thus, several compounds, all belonging to the amine type of CAAs, such as **11–14**, **16** and **18**, showed effective CA activating properties, with K_As in the range of 0.97–18.4 μM. The best Co-Cam activator was serotonin **13** (submicromolar activity) whereas many of the remaining amones had K_As < 10 μM. Thus, the two metal derivatives of Cam show a very different behavior towards activators of the amine and amino acid type, with Co-Cam being generally better activated as compared to Zn-cam (except for compounds **1, 2**, and **6–9**) which are better Zn-Cam than Co-Cam activators. It is rather difficult to interpret these data, since no X-ray crystal structures for adducts of Cab/Cam with activators are available up until now, and also homology modeling with α-CA-activator adducts are not useful due to the very different structures of the various families of such enzymes.

We do not know what is the relevance of these phenomena in vivo, in archaea in which these enzymes are present. In aerobic prokaryotes, β-CAs are implicated in maintaining internal pH and CO_2/bicarbonate balances required for biosynthetic reactions, in anaerobic prokaryotes, in the transport of CO_2 and bicarbonate across the cytoplasmic membrane that regulates pH and facilitates acquisition of substrates and product removal required for growth,

Table 1
Activation of human (hCA) isozymes I, II, as well as Cab and Zn/Co-Cam with L-Tyr, at 25 °C, for the CO_2 hydration reaction.

Isozyme	k_{cat}* (s^{-1})	K_M* (mM)	(k_{cat}) L-Tyr** (s^{-1})	K_A*** (μM) L-Tyr
hCA I[a]	2.0×10^5	4.0	13.9×10^5	0.02
hCA II[a]	1.4×10^6	9.3	12.8×10^6	0.011
Cab[b]	3.1×10^4	1.7	6.8×10^4	10.5
Zn-Cam[b]	7.1×10^4	1.8	10.5×10^4	24
Co-Cam[b]	6.5×10^4	1.5	9.7×10^4	53

* Observed catalytic rate without activator. K_M values in the presence and the absence of activators were the same for the various CAs (data not shown).
** Observed catalytic rate in the presence of 10 μM activator.
*** The activation constant (K_A) for each enzyme was obtained by fitting the observed catalytic enhancements as a function of the activator concentration.[16] Mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[16] Standard errors were in the range of 5–10% of the reported values.
[a] Human recombinant isozymes.[1]
[b] Archaeal recombinant enzymes.[2]

Table 2
Activation constants of hCA I, hCA II, hCA III (cytosolic isozymes) and archaeal β- (Cab) and γ-CAs (Zn-Cam and Co-Cam) with amino acids and amines **1–18**. Data for hCA I–III activation with these compounds are from Refs. 11–14.

No.	Compound	hCA I[a]	hCA II[a]	K_A (μM)* hCA III[a]	Cab[b]	Zn-Cam[c]	Co-Cam[c]
1	L-His	0.03	10.9	35.9	69	68	135
2	D-His	0.09	43	1.12	57	46	73
3	L-Phe	0.07	0.013	34.7	70	68	70
4	D-Phe	0.035	86	15.4	10.3	42	24
5	L-DOPA	3.1	11.4	13.5	11.4	39	38
6	D-DOPA	4.9	7.8	28.7	15.6	37	41
7	L-Trp	44	27	20.5	16.9	38	47
8	D-Trp	41	12	19.0	41	33	68
9	L-Tyr	0.02	0.011	34.1	10.5	24	53
10	4-H_2N-L-Phe	0.24	0.15	43.2	89	72	22
11	Histamine	2.1	125	36.9	76	63	9.2
12	Dopamine	13.5	9.2	33.2	51	54	18.4
13	Serotonin	45	50	0.78	62	38	0.97
14	2-Pyridyl-methylamine	26	34	1.03	18.7	11.4	8.7
15	2-(2-Aminoethyl)pyridine	13	15	1.10	40	24	18.5
16	1-(2-Aminoethyl)-piperazine	7.4	2.3	0.32	13.8	10.1	16.1
17	4-(2-Aminoethyl)-morpholine 0.14	0.14	0.19	0.091	18.5	45	38
18	L-Adrenaline	0.09	96	36.4	11.5	39	8.9

* 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.
[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method.[24]
[b,c] Stopped flow CO_2 hydrase assay method.[24]

whereas in phototrophic organisms, they are particularly important for transport and concentration of CO_2 and bicarbonate for photosynthesis. Thus, we hypothesize that amino acids and amines as those investigated here may considerably influence these processes. Furthermore, as the physiological roles for the γ-class CAs are not as well documented for the moment, understanding the activation patterns by compounds as those investigated here may shed some light on their in vivo role(s). We also prove here for the first time that the activation mechanisms of the α-, β-, and γ-class CAs are similar, although the activation profiles with various compounds differ dramatically between the diverse enzymes.

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constant is given by eq. *3*:[10–14]where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[10–14].

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