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Short communication

The zinc – but not cadmium – containing ζ-carbonic from the diatom *Thalassiosira weissflogii* is potently activated by amines and amino acids

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ABSTRACT

The activation of the ζ-class carbonic anhydrase (CAs, EC 4.2.1.1) from the diatom *Thalassiosira weissflogii* (TweCAζ) incorporating both Zn(II) and Cd(II) at the active site, was investigated for the first time, using a panel of natural and non-natural amino acids and amines. CdTweCAζ was completely insensitive to activation, whereas all these compounds were effective activators of the zinc-containing enzyme ZnTweCAζ, with activation constants ranging between 92 nM and 37.9 μM. The most effective ZnTweCAζ activators were L-adrenaline, 1-(2-aminoethyl)-piperazine and 4-(2-aminoethyl)-morpholine, with K_As in the range of 92–150 nM. L-His, L- and D-Tyr and some pyridyl-alkylamines, had K_As in the range of 0.62–0.98 μM, whereas L-/D-DOPA, D-Trp, histamine, serotonin and L-Asn were the next most efficient activators, with K_As in the range of 1.27–3.19 μM. The least effective activators were L-Phe (K_A of 15.4 μM) and L-Asp (K_A of 37.9 μM). This in vitro study may be useful for a more complete understanding of the activation processes of various CA enzyme families, of which the ζ-class was scarcely investigated.

1. Introduction

Morel's group discovered two new carbonic anhydrase (CA, EC 4.2.1.1) genetic families in the diatom *Thalassiosira weissflogii*, δ-CAs [1], and ζ-CAs [2]. A number of orthologues of these enzymes were thereafter identified in most diatoms from natural phytoplankton assemblages, in which they are responsible for CO_2 fixation (together with ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), to which they furnish one of the substrates) [1–3]. A carbon concentration mechanisms (CCM) also involves CAs as one of the machinery leading to the achievement of high enough CO_2 concentrations at the site of photosynthesis, and this is quite relevant in the CO_2-poor habitats of cyanobacteria, aquatic microalgae, and macrophytes [3]. Indeed, in seawater the supply of inorganic carbon (Ci) to the chloroplasts is diffusion limited, and therefore, the supply of CO_2 and bicarbonate across the diffusive boundary layer on the outer side of the epidermis is often a limiting factor, and needs the presence of CAs [3]. This is probably the reason why so many CAs are present in marine organisms among which diatoms.

A related species of the original diatom in which CAs were originally reported, *T. pseudonana*, was in fact demonstrated to possess genes for three α-, five γ-, four δ-, and one ζ-CAs, making the understanding of the functions and roles of these 13 enzymes rather challenging [3]. In fact CAs are ubiquitous enzymes all over the tree of life (as seven genetically distinct families, the α-, β-, γ-, δ-, ζ-, η- and θ-CAs) [4–6], and they catalyze the rapid interconversion between CO_2 and water to bicarbonate and protons, by using a metal hydroxide nucleophilic species [7]. Indeed, all seven CA families belong to the metalloenzyme proteins, and the metal ion is crucial for the catalytic activity, since the apoenzymes are totally devoid of catalytic power [4–7]. Most classes use Zn(II) ion within the active site with a zinc hydroxide species acting as nucleophile [4–7], but the ζ-CAs are quite particular, as it has been shown that they are cambialistic enzyme, active with both Zn(II) and Cd(II) ions within the active site. it seems that in the marine environment, due to a shortage of zinc ions availability, these enzymes are cadmium proteins, showing thus that at least for diatoms, cadmium is not a toxic metal ion [2,8]. The best characterized ζ-CA is the enzyme from *T. weissflogii*, TweCAζ (which can exist both with Zn(II) and Cd(II) at the active site, ZnTweCAζ, and CdTweCAζ, respectively) [2,8]. The X-ray crystal structure of all its three slightly different

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R1, R2 and *R*3) fragments were reported (with Cd(II) and Zn(II) within the active site), and inhibition studies with anions and sulfonamides are also available [2,8]. However, no activators of this CA genetic family were investigated so far.

The CA activators (CAAs) have been demonstrated to participate in the CA catalytic cycle [9], which is shown schematically in Eqs. (1) and (2) below, with M being Zn(II) or Cd(II):

$$EM^{2+}-OH^{-} + CO_2 \Leftrightarrow EM^{2+}-HCO_3^{-} \overset{H_2O}{\Leftrightarrow} EM^{2+}-OH_2 + HCO_3^{-} \quad (1)$$

$$EM^{2+}-OH_2 \Leftrightarrow EM^{2+}-HO^{-} + H^{+} \quad (2)$$

The metal-bound hydroxide species of the enzyme nucleophilically attacks the CO_2 substrate, bound in a hydrophobic pocket nearby and is optimally orientated for the hydration reaction to occurs (Eq. (1) [4–8]. The second part of the process involves the replacement of bicarbonate formed in the hydration reaction by an incoming water molecule to form the acidic enzymatic species, EM^{2+}–OH_2 (Eq. (1). In order to regenerate the metal hydroxide species, a proton is transferred from the metal ion-bound water molecule to the external medium (Eq. (2), which is the rate-determining step of the entire catalytic cycle, and is assisted in α-CAs by a His residue placed in the middle of the active site cavity, which acts as a proton shuttle residue [4–8]. The activators were shown to participate in this rate-determining step, by forming enzyme-activator complexes, in which the proton transfer is favoured due to the fact that it is an intra- and not intermolecular process, with Eq. (2) being transformed to Eq. (3), in which the enzyme-activator complexes are shown, and they behave as supplemental proton shuttling molecules, in addition to the wild type residues, which as mentioned above, is a His in α-CAs [9–12]:

$$EM^{2+}-OH_2 + A \Leftrightarrow [EM^{2+}-OH_2-A] \underset{\text{enzyme - activator complexes}}{\Leftrightarrow} [EM^{2+}-OH^{-} -AH^{+}] \Leftrightarrow EM^{2+}-OH^{-} + AH^{+} \quad (3)$$

This mechanism was thoroughly demonstrated by X-ray crystallographic and kinetic experiments mainly for human (h) CAs (belonging to the α-class) [9–13], but presumably the other CA families possess a similar activation mechanism, although the proton shuttle may be not His but a diverse residue. In fact, this proton-transfer process is less well understood in all other CA classes except the α-CAs. For β-CAs, His and Tyr residues (His92 and Tyr88, *Coccomyxa* CA numbering) [14] may act as proton shuttle residues, whereas for γ-CAs, Ferry's group [15] reported that one or two Glu residues (Glu84 and Glu62, Cam numbering system; Cam is the enzyme from *Methanosarcina thermophila*) [15] act as proton shuttles in the catalytic cycle. Nothing is known regarding the δ-, ζ-, η-, and θ-CAs in this regard, and these enzymes were in fact hardly been investigated for their activation only in the last period [16,17]. In fact very recently the first activation study of the δ-CA from *T. weissflogii* has been reported by our groups [18], but as already mentioned, no ζ-CA activation studies are available in the literature so far. Here we show that the zinc-containing ζ-CA is highly activated by amino acid and amine CAAs, whereas the cadmium-containing enzyme is not affected by these modulators of activity.

2. Results and discussion

Natural and non-natural amino acids and amines **1–24** were included among the investigated compounds as activators of Zn/CdTweCAζ (Fig. 1). These compounds were employed for investigations as CAAs against many classes of CAs, including the bacterial, archaeal and mammalian ones mentioned earlier [9–12,16–18]. The presence of protonatable moieties of the amine, carboxylate or imidazole type present in these derivatives makes them appropriate for participating in the proton shuttling processes between the active site and the reaction medium, as described by Eq. (3).

Data of Table 1 shows that both zinc- or cadmium-containing TweCAζ show a CO_2 hydrase activity similar to hCA II, one of the most efficient CAs known to date and a catalytically highly performant enzyme, with k_{cat} values of $> 10^6\ s^{-1}$, and K_M values in the range of 8.7–10.7 mM (the same range as hCA II) [6–8]. Both the first order kinetic constant (k_{cat}) and the K_M of the three enzymes are very similar, as already reported earlier [8]. In the presence of 10 μM L-Tyr as activator, the K_M of ZnTweCAζ remained unchanged (data not shown) but the k_{cat} was 4.9 times higher than in the absence of the activator (Table 1). This situation has been observed for other CAs belonging to various genetic families investigated to date, proving that presumably the CA activation mechanism is similar for all enzyme classes, i.e., a facilitation of the proton transfer process by the activator molecule bound within the enzyme active site in the enzyme-activator complex. We have performed the same type of detailed kinetic analysis in the presence of 10 μM 2-pyridyl-methylamine, and similar observations (as fopr L-Tyr) were obtained: the measured k_{cat} of ZnTweCAζ was of $7.3 \times 10^6\ s^{-1}$, whereas K_M remained the same as for the pure enzyme (K_M = 8.7 mM). Unexpectedly, the k_{cat} of CdTweCAζ was not affected by the presence of 10 μM L-Tyr or amine **15** (and also its K_M), and as it will be discussed shortly, the cadmium-containing enzyme was totally insensitive to activators (Table 2).

Data of Table 2 shows the Zn/CdTweCAζ activation with amino acids and amines **1–24**. The activation profile with the same compounds for the widespread, physiologically relevant isoforms hCA I and II (belonging to the α-CA family) are also shown for comparison reasons. The following structure-activity relationship can be inferred for Zn/CdTweCAζ activation with these compounds:

(i) The cadmium-containing TweCAζ, although possessing a very similar catalytic activity with its zinc-containing counterpart (Table 1. and Ref. [8]) was not at all activated by amino acids and amines **1–24**, up to 50 μM activator in the assay system (which is a very high concentration), unlike ZnTweCAζ, for which the activation constants ranged between 92 nM and 37.9 μM (Table 2). spanning thus on several orders of magnitude. This is a very unexpected and surprising finding, considering the fact that, apart for the ionic radius, which is higher for Cd(II) compared to Zn(II), there are no significant differences of behaviour between these two d [10] metal ion in metalloproteins. Thus, our finding that the Cd-CA is not activated remains unexplained for the moment. This is also the first time that a CA protein of any genetic family shows a behaviour of this type. In fact, representatives all other CA families were sensitive to activators f the amine and amino acid type [9–13].

(ii) The most effective ZnTweCAζ activators belonged to the amine chemotype, i.e., L-adrenaline **19** and the heterocyclic amines **17** and **18**, showing K_As in the range of 92–150 nM (Table 2). Thus, both compounds incorporating aminoethyl- (**17** and **18**) or methylamino-hydroxyethyl (adrenaline) show a potent activating effect, and presumably these primary/secondary amine functionalities are involved in the proton shuttling favouring catalysis.

(iii) Effective ZnTweCAζ activation was also observed with L-His, L- and D-Tyr and the pyridyl-alkylamines **15** and **16**, which had K_As in the range of 0.62–0.98 μM (Table 2). L-/D-DOPA, D-Trp, histamine, serotonin and L-Asn were the next most efficient activators, with K_As in the range of 1.27 – 3.19 μM. Thus, both amines and amino acids may show this behaviour of medium potency activator against ZnTweCAζ.

(iv) Weaker activation against ZnTweCAζ was observed for D-His, D-Phe, L-Trp, 4-amino-l-Phe, dpamine, L-Glu, D-Glu and L-Gln, which showed K_As in the range of 6.43–10.1 μM. Generally, the D-amino acids were more effective activators compared to the L-enantiomers, except for His, for which L-His was 8.8 times a more effective CAA compared to the D-enantiomer (Table 2). For the two Trp enantiomers, the D-one was 4.77 times a more effective activator compared to L-Trp.

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