The first activation study of a δ-carbonic anhydrase: TweCAδ from the diatom *Thalassiosira weissflogii* is effectively activated by amines and amino acids

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**ABSTRACT**

The activation of the δ-class carbonic anhydrase (CAs, EC 4.2.1.1) from the diatom *Thalassiosira weissflogii* (TweCAδ) was investigated using a panel of natural and non-natural amino acids and amines. The most effective activator of TweCAδ was D-Tyr (K_A of 51 nM), whereas several other amino acids and amines, such as L-His, L-Trp, d-Trp, dopamine and serotonin were submicromolar activators (K_A from 0.51 to 0.93 μM). The most ineffective activator of TweCAδ was 4-amino-L-Phe (18.9 μM), whereas L-His, l-/d-Phe, l-/d-DOPA, l-Tyr, histamine, some pyridyl-alkylamines, L-adrenaline and aminoethyl-piperazine/morpholine were moderately potent activators (K_A from 1.34 to 8.16 μM). For any δ-CA, there are no data on the crystal structure, homology modelling and the amino acid residues that are responsible for proton transfer to the active site are currently unknown making it challenging to provide a detailed rationale for these findings. However, these data provide further evidence that this class of underexplored CA deserves more attention.

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1. Introduction

Carbonic anhydrases are an ubiquitous family of enzymes that catalyse the rapid interconversion between CO_2 and water to bicarbonate and protons. Of the seven genetically distinct families of CA enzymes known to date, the δ-class carbonic anhydrases (CAs, EC 4.2.1.1) are the least investigated. In 2000, Morel’s group discovered δ-CAs in the diatom *Thalassiosira weissflogii*, which was initially denominated TWCA1. Subsequently, a number of orthologues of this specific enzymes have been identified in most diatoms from natural phytoplankton assemblages and are responsible (along with other CAs) for CO_2 fixation by marine organisms. A related species of the original diatom in which these enzymes were reported, *T. pseudonana*, was demonstrated to possess genes for three δ-, five γ-, four α- and one ζ-CAs. However, none of these enzymes have been cloned and characterised in detail to date. Thus, diatoms can be considered the organisms with the most intricate and poorly understood distribution of CAs, and the roles of CAs are far from being well understood with the exception of their important role in CO_2 fixation and photosynthesis, as they provide bicarbonate or CO_2 to ribulose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO).

In 2013, Lee et al. cloned and purified the δ-CA from *T. weissflogii* and investigated its esterase activity (and not its CO_2 hydrase activity) using the substrate, 4-nitrophenyl acetate. Our group demonstrated that such esterase activity is artefactual; i.e. the activity does not result from hydrolysis of the ester at the zinc hydroxide active site of the enzyme. This was confirmed by performing the esterase hydrolysis catalysed reaction in the presence of the enzyme with and without a potent CA inhibitor (CAI) that selectively binds to the zinc active site. This highlights the importance of performing control experiments to confirm CA enzymatic activity. Our group characterised the CO_2 hydrase activity of this enzyme (denominated by us TweCAδ) and reported the first anion and sulphonamide inhibition studies for any δ-class enzyme. These data demonstrate that TweCAδ is similar to other CAs belonging to the α-, β-, γ-, ζ-, η- and θ-CAs, i.e. TweCAδ is an excellent catalyst for the hydration of CO_2 to bicarbonate and hydronium ions, and that its activity may be inhibited by anions and sulphonamides, the two main classes of simple CAs. However, no activation studies of this enzyme have been reported to date, although the CA activators (CAAs) are an important class of modulators for the activity of CA enzymes.

CAAs have been demonstrated to participate in the CA catalytic cycle, which is shown schematically in the following equations:

\[ \text{H}_2\text{O} \rightarrow \text{EZn}^{2+} + \text{OH}^- + \text{CO}_2 \leftrightarrow \text{EZn}^{2+} + \text{HCO}_3^- \leftrightarrow \text{EZn}^{2+} + \text{OH}^- + \text{H}^+ \]

In the first step, a zinc-bound hydroxide species of the enzyme nucleophilically attacks the CO_2 substrate, which is bound in a hydrophobic pocket nearby and is optimally orientated for the hydration reaction to occur (Equation (1)). 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, EZn^{2+} + OH^- (Equation (1)). In order to
regenerate the zinc hydroxide species, a proton is transferred from the Zn(II)-bound water molecule to the external medium (Equation (2)), which is the rate-determining step of the entire catalytic cycle: 9:

\[
\text{EZn}^{2+} + \text{OH}_2^+ + \text{A} \rightleftharpoons [\text{EZn}^{2+} - \text{OH}_2^- - \text{A}] \\
\rightleftharpoons [\text{EZn}^{2+} - \text{HO}^- - \text{AH}^+] \rightleftharpoons \text{E}^\text{−} + \text{HO}^- + \text{AH}^+ \quad (3)
\]

enzyme – activator complexes

In the presence of activators (A in Equation (3)), this rate-determining step is facilitated by an additional proton release pathway, which involves the activator A bound within the enzyme active site. By forming an enzyme-activator complex, the proton transfer reaction becomes intramolecular and thus more rapid compared to the intermolecular process in which for example buffers can take part.5,10 The enzyme–activator complexes were thoroughly characterised for α-CAs of human (h) origin, such as hCA I and II, by means of kinetic and X-ray crystallographic techniques, which allowed the activator-binding site within the CA cavity and the structure–activity relationship governing these processes to be determined.5,10 However, CAA research has been relatively neglected compared with that for CAI. Inhibitors of the sulphonamide type5,11–13 that target CAs belonging to various classes and from various organisms have been extensively studied, and possess clinical applications as drugs for the treatment of oedema, glaucoma, epilepsy, obesity and cancer.14,15 Recently, CAs were also proposed as an alternative for the management of neuropathic pain,16 cerebral ischemia,17 arthritis17 and potentially as anti-infectives18. In contrast, the activation of CAs by naturally occurring amines and amino acids may play a role in increasing the activity of CAs in pathogens.19 In this paper, we report the first activation study of a δ-CA, investigating the activation profile with amines and amino acids of TweCAδ.

2. Materials and methods

2.1. Materials

Amino acids and amines (1–19) were commercially available, highest purity reagents from Sigma-Aldrich, Milan, Italy. TweCAδ was a recombinant protein produced as reported earlier by our group.5

2.2. CA enzyme activation assay

An SX.18Mw-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic activity of various CA isozymes for CO2 hydration reaction.18 Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, and 0.1 M Na2SO4 (for maintaining constant ionic strength, absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as concentration of 0.2 mM) was used as indicator, working at the instrument has been used to assay the catalytic activity of various enzymes are very similar. In the presence of 10 μM L-Trp as activator, the KM of TweCAδ remained unchanged (data not shown) but the kcat remained 8.15 times higher than in the absence of the activator (Table 1). This situation has been observed for all CAs investigated to date, belonging to all known CA genetic families, proving that presumably the CA activation mechanism is similar for all enzyme classes, involving facilitation of the proton transfer process by the activator molecule bound within the enzyme active site in the enzyme-activator complex.

Data of Table 2 show the TweCAδ activation with amino acids and amines (1–19). 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 TweCAδ activation with these compounds: (i) the most effective TweCAδ activator was d-Tyr, with an activation constant of 51 nM, whereas several other amino acids and amines, such as L-His, L-Trp, D-Trp, dopamine and serotonin were submicromolar activators, with KM ranging between 0.51 and 0.93 μM; (ii) the most ineffective activator of TweCAδ was 4-amino-L-Phe, with an activation constant of 18.9 μM; (iii) the remaining derivatives investigated were effective to moderately potent activators, with KM ranging between 1.34 and 8.16 μM. Thus, the SAR for these compounds is rather “flat” because most were rather effective activators of this enzyme. However, some features will be discussed. The stereochemistry of the chiral centre for the amino acid derivatives seems to not be very important, since both L- (e.g. L-His, L-Trp) and D-amino acid derivatives (e.g. D-Trp, D-Tyr) showed effective TweCAδ activation (Table 2). Small changes in the scaffold of an activator led to important differences of activity. For example, introduction of an amino moiety in the 4 position of the phenyl ring in L-Phe (a rather effective activator) led to a massive loss of efficacy in compound 11, which was 8.8 times a less efficient

\[
v = \frac{v_{\text{max}}}{1 + \text{KM}/(1 + [\text{A}]_f/\text{KM})} \quad (4)
\]

where [A]f is the free concentration of activator.

Working at substrate concentrations considerably lower than KM ([S] < KM), 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 the following equation:

\[
v = v_0 \cdot K_a - \left( [\text{A}]_f - 0.5 \left( (\frac{[\text{A}]_f + [\text{E}]_f + \text{KM}) - ([\text{A}]_f + [\text{E}]_f + \text{KM})^2 - 4\text{[A]}_f[\text{E}]_f \right)^{1/2} \right) \quad (5)
\]
Amino acids 1–11 and amines 12–19 investigated as TweCAα activators.

Table 1. Activation of human carbonic anhydrase (hCA) isozymes I, II, and TweCAα with l-Trp, at 25 °C, for the CO2 hydration reaction.

| Isozyme   | $K_a$ (s$^{-1}$) | $K_a^{a}$ (mM) | $K_{cat}$ (s$^{-1}$) | $K_s$ (µM) | l-Trp |
|-----------|------------------|----------------|-----------------------|--------------|-------|
| hCA I$^a$ | 2.0 × 10$^4$     | 4.0            | 3.4 × 10$^5$          | 44           |       |
| hCA II$^a$| 1.4 × 10$^6$     | 9.3            | 4.9 × 10$^6$          | 27           |       |
| TweCAα$^d$| 1.3 × 10$^5$     | 3.9            | 10.6 × 10$^5$         | 0.93         |       |

$^a$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).

$^b$Observed catalytic rate in the presence of 10 µM activator.

$^c$The activation constant ($K_a$) for each enzyme was obtained by fitting the observed catalytic enhancements as a function of the activator concentration.

$^d$Mean from at least three determinations by a stopped-flow CO2 hydrase method. Standard errors were in the range of 5–10% of the reported values (data not shown).

Table 2. Activation constants of hCA I, hCA II and the bacterial TweCAα with amino acids and amines 1–19, by a stopped-flow CO2 hydrase assay.

| No. | Compound                      | hCA I$^b$ | hCA II$^b$ | TweCAα$^d$ |
|-----|-------------------------------|-----------|------------|------------|
| 1   | L-His                         | 0.03      | 10.9       | 0.75       |
| 2   | D-His                         | 0.09      | 43         | 4.90       |
| 3   | L-Phe                         | 0.07      | 0.013      | 2.15       |
| 4   | D-Phe                         | 86        | 0.035      | 1.96       |
| 5   | L-DOPA                        | 3.1       | 11.4       | 2.11       |
| 6   | D-DOPA                        | 4.9       | 7.8        | 6.24       |
| 7   | L-Trp                         | 41        | 12         | 0.69       |
| 8   | D-Trp                         | 0.02      | 0.011      | 1.52       |
| 9   | L-Tyr                         | 0.04      | 0.013      | 0.051      |
| 10  | D-Tyr                         | 0.24      | 0.15       | 18.9       |
| 11  | 4-H$_2$N-L-Phe                | 2.1       | 125        | 1.34       |
| 12  | Histamine                     | 13.5      | 9.20       | 0.51       |
| 13  | Dopamine                      | 45        | 50         | 0.90       |
| 14  | Serotonin                     | 2.5       | 34         | 5.28       |
| 15  | 2-Pyridy-methylamine          | 26        | 34         | 5.28       |
| 16  | (2-Amino-ethyl)pyridine       | 17        | 15         | 8.16       |
| 17  | 1-(2-Aminoethyl)-piperazine   | 7.4       | 2.30       | 4.37       |
| 18  | 4-(2-Aminoethyl)-morpholine   | 0.19      | 7.39       | 2.43       |

$^a$Mean from three determinations by a stopped-flow CO2 hydrase method. Standard errors were in the range of 5–10% of the reported values (data not shown).

$^b$Human recombinant isozymes, stopped flow CO2 hydrase assay method.

$^d$Diatom enzyme, this work.

4. Conclusions
The first activation study of a δ-class CA is reported. The most effective TweCAα activator was δ-Tyr, with an activation constant of 51 nM, whereas several other amino acids and amines, such as

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**Figure 1.** Amino acids 1–11 and amines 12–19 investigated as TweCAα activators.
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