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Carbonic anhydrase activators: Activation of the β-carbonic anhydrases from the pathogenic fungi *Candida albicans* and *Cryptococcus neoformans* with amines and amino acids

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ABSTRACT

The proteins encoded by the *Nce103* genes of *Candida albicans* and *Cryptococcus neoformans* are catalytically active β-carbonic anhydrases (CAs, EC 4.2.1.1) playing various roles in the life cycle of these fungal pathogens, such as CO_2 sensing, regulation of capsule biosynthesis, filamentation, and adaptation of the organism to various pH and CO_2 conditions in various niches where the fungi grow. Here, we report the first activation study of these two enzymes, CaNce103 and Can2, respectively, with amines and amino acids. The *C. albicans* enzyme, CaNce103 was activated by amino acids such as L-/D-His, L-D-Trp, L-Tyr with K_As in the range of 19.5–46 μM. More effective activators were some amines such as histamine, dopamine, 2-aminoethyl-piperazine, and L-adrenaline (K_As of 13.2–18.5 μM). The best CaNce103 activators were L- and D-Dopa, with K_As of 0.96–2.5 μM. The *C. neoformans* enzyme, Can2, showed much lower propensity to be activated by all these amino acids and amines, which had activation constants in the range of 28.7–47.2 μM. The best Can2 activator was L-Trp. This study may help to better understand the catalytic/activation mechanisms of the β-CAs and eventually to design CA activity modulators of such widespread enzymes in pathogenic fungi.

1. Introduction

In previous work from these laboratories we have investigated the activation of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) with various classes of compounds, such as amines, amino acids, oligopeptides, and some of their derivatives.[1–4] Among the five genetically distinct CA families (α, β, γ, δ, and ζ classes), the α-CAs of mammalian (human or murine) origin were the most thoroughly investigated, with all 16 isoforms (CAI–XV) characterized for their activation profile with amines and amino acids.[1–4] Furthermore, we have reported several X-ray crystal structures for adducts of the human isoforms hCAI and II with histamine, L-/D-His, L-/D-Phe, D-Trp, L-adrenaline, and other such activators,[5,6] which allowed an understanding at the molecular level of the CA activation mechanism for the α-class enzymes. However, very recently the activation of CAs belonging to other classes, such as the β- and γ-CAs have started to be investigated.[7,8] Indeed, we have reported the first activation study of Cab (a β-CA from the archaeon *Methanobacterium thermoautotrophicum*)[7] and Cam (a γ-CA from another archaeon, *Methanosarcina thermophila*)[7] with amines and amino acids. More recently we have also investigated the activation of the β-CA from the yeast *Saccharomyces cerevisiae* with the same set of compounds.[8] These are the only data available in the literature regarding the activation of β- and γ-CAs, even though these enzymes are widespread in the phylogenetic tree.[9,10] The recent characterization of CAs in fungi[11–14] led us to initiate a systematic search of modulators of these enzymes activity, that is, inhibitors and activators, which might show therapeutic potential.[15,16]

Cryptococcus neoformans contains two β-CAs, Can1 and Can2, which share homology to the *S. cerevisiae* gene *NCE103*.[8,13,14] *CAN2* is expressed at high levels, while low expression levels of *CAN1* can only be detected by RT-PCR, suggesting that *CAN2* is the major CA isoform in *C. neoformans*.[13,14] This was further confirmed in disruption mutants where mutation of Can1 failed to produce any observable phenotype, while Can2 was shown to be essential for growth in CO_2 limiting atmospheres.[13,14] One of the major virulence determinates of *C. neoformans* is its ability to synthesize a capsule, protecting itself from the host's immune system.[13,14] Capsule biosynthesis is up-regulated upon exposure to environments with high CO_2 concentrations and Can2 together with a soluble adenylyl cyclase (sAC) were shown to be essential for capsule biosynthesis (Fig. 1). Indeed, Can2 is required to produce bicarbonate for increasing intracellular cAMP concentrations, through direct interaction with adenylyl cyclase, which is essential

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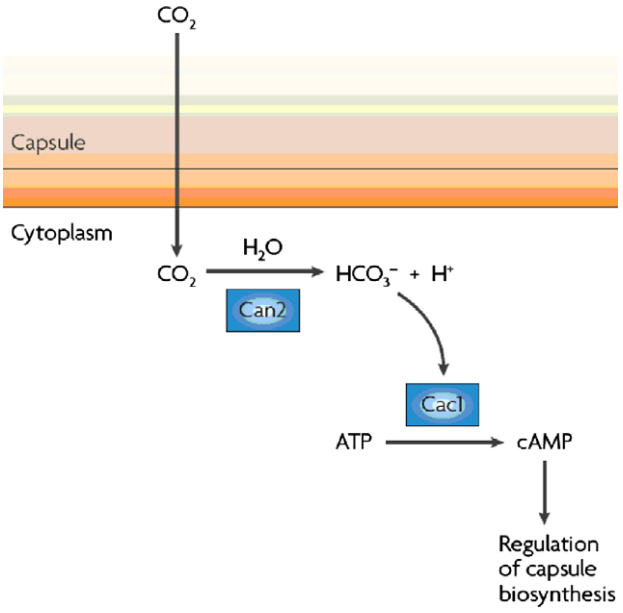


Figure 1. Regulation of capsule biosynthesis in *C. neoformans*, in the presence of the β-CA Can2 and the soluble adenylyl cyclase Cac1.

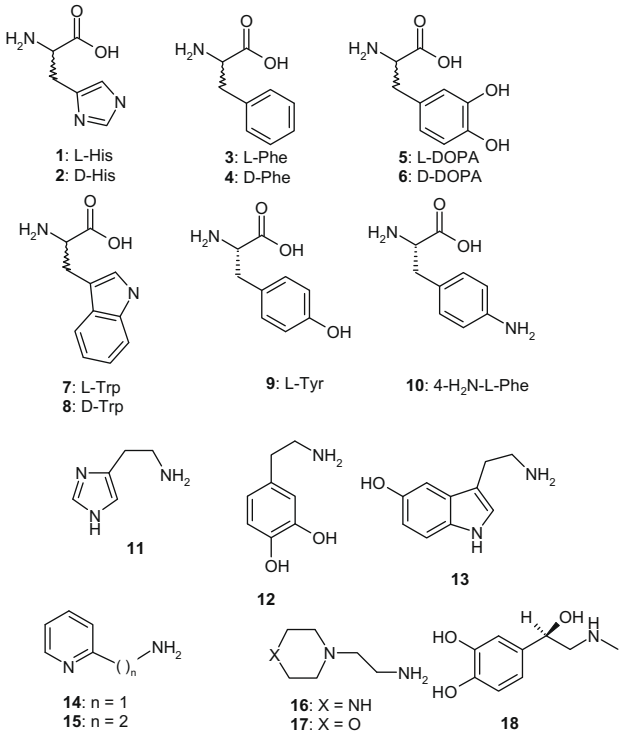
for spore formation.[13,14] The involvement of this enzyme in essential pathogenesis traits has led to significant studies on the activity and inhibition profile of Can2, as well as structural studies.[12,15,16]

CO_2 is also a strong promoter of filamentation in *Candida albicans*, a ubiquitous pathogenic fungus/yeast in humans.[17] In systemic infections *C. albicans* disseminates into the blood stream. Here, in addition to serum, *C. albicans* is exposed to high concentrations of CO_2 (5.5%). This phenomenon has been dubbed 'CO_2 sensing' and has opened up a wide and expansive new field in *C. albicans* research.[18] Attempts to characterize the CO_2 sensing pathway have so far highlighted two proteins that play a significant role.[14,18] Again, the first is an adenylyl cyclase which produces the secondary messenger, cAMP, which is required for filamentation in *C. albicans*.[19] The sAC is directly activated by CO_2/HCO_3^-.[14] The second protein is the β-carbonic anhydrase, Nce103p, designated here as CaNce103. CaNce103 was identified through its similarity to *S. cerevisiae* Nce103p from global transcriptional approaches. *NCE103* is significantly up-regulated upon immediate adhesion to surfaces and during the formation of biofilms.[20,21] The exact function of *NCE103* in biofilm formation in *C. albicans* has not been fully investigated but it may be required to provide bicarbonate ions for intermediate metabolism.[12a] Identifying and comparing how each of the CAs from different fungal species are regulated, could be an important aim for future research.

Here, we report the first activation study of CaNce103 and Can2 with a series of amines and amino acids (of types **1**–**18**), investigated earlier[10–14] for their interaction with mammalian α-CAs as well as very recently[15] with the β- and γ-class enzymes from the *Archaea* domain and the common yeast. Such a study may help a better understanding of the β-CA catalytic/activation mechanism (the natural proton shuttling residue in this class of enzymes has not been yet identified), as well as the design of CAAs targeting β-CAs from these or other pathogenic fungi.

2. Results and discussion

The rate-determining step in the CA catalytic cycle for CO_2 hydration to bicarbonate is the formation of the zinc hydroxide species of the enzyme.[1–4] This involves the transfer of a proton from a Zn(II)-coordinated water molecule to the environment,



which can be assisted by amino acid residues from the enzyme active site (such as His64 in CA II, IV, VII, IX, XIII, XIII, and XIV) or by an activator molecule bound within the cavity.[1–4] Such phenomena are now well understood for the α-CAs (with many X-ray crystal structures of enzyme–activator adducts available)[1–6] but are not yet fully elucidated for enzymes belonging to other classes, such as the β- and γ-CAs. We recently showed that the activation profile of the β-CA from *M. thermoautotrophicum* (Cab) and the γ-class enzyme from *M. thermophila* (Cam) with a series of amine and amino acids is very different as compared to those of the mammalian α-CAs,[7] but seems to operate via the same mechanism of action, that is, the activator bound within the enzyme active site facilitates the shuttling of protons between the Zn(II) ion-coordinated water molecule and the environment, with generation of the nucleophilic zinc hydroxide, catalytically active species of the enzymes.[7] The same seems to be true for the *S. cerevisiae* β-CA (ScCA) encoded by the *Nce103* gene.[8,21]

Similarly to other CAs belonging to the α- or β-class, the fungal enzymes CaNce103 and Can2 possess appreciable CO_2 hydrase activity (Table 1).[15a] Data of Table 1[22] show that histamine, Hst (at 10 μM concentration), which is an effective CAA for all these enzymes except hCA II (for which it is a weak activator),[1] enhances k_{cat} values for all these enzymes, whereas K_M remains unchanged. Hst is a millimolar activator for the α-class enzyme (hCA II), with K_A of 125 μM,[11–14] being a more effective, micromolar one for the archaeal one Cab (K_A of 76 μM) and a better activator for the yeast/fungal enzyme investigated here (K_As of 18.4–33.2 μM, see discussion later in the text). It is thus obvious that the activation mechanism of the α- and β-CAs (of archaeal, yeast or fungal origin) seems to be similar, that is, the activator enhances k_{cat} with no influence on K_M, facilitating the release of the proton from the water coordinated to the catalytic zinc ion.

Data of Table 2 present the activation constants for a set of amino acids and amines **1**–**18** against the two fungal enzymes CaNce103 and Can2 (activation of the α-class enzymes hCA II and the β-one Cab, investigated earlier[1,7] are included in Table 2 for comparison reasons). The following structure–activity relationship (SAR) can be observed for the activation of the fungal CAs with compounds **1**–**18**:

Table 1
Kinetic parameters for the activation of human (hCA) isozyme II, Cab, ScCA and CaNce103 and Can2 with histamine (Hst), measured at 25 °C, pH 8.3 in 20 mM Tris buffer and 20 mM $NaClO_4$, for the CO_2 hydration reaction[22]

Isozyme	k_{cat}* (s^{-1})	K_M* (mM)	$(k_{cat})_{Hst}$** (s^{-1})	K_A*** (μM) Hst
hCA II[a]	1.4×10^6	9.3	2.0×10^6	125
Cab[b]	3.1×10^4	1.7	4.5×10^4	76
ScCA[c]	9.4×10^5	9.5	19.6×10^5	20.4
CaNce 103[d]	8.0×10^5	8.2	20.1×10^5	18.4
Can2[d]	3.9×10^5	9.0	6.8×10^5	33.2

* 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. Mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[22] Standard errors were in the range of 5–10% of the reported values.
[a] Human recombinant enzyme, data from Ref. 1.
[b] Archaeal recombinant enzyme, data from Ref. 7.
[c] Yeast recombinant enzyme, from Ref. 8.
[d] Fungal recombinant enzymes, this work.

(i) Against the *C. albicans* enzyme, CaNce103, the first group of compounds, including: L-His **1**, D-Trp **8**, L-Tyr **9**, 4-amino-L-Phe **10**, serotonin **13**, the pyridyl-alkylamines **14** and **15**, as well as the morpholine derivative **17**, showed medium potency as CAAs, with activation constants in the range of 24.1–46.1 μM. On the other hand, structurally similar compounds (or even enantiomers of some of these amino acid derivatives), such as D-His **2**, L-Phe **3**, L-Trp **7**, histamine **11**, dopamine **12**, the piperazine **16**, and L-adrenaline **18**, showed a better affinity for the *C. albicans* enzyme, with K_As in the range of 13.2–19.5 μM (Table 2). As for other CAs investigated earlier, small structural changes in the activator molecule led to very different activating properties. For example, L-Trp is a 2.2 times better activator compared to its enantiomer D-Trp.

Table 2
Activation constants of hCA II (cytosolic α-isozyme), Cab (archaeal β-CA) and fungal β-CAs from *C. albicans* (CaNce 103) and *C. neoformans* (Can2) with amino acids and amines **1**–**18**

No.	Compound	K_A (μM)*			
		hCA II[a]	Cab[b]	CaNce 103[c]	Can2[c]
1	L-His	10.9	69	24.1	45.0
2	D-His	43	57	19.5	47.2
3	L-Phe	0.013	70	15.5	44.1
4	D-Phe	0.035	10.3	8.4	45.2
5	L-DOPA	11.4	11.4	0.96	43.3
6	D-DOPA	7.8	15.6	2.5	35.1
7	L-Trp	27	16.9	19.2	28.7
8	D-Trp	12	41	43.0	42.1
9	L-Tyr	0.011	10.5	46.1	29.5
10	4-H_2N-L-Phe	0.15	89	23.7	30.4
11	Histamine	125	76	18.4	33.2
12	Dopamine	9.2	51	18.5	34.6
13	Serotonin	50	62	28.6	46.7
14	2-Pyridyl-methylamine	34	18.7	29.1	47.0
15	2-(2-Aminoethyl)pyridine	15	40	30.2	46.3
16	1-(2-Aminoethyl)-piperazine	2.3	13.8	17.3	44.9
17	4-(2-Aminoethyl)-morpholine	0.19	18.5	25.4	40.1
18	L-Adrenaline	96	11.5	13.2	32.8

Data for hCA II and Cab activation with these compounds are from Ref. 15.
* Mean from three determinations by a stopped-flow, CO_2 hydrase method.[22] Standard errors were in the range of 5–10% of the reported values.
[a] Human recombinant isozyme, from Ref. 15.
[b] Recombinant archaeal enzyme, from Ref. 15.
[c] Recombinant fungal enzyme, this study.

The difference in activity between L-Phe, L-Tyr, and L-DOPA (which progressively contain more OH groups on the phenyl ring of Phe) is even more dramatic, with L-DOPA activating CAs 16.1 and 48 times more than L-Phe and L-Tyr, respectively. Indeed, the best activators among the investigated compounds are D-Phe **4**, L-DOPA **5**, and D-DOPA **6**, with activation constants of 0.96–8.4 μM (Table 2). It should be also noted that L-DOPA **5** is 2.6 times a better CaNce103 activator compared to its D-enantiomer **6**. The absence of the COOH moiety of **5**/**6** (as in dopamine **12**) also leads to a diminished activating property of the amine, which is 22 times less efficient compared to L-DOPA and 7.4 times less efficient than D-DOPA as a CA activator. Thus, the SAR in this small set of activators is rather complex and as this enzyme has not yet been crystallized (alone or complexed with inhibitors/activators) it is also difficult to hypothesize how such minor structural changes affect potency of the activator. However, one must note that many of the amines/amino acids investigated here are present in rather high amounts in body fluids[1–3] in which *C. albicans* is present as a commensal or as an opportunistic pathogen, warranting further studies for understanding whether its CA activation may have physiologic/pathological significance.

(ii) For the *C. neoformans* enzyme, Can2, a much more flat SAR has been observed with the investigated activators **1**–**18**, compared to its *C. albicans* orthologue discussed above (Table 2). Thus, Can2 was activated by all compounds **1**–**18** with medium efficacy, with activation constants in the range of 28.7–47.2 μM. The best activators were L-Trp **7**, L-Tyr **9**, and its amino analogue **10** (K_As of 29.5–30.4 μM). Smaller differences of activity were observed in this case for enantiomers of the same amino acid (e.g., L- and D-His, or L- and D-Phe), compared to the same activator pairs for CaNce103 (or other CA isoforms, such as hCA II or Cab, shown for comparison reasons in Table 2). However, this is not true for L- and D-Trp, with the first amino acid being 1.46 times a better Can2 activator compared to the second one. Amines **11**–**18** showed after all comparable Can2 activating properties with amino acids **1**–**10** (Table 2).

(iii) The activation profile of amines and amino acids **1**–**18** against the two fungal enzymes are very different between them, and also in comparison with other α- and β-CAs investigated earlier. For example, L-DOPA is a submicromolar inhibitor of CaNce103 (K_A of 0.96 μM) having a worse activating effect on both hCA II as well as the β-class enzymes Cab and Can2 (K_As of 11.4–43.3 μM).
D-Phe, for example, is a low nanomolar activator of hCA II (K_A of 13 nM) but has micromolar affinity for Cab, CANce103, and Can2 (K_As of 8.4–45.2 μM).

3. Conclusions

In conclusion, we report the first activation study of the β-CA from the fungal pathogens *C. albicans* and *C. neoformans* with amines and amino acids. The two enzymes play various roles in the life cycle of these fungal pathogens, such as CO_2 sensing, regulation of capsule biosynthesis, filamentation, adaptation of the organism to various pH and CO_2 conditions in various niches where the fungi grow. The *C. albicans* enzyme, CaNce103 was activated by amino acids such as L-/D-His, L-D-Trp, L-Tyr with K_As in the range of 19.5–46 μM. More effective activators were some amines such as histamine, dopamine, 2-aminoethyl-piperazine, and L-adrenaline (K_As of 13.2–18.5 μM). The best CaNce103 activators were L- and D-Dopa, with K_As of 0.96–2.5 μM. The *C. neoformans* enzyme, Can2, showed much lower propensity to be activated by all these amino acids and amines, which had activation constants in the range of 28.7–47.2 μM, with the best Can2 activator being L-Trp.

This study may help to better understand the catalytic/activation mechanisms of the β-CAs and eventually to design CA activity modulators of such widespread enzymes in pathogenic fungi.

4. Experimental

4.1. Chemistry

Compounds **1**–**18** are commercially available, highest pure derivatives from Sigma–Aldrich (Milan, Italy) and were used without further purification.

4.2. CA activation assay

zAn Applied Photophysics stopped-flow instrument was used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, 10–20 mM Hepes (pH 7.5) or Tris (pH 8.3) as buffers, and 20 mM Na_2SO_4 or 20 mM $NaClO_4$ (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s at 25 °C. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation 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 activators **1**–**18** (10 mM) were prepared in distilled–deionized water and dilutions up to 0.001 μM 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,[1–3] can 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:[10–14]

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

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[1–4] Can2 and Nce103 were recombinant enzymes obtained as reported earlier by our groups.[12,15]

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