Carbonic anhydrase activators: Activation of the β-carbonic anhydrase Nce103 from the yeast *Saccharomyces cerevisiae* with amines and amino acids

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ABSTRACT

The protein encoded by the Nce103 gene of *Saccharomyces cerevisiae*, a β-carbonic anhydrase (CA, EC 4.2.1.1) designated as scCA, was investigated for its activation with amines and amino acids. scCA was poorly activated by amino acids such as L-/D-His, Phe, DOPA, Trp (K_As of 82–90 μM) and more effectively activated by amines such as histamine, dopamine, serotonin, pyridyl-alkylamines, aminoethyl-piperazine/morpholine (K_As of 10.2–21.3 μM). The best activator was L-adrenaline, with an activation constant of 0.95 μM. This study may help to better understand the catalytic/activation mechanisms of the β-CAs and eventually to design modulators of CA activity for similar enzymes present in pathogenic fungi, such as *Candida albicans* and *Cryptococcus neoformans*.

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Modulation of the enzyme activity of carbonic anhydrases (CAs, EC 4.2.1.1) represents a means of therapeutic intervention[1–3] for a variety of diseases due to the fact that these metalloenzymes are involved in critical physiologic processes in organisms all over the phylogenetic tree, from bacteria and archaea to plants, fungi, and animals.[1–5] Indeed, there are five independently-evolved (α, β, γ, δ, and ζ) classes of CAs reported up to date, of which the α-class from mammalian sources has been studied to a far greater extent than the other four classes.[1–3] Yet, CAs other than those belonging to the α-class are widely distributed in nature, with the β-CAs being the most abundant such catalysts for the interconversion between carbon dioxide and the bicarbonate ions.[1,2,5] Recent work has shown that various CAs are widespread in metabolically diverse species from both the *Archaea* and *Bacteria* but also in microscopic eukaryotes, such as yeast or pathogenic fungi, indicating that these enzymes have a more extensive and fundamental role than originally recognized.[1,6,7]

Whereas inhibition of CAs was investigated in great detail (again, mainly for the α-CAs from mammals)[1,3,8,9] with several CA inhibitors (CAIs) in clinical use as diuretics, antiglaucoma, anti-obesity or anticancer agents/diagnostic tools,[1,3,8] CA activators (CAAs) received less attention and only in the last 10 years this class of enzyme modulators started to be investigated systematically for their interaction with mammalian α-CAs.[10] Indeed, our group reported several kinetic and X-ray crystallographic studies regarding the interaction of all mammalian isoforms (CA I–XIV) with amino acid and amine types of activators, also unraveling the activation mechanism of these enzymes.[10–14]
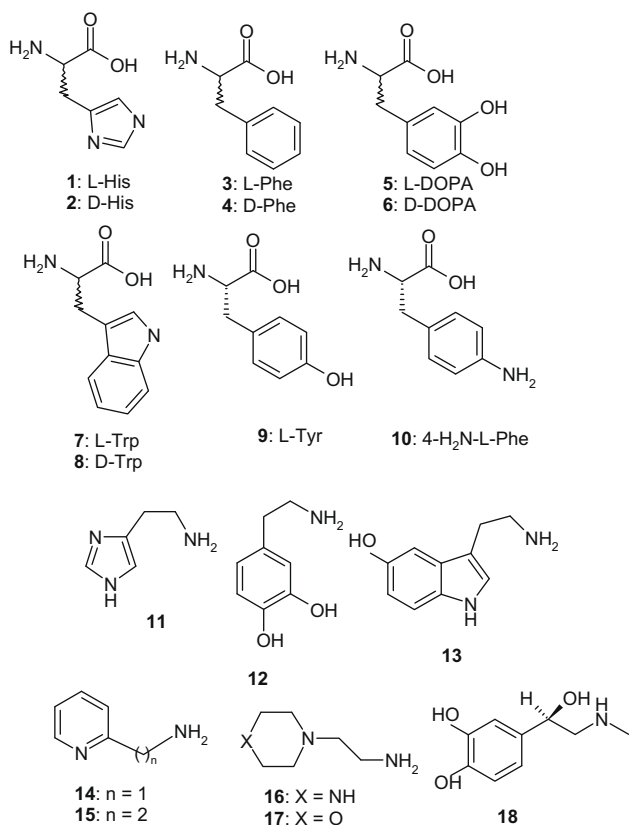
The rate-determining step in the CA catalytic cycle for CO_2 hydration to bicarbonate is the formation of the zinc hydroxide species of the enzyme.[1–5] This involves the transfer of a proton from a Zn(II)-coordinated water molecule to the environment, which can be assisted by amino acid residues from the enzyme active site (such as His64 in CA II, IV, VII, IX, XII, XIII, and XIV)[10] or by an activator molecule bound within the cavity.[10–14] Such phenomena are now well understood for the α-CAs (with many X-ray crystal structures of enzyme-activator adducts available)[10–14] but started to be investigated only recently for enzymes belonging to the β- and γ-classes. Indeed, recently a first activation study of the β- and γ-CAs from some Archaea was reported by this group.[15] The activation profile of the β-CA from *Methanobacterium thermoautotrophicum* (Cab) and the γ-class enzyme from *Methanosarcina thermophila* (Cam) with a series of amine and amino acids is very

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different as compared to those of the mammalian α-CAs,[15] 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.[15]

Recently we have cloned and characterized kinetically a β-CA encoded by the Nce103 gene of the yeast *Saccharomyces cerevisiae*, denominated scCA.[16] Its inhibition with inorganic metal-complexing anions and sulfonamides has also been investigated.[16] Indeed, *S. cerevisiae*, one of the most studied budding yeasts and a widely used model of eukaryotic organisms has a genome comprising 6275 genes condensed into 16 chromosomes, which was completely sequenced in 1996.[17] The gene Nce103 (from non-classical export), was originally reported by Cleves et al. to encode for a protein involved in a non-classical protein secretion pathway.[18] Subsequently, it has been shown by several groups that this protein is a β-CA required to provide sufficient bicarbonate for essential metabolic carboxylation reactions of the yeast metabolism, such as those catalyzed by pyruvate carboxylase (PC), acetyl-CoA carboxylase (ACC), carbamoyl phosphate synthase (CPSase) and phosphoribosylaminoimidazole (AIR) carboxylase.[19,20]

1: L-His
2: D-His
3: L-Phe
4: D-Phe
5: L-DOPA
6: D-DOPA
7: L-Trp
8: D-Trp
9: L-Tyr
10: 4-H$_2$N-L-Phe
11
12
13
14: n = 1
15: n = 2
16: X = NH
17: X = O
18

Here we report the first activation study of scCA 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. 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 other β-CAs from pathogenic organisms, such as the closely related enzymes from *Candida albicans* (Nce103, that is, encoded by the orthologue gene of *S. cerevisiae* present in the path-

ogenic fungus) and *Cryptococcus neoformans* (Can2).[21] The cloning, kinetic characterization and inhibition with anions and sulfonamides of these enzymes present in pathogenic fungi were recently reported by this group.[21]

scCA has been overexpressed[16] in *Escherichia coli* and purified by an original procedure leading to high amounts of pure protein possessing a good enzyme activity for the physiological reaction, that is, CO$_2$ hydration to bicarbonate. Similarly to other CAs belonging to the α- or β-class, the yeast CAs enzyme scCA possesses appreciable CO$_2$ hydrase activity, with a k_{cat} of 9.4×10^5 s^{-1}, and k_{cat}/K_m of 9.8×10^7 M^{-1} s^{-1}.[16] Data of Table 1[22] show that histamine, Hsn (at 10 μM concentration), which is an effective CAA for scCA (see later in the text) and a less effective one for Cab and hCA II,[15] enhances k_{cat} values for all these enzymes, whereas K_M remains unchanged. Hsn is a micromolar 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 yeast enzyme scCA investigated here (K_A of 20.4 μM, see discussion later in the text). It is thus obvious that the activation mechanism of the α- and β-CAs seems to be similar, that is, the activator enhances k_{cat} with no influence on K_M, facilitating thus the release of the proton from water coordinated to the catalytic zinc ion.

Data of Table 2 show that all amino acids and amines **1–18** investigated here act as CAAa against the yeast enzyme scCA, but with very different potencies (activation of the α-class enzymes hCA II and the β-one Cab, investigated earlier[10–15] are included in Table 2 for comparison reasons). The following structure activity relationship (SAR) can be observed for the activation of these CAs with compounds **1–18**:

(i) scCA[23] was activated rather inefficiently by amino acids **1–9**, which showed activation constants[22] in the range of 82–90 μM. It may be observed that all these aromatic/heterocyclic amino acids show a rather flat SAR, being weak scCA activators, whereas some of them are much more effective Cab (D-Pe, L-DOPA, D-DOPA and L-Trp) or hCA II (L- and D-Phe, L- and D-DOPA, L-Tyr) activators (Table 2). The enantiomeric form (L- or D-) as well as the substitution pattern (in β to the carboxyl group) of these amino acid derivative were non-influential to their activating efficacy.

(ii) A second group of derivatives, including 4-amino-phenylalanine **10**, amines **11–15** and **17**, showed more effective scCA activating power as compared to the previously discussed compounds, with K_As in the range of 10.2–21.3 μM. The main difference between amino acid **10** and derivatives **1–9** mentioned above, is the presence of the supplementary

Table 1
Kinetic parameters for the activation of human (hCA) isozyme II, Cab and scCA 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

* 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.[22] 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. 10.
  [b] Archaeal recombinant enzyme, data from Ref. 15.
  [c] Yeast recombinant enzyme.

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Table 2

Activation constants of hCA II (cytosolic α-isozyme), Cab (archaeal β-CA) and yeast β-CA from *S. cerevisiae* (scCA) with amino acids and amines **1–18**. Data for hCA II and Cab activation with these compounds are from Ref. 15

No.	Compound	K_A (μM)[*]		
		hCA II[a]	Cab[b]	scCA[c]
1	L-His	10.9	69	82
2	D-His	43	57	85
3	L-Phe	0.013	70	86
4	D-Phe	0.035	10.3	86
5	L-DOPA	11.4	11.4	90
6	D-DOPA	7.8	15.6	89
7	L-Trp	27	16.9	91
8	D-Trp	12	41	90
9	L-Tyr	0.011	10.5	85
10	4-H₂N-L-Phe	0.15	89	21.3
11	Histamine	125	76	20.4
12	Dopamine	9.2	51	13.1
13	Serotonin	50	62	15.0
14	2-Pyridyl-methylamine	34	18.7	16.2
15	2-(2-Aminoethyl)pyridine	15	40	11.2
16	1-(2-Aminoethyl)-piperazine	2.3	13.8	9.3
17	4-(2-Aminoethyl)-morpholine	0.19	18.5	10.2
18	L-Adrenaline	96	11.5	0.95

[*] 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 yeast enzyme, this study.[23,24]

amino moiety in **10**, attached to the aromatic ring, whereas derivatives **1–9** possess only the aliphatic amine moiety. On the other hand, histamine **11** is quite structurally similar to **1** and **2**, from which it can be formed by a decarboxylation reaction. The same is true for dopamine **12** and L-/D-DOPA **5** and **6**. It may be observed that the biogenic amines **11** and **12** are around 4.1–6.9 times more effective scCA activators as compared to the structurally related amino acids **1/2** and **5/6**, respectively. The pyridyl-alkylamino derivatives **14** and **15** are also effective scCA activators, with the amino-ethyl derivative **15** being slightly more effective than the aminomethyl one **14**. It is thus clear that minor structural variations in the scaffold of an amine/amino acid, strongly influence their interaction with scCA active site and as a consequence, their activation properties. It is also obvious from data of Table 2 that the activation profiles of hCA II and Cab with these compounds is quite different from those of scCA. Generally amines **10–18** act as effective scCA activators being less effective as hCA II or Cab activators. The reverse is true for many amino acid derivatives **1–9**, as mentioned above.

(iii) The most potent scCA activators were the piperazine derivative **16** (K_A of 9.3 μM) and L-adrenaline **18**, the only compound showing a submicromolar activation constant (K_A of 0.95 μM). Whereas **16** is a medium potency activator of Cab and an efficient hCA II activator, L-adrenaline **18** shows very weak hCA II activating properties (K_A of 96 μM) being a more effective Cab activator. The weak hCA II activating properties of L-adrenaline were explained by us after the report of the X-ray crystal structure of the hCA II–**18** adduct,[14a] in which the activator was seen bound in an unexpected region of the active site, plugging the entrance to it, and being unable to favorably shuttle protons, unlike histamine,[10b] L-/D-His[13c] or L-/D-Phe,[11e] which bind in different regions of the CA II active site and actively participate to the transfer of protons between the active site and the environment. Thus, L-adrenaline may be considered a potent and also rather selective scCA activator. At this moment it is

unclear whether activation of scCA with this type of compounds may have physiological relevance but studies in this field are clearly warranted. Considering the fact that scCA is involved in carboxylation reactions of the yeast metabolism,[19,20] it is possible that its activation may have relevant consequences for the growth of *S. cerevisiae* and might be exploited biotechnologically.

A possible activation mechanism of the β-CAs is depicted schematically in Figure 1. As for other fungal β-CAs, the catalytic Zn(II) ion in the scCA active site is coordinated to residues Cys106, His161 and Cys164 (Nce103 of *C. albicans* numbering system).[16,21] A second pair of conserved amino acid residues in all sequenced β-CAs known to date,[2,16,21] is constituted by the dyad Asp108–Arg110 (Nce103 of *C. albicans* numbering, Fig. 1). These amino acids are close[21] to the zinc-bound water molecule, which is the fourth Zn(II) ligand in this type of open active site β-CAs,[21] participating in a network of hydrogen bonds with it, which probably assist water deprotonation and formation of the nucleophilic, zinc hydroxide species of the enzyme. The active site channel of β-CAs (as exemplified by the recently determined X-ray crystal structure of the *C. neoformans* enzyme Can2)[21b] is a channel which can accommodate elongated molecules such as the aromatic amino acids/amines investigated here. Thus, we hypothesize that the activators bind nearby the pocket defined by Asp108/Arg110, establishing supplementary hydrogen bonds with the polar moieties of these amino acids or with the zinc-bound water molecule (directly or through a relay of several other water molecules, as demonstrated for the interaction of α-CAs with this type of activator)[10–14] assisting thus water deprotonation and facilitating the catalytic turnover. Indeed, both the amino or carboxyl moieties of these activators can establish hydrogen bonds with these structural elements, due to the presence of many heteroatoms in their molecules. Figure 1 shows schematically a putative binding mode of L-adrenaline **18** within the active site of scCA. This hypothesis should be checked by X-ray crystallography, but the structure of scCA is not yet reported.

In conclusion, we report the first activation study of the β-CA from the yeast *S. cerevisiae* with amines and amino acids. scCA was poorly activated by amino acids such as L-/D-His, Phe, DOPA, Trp (K_As of 82–90 μM) and more effectively activated by amines such as histamine, dopamine, serotonin, pyridyl-alkylamines, aminoethyl-piperazine/morholine (K_As of 10.2–21.3 μM). The best activator was L-adrenaline, with an activation constant of
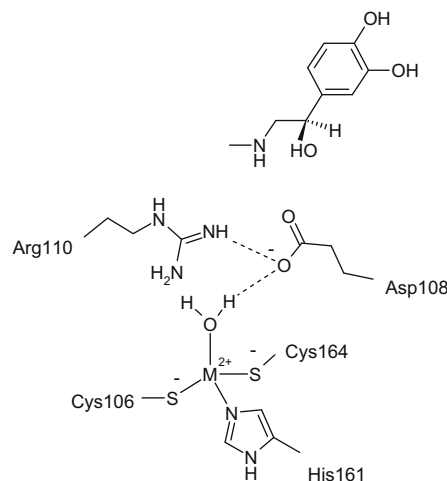

Figure 1. Proposed schematic interactions between an activator (L-adrenaline **18**) and the scCA active site.

0.95 µM. This study may help to better understand the catalytic/activation mechanisms of the β-CAs and eventually to design modulators of CA activity for similar enzymes present in pathogenic fungi, such as *C. albicans* and *C. neoformans*.

Acknowledgments

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22. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An 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:

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

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]

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

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[10–14]
23. Yeast genomic DNA was isolated using the Johnston's procedure (Johnston, J. R. *Molecular Genetics of Yeast*; Oxford University Press: New York, 1994). The Nce103 gene was amplified from genomic DNA by PCR based strategies using the following oligonucleotides; NCE103ORF-for (5′-AGGATCCATGAGCGCTACCGAA-3′) and NCE103ORF-rev (5′-AGAGCTCCTATTTTGGGGTAAC-3′). PCR conditions were: 94 °C for 2 min, 35 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min and a final step of 72 °C for 10 min. The amplified band containing Nce103 ORF was inserted into the pGEM-T (PROMEGA) vector with T:A strategy.[24] Automated sequencing of the clone was performed in order to confirm the gene and the integrity of amplified gene. The construct was then excised with BamH I and Sac I restriction enzymes and subcloned into pET21a(+) expression vector. The vectors were transformed into *E. coli* BL21 (DE3) competent cells. The enzyme was purified as reported earlier[16].
24. Promega Technical Manual 'pGEM-T and pGEM-T Easy Vector Systems', available at www.promega.com.