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Carbonic anhydrase inhibitors. Inhibition of the β-class enzyme from the yeast *Saccharomyces cerevisiae* with anions

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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, has been cloned, purified, characterized kinetically, and investigated for its inhibition with a series simple, inorganic anions such as halogenides, pseudohalogenides, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, perchlorate, sulfate, and some of its isosteric species. The enzyme showed high CO_2 hydrase activity, with a k_{cat} of $9.4 \times 10^5\ s^{-1}$ and k_{cat}/K_m of $9.8 \times 10^7\ M^{-1}\ s^{-1}$. scCA was weakly inhibited by metal poisons (cyanide, azide, cyanate, thiocyanate, K_Is of 16.8–55.6 mM) and strongly inhibited by bromide, iodide, and sulfamide (K_Is of 8.7–10.8 μM). The other investigated anions showed inhibition constants in the low millimolar range.

In a preceding letter,[1] we have reported the cloning, purification, kinetic properties, and inhibition by simple anions of two β-carbonic anhydrases (CAs, EC 4.2.1.1) from the fungal pathogens *Candida albicans* (denominated Nce103) and *Cryptococcus neoformans* (denominated Can2). Indeed, there are five independently-evolved (α, β, γ, δ, and ξ) 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.[2–5] Yet, CAs other than 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.[1–5] Although ubiquitous in highly evolved organisms from the Eukarya domain, these enzymes have received scant attention in prokaryotes from the Bacteria and Archaea domains.[1,5,6] 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 pathogenic fungi, indicating that these enzymes have a more extensive and fundamental role than originally recognized.[1–9]

Saccharomyces 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.[10] 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.[11] Subsequently, it has been shown by several groups[12–14] 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.[12–14] Although several transcriptional analysis studies involving Nce103 of *S. cerevisiae* have been reported,[11–14] and the CO_2 hydrase activity of the β-CA encoded by the Nce103 gene has been measured by Amoroso et al.,[13] the kinetic parameters of this enzyme as well as inhibition studies with various classes of inhibitors are missing at this moment in the literature. Indeed, Amoroso et al.[13] measured the activity of scCA by an ^{18}O exchange technique (but no kinetic parameters were provided) and also showed that the enzyme is prone to be inhibited by the sulfonamides acetazolamide and ethoxzolamide (with K_Is in the range of 16–19 μM) as well as by the inorganic anion nitrate (K_I of 0.9 mM). Since the related fungal species *Candida albicans* investigated earlier[1,7–9] also has a β-CA encoded by the Nce103 gene (the ortholog of the *S. cerevisiae* Nce103 gene), the yeast enzyme investigated by us here will be denominated scCA (i.e., the β-CA from *S. cerevisiae*), in order to distinguish

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it from the *C. albicans* β-CA, which has been denominated in earlier publications as Nce103,[1,7–9] and we shall maintain this nomenclature here too.

In this letter, we report a method for the cloning and purification of high enough amounts of scCA[15–17] in order to investigate its kinetic properties for the physiologic reaction (i.e., CO_2 hydration to bicarbonate and protons), as well as its inhibition by anions, known to interact with most metal centers of such metalloenzymes.[1,3,4] The aim of this study is thus to understand the catalytic efficiency of an enzyme essential for the metabolism of *S. cerevisiae*, as it has been demonstrated[14] that scCA provides bicarbonate to carboxylating enzymes such as PC, ACC, CPSase, and AIR, a function similar to that played by CA VA and CA VB in the mammalian cells [3,18] (these last enzymes belong to the α-CA class, unlike scCA which is a β-CA). As a second goal, we also investigate the simplest class of CA inhibitors (CAIs), that is, the inorganic anions,[3] for their interaction with scCA. Several such simple chemical species are fundamental in many physiologic processes and are found in relevant concentrations in many eukaryotic cell compartments (e.g., Cl^-, bicarbonate, sulfate, etc.) whereas others are 'metal poisons' (CN^-, N_3^-, thiocyanate, etc.) and their interaction with this enzyme may shed some light regarding the design of CAIs, with potential biomedical or environmental applications. Furthermore, as the Nce103 gene is also present in many other fungal species,[1,7–9] some of which are pathogenic (e.g., *Candida albicans*, *Candida glabrata*, etc.), this inhibition study may have relevance for designing novel antifungal/anti-yeast therapies.

Although scCA has been cloned and purified earlier,[13] its kinetic parameters for the catalyzed physiological reaction, i.e., CO_2 hydration to bicarbonate and a proton, are not available in literature. Therefore, we performed a detailed kinetic investigation of purified scCA, comparing its kinetic parameters (k_{cat} and k_{cat}/K_m) with those of thoroughly investigated CAs, such as the cytosolic, ubiquitous human isozymes hCA I and II (α-class CAs) as well as Can2 and Nce103, the β-CAs from the pathogenic fungi *C. neoformans* and *C. albicans*, investigated earlier by us[1a] (Table 1).

Data from Table 1 show that 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 $9.4 \times 10^5\ s^{-1}$, and k_{cat}/K_m of $9.8 \times 10^7\ M^{-1}\ s^{-1}$. Data of Table 1 also show that these enzymes are inhibited appreciably by the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), with an inhibition constant of scCA of 82 nM. Thus, our data prove that scCA has an excellent catalytic efficiency for the physiologic reaction, quite similar to that of the ortholog enzyme (Nce103) from *C. albicans*, and that these two β-CAs are better catalysts for CO_2 conversion to bicarbonate than the highly abundant and widspread human isoform hCA I, being only slightly less effective than the most efficient mammalian isozyme, hCA II.[3] Furthermore, scCA has an affinity for the clinically used sulfonamide acetazolamide intermediate between that of very sulfonamide-avid isoforms (hCA II and Can2) and those enzymes with less susceptibility to be inhibited (e.g., hCA I and Nce103).

Table 2 shows the scCA inhibition data with anionic, physiological species (such as chloride, bicarbonate, sulfate, etc.) as well as other non-physiologic anions.[19,20] Here we also include inhibition data for hCA I and II as well as Can2 and Nce103, reported earlier.[1a] This helps to compare the newly generated data with those of the better investigated CAs belonging to the α- and β-CA families. The following should be noted regarding the yeast β-CA inhibition data of Table 2:

(i) scCA was not inhibited by perchlorate, similarly to all other α- and β-CAs investigated up to now, and it was weakly inhibited by cyanate, thiocyanate, cyanide, azide, nitrate, and phenylboronic acid, with inhibition constants in the range of 13.9–55.6 mM. It is very interesting to note that the 'metal poisons' cyanate, thiocyanate, cyanide, and azide, which usually have submicromolar affinity for hCA I (and many other CA isozymes)[3] are ineffective inhibitors of scCA, whereas these same anions show much higher inhibitory activity towards the *C. albicans*-related enzyme, Nce103 (Table 2). It is difficult to rationalize these data since the X-ray crystal structures of these two enzymes are not known for the moment.

(ii) A second group of anions, including fluoride, chloride, bicarbonate, carbonate, nitrite, hydrogen sulfide, bisulfite, sulfate, sulfamate, and phenylarsonic acid show a much better scCA inhibitory activity as compared to the anions mentioned above, with a compact behavior of low millimolar inhibitors (K_Is in the range of 0.33–2.85 mM). Many of these anions showed a similar behavior also towards the *C. albicans* enzyme, Nce103 (e.g., chloride, bicarbonate, nitrite, hydrogen sulfide, bisulfite) whereas others possessed a distinct inhibition profile for the two enzymes. For example, carbonate was 76 times a better Nce103 than scCA inhibitor, whereas sulfate 24.4 times a better scCA than Nce103 inhib-

Table 1
Kinetic parameters for the CO_2 hydration reaction catalyzed by the human cytosolic isozymes hCA I and II (α-class CAs) at 20 °C and pH 7.5 in 10 mM HEPES buffer and 20 mM Na_2SO_4, and the β-CAs Can2 and Nce103 (from *C. neoformans* and *C. albicans*, respectively) and scCA (from *Saccharomyces cerevisiae*) measured at 20 °C, pH 8.3 in 20 mM Tris buffer and 20 mM $NaClO_4$.[17] Inhibition data with the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also provided.

Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1}$)	K_I (acetazolamide) (nM)
hCA I[a]	Moderate	2.0×10^5	5.0×10^7	250
hCA II[a]	Very high	1.4×10^6	1.5×10^8	12
Can2[a]	Moderate	3.9×10^5	4.3×10^7	10.5
Nce103[a]	High	8.0×10^5	9.7×10^7	132
scCA[b]	High	9.4×10^5	9.8×10^7	82

[a] Data from Ref. 1a.
[b] This work.

Table 2
Inhibition constants of anionic inhibitors against isozymes hCA I, and II (α-CA class), and β-isozymes Nce103 (from *Candida albicans*)[1a] and scCA (from *S. cerevisiae*), for the CO_2 hydration reaction, at 20 °C.[19]

Inhibitor	K_I (mM)[b]			
	hCA I	hCA II	Nce103 (*C. albicans*)	scCA
F^-	>300	>300	0.69	2.85
Cl^-	6	200	0.85	0.85
Br^-	4	63	0.94	0.0108
I^-	0.3	26	1.40	0.0103
CNO^-	0.0007	0.03	1.18	31.7
SCN^-	0.2	1.6	0.65	55.6
CN^-	0.0005	0.02	0.011	16.8
N_3^-	0.0012	1.5	0.52	27.9
HCO_3^-	12	85	0.62	0.78
CO_3^{2-}	5	73	0.010	0.76
NO_3^-	7 35	0.69	13.9	
NO_2^-	8.4	63	0.53	0.46
HS^-	0.0006	0.04	0.37	0.33
HSO_3^-	18	89	0.54	0.33
SO_4^{2-}	63	>200	14.15	0.58
ClO_4^-	>200	>200	>200	>200
$H_2NSO_2NH_2$	0.31	1.13	0.30	0.0087
H_2NSO_3H[a]	0.021	0.39	0.70	0.84
Ph-$B(OH)_2$	58.6	23.1	30.85	38.2
Ph-AsO_3H_2[a]	31.7	49.2	30.84	0.40

[a] As sodium salt.
[b] Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[19]

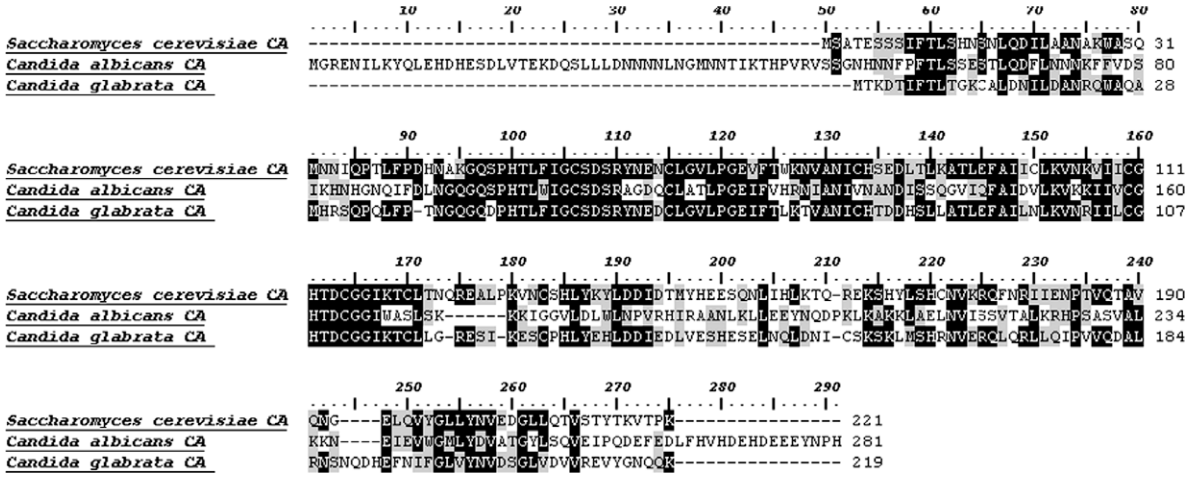

Figure 1. Alignment of scCA, Nce103 (from *C. albicans*) and Nce103 (from. *C. glabrata*) amino acid sequences. The three zinc ligands are conserved in all these three enzymes (Cys106, His161 and Cys164) whereas the other conserved/semiconserved amino acid residues between the three β-CAs are evidenced by black boxes. The two residues Asp108 and Arg110, thought to be involved in the β-CA catalytic cycle[1] are also conserved in the three enzyms (the numbering system used here corresponds to the Nce103 of *C. albicans* amino acid sequence).[7–9]

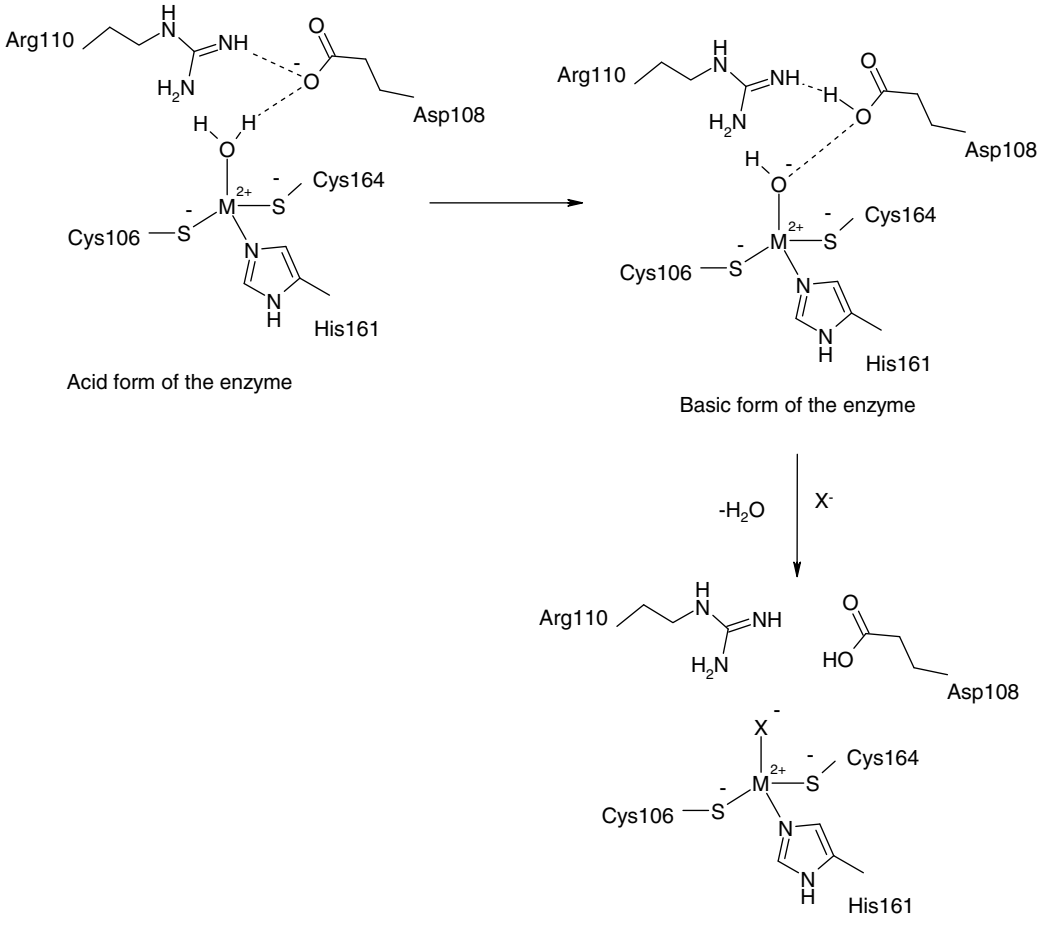

Figure 2. Proposed zinc water activation and inhibition mechanisms of β-CAs (*C. albicans* numbering of amino acid residues). The transfer of the proton from the acidic form of the enzyme (with water coordinated to the zinc ion) is assisted by the conserved dyad Asp108–Arg110, which leads to the catalytically active, nucleophilic species (with hydroxide coordinated to zinc). Inhibitors (X^-) may display either the hydroxide (as depicted above) or the zinc bound water, leading to tetrahedral Zn(II), inhibited forms of the enzyme. The mechanism is supported by the recent X-ray crystal structure of the adduct of Can2 with acetate, in which acetate is bound as depicted for X^- above, as the fourth zinc ligand.[1b]

itor. The same is true for phenylarsonic acid, which is 77 times more potent a scCA than Nce103 inhibitor. All these data reflect the fact that it might probably be possible to detect or design scCA-specific inhibitors.

(iii) Very strong inhibition has been detected for three investigated chemical species, that is, bromide, iodide and sulfamide, which showed K_Is in the range of 8.7–10.8 μM. Thus, it may be observed that for halogenides the inhibitory power increased with the increase of the atomic weight. Sulfamide is on the other hand a particular case, since this compound is an extremely potent inhibitor of only scCA, its affinity for other β- or α-CAs being in the millimolar range. There is also a net difference of inhibitory activity between the isoelectronic and isostructural sulfate, sulfamic acid and sulfamide, with the last compound being 66.6–96.5 times more effective than sulfate and sulfamate against scCA (Table 2). All these data prompt us to propose sulfamide as a potent, scCA rather specific CAI.

In order to try to rationalize the kinetic and inhibition data reported here, an alignment of the amino acid sequences of scCA, Nce103 and the corresponding gene product of *C. glabrata* is shown in Figure 1. We chose these fungal β-CAs for comparison since they are encoded by the same Nce103 (yeast) ortholog genes.[7,8,13,14] Furthermore, the fungal enzyme from *C. albicans* is relatively better investigated as compared to scCA, even if an X-ray crystal structure is not yet available.[1a,7–9]

Data from Figure 1 show that the putative zinc ligands of these fungal β-CAs are all conserved, corresponding to residues Cys106, His161 and Cys164 (Nce103 of *C. albicans* numbering system, see Fig. 1).[7–9] A second pair of conserved amino acid residues in all sequenced β-CAs, known to date,[1,2,6] is constituted by the dyad Asp108–Arg110 (Nce103 of *C. albicans* numbering, Fig. 1). These amino acids are close[1b] to the zinc-bound water molecule, which is the fourth zinc ligand in this type of open active site β-CAs, participating in a network of hydrogen bonds with it, which probably assists water deprotonation and formation of the nucleophilic, zinc hydroxide species of the enzyme (Figure 2). Indeed, in β-CAs, unlike the α-class enzymes, the formal zinc charge is zero (the two cysteinates ligands 'neutralize' the +2 charge of the zinc ion), and as a consequence the activation of the zinc-coordinated water molecule (for the hydration of CO_2 to bicarbonate, Fig. 2) needs the assistance of additional amino acids. The pair Asp108–Arg110 probably has this activation function, as it is conserved in all β-CAs.[1,2,6,21] As a consequence, the catalytic water molecule is activated both by the metal ion (as in metalloproteases[22] and α-CAs[1,23]), but also by an aspartic acid residue, as in aspartic proteases.[24] This particular mechanism makes the β-CAs, including scCA, very different as compared to all other known enzyme classes involved in hydrolytic or hydration processes. Furthermore, strong scCA inhibitors, such as heavy halides (X^-, X = Br, I), probably bind to the metal ion within the enzyme active site as depicted schematically in Figure 2.

In conclusion, we investigated the catalytic activity and inhibition of the β-CAs from the yeast *S. cerevisiae* (encoded by the Nce103 gene) with simple inorganic anions such as halogenides, pseudohalogenides, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, perchlorate, sulfate, and some of its isosteric species. The enzyme showed high 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}. scCA was weakly inhibited by metal poisons (cyanide, azide, cyanate, thiocyanate, K_Is of 16.8–55.6 mM) and strongly inhibited by bromide, iodide, and sulfamide (K_Is of 8.7–10.8 μM). The other investigated anions showed inhibition constants in the low millimolar range.

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19. Khalifah, R.G. *J. Biol. Chem.* **1971**, *246*, 2561. An applied photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 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 initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor 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 inhibitor (100 mM) were prepared in distilled-deionized water and dilutions up to 0.01 μM were done thereafter with distilled-deionized water. Inhibitor 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-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier,[1a] and represent the mean from at least three different determinations.
20. Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, hydrogen sulfite, sulfate, and perchlorate) were of highest purity available, and were used without further purification. Sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid were from Sigma–Aldrich.
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