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Carbonic anhydrase inhibitors: Inhibition of the β-class enzyme from the yeast *Saccharomyces cerevisiae* with sulfonamides and sulfamates

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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 of sulfonamides and one sulfamate. 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}. Simple benzenesulfonamides substituted in 2-, 4- and 3,4-positions of the benzene ring with amino, alkyl, halogeno and hydroxyalkyl moieties were weak scCA inhibitors with K_Is in the range of 0.976–18.45 μM. Better inhibition (K_Is in the range of 154–654 nM) was observed for benzenesulfonamides incorporating aminoalkyl/carboxyalkyl moieties or halogenosulfanilamides; benzene-1,3-disulfonamides; simple heterocyclic sulfonamides and sulfanilyl-sulfonamides. The clinically used sulfonamides/sulfamate (acetazolamide, ethoxzolamide, methazolamide, dorzolamide, topiramate, celecoxib, etc.) generally showed effective scCA inhibitory activity, with K_Is in the range of 82.6–133 nM. The best inhibitor (K_I of 15.1 nM) was 4-(2-amino-pyrimidin-4-yl)-benzenesulfonamide. These inhibitors may be useful to better understand the physiological role of β-CAs in yeast and some pathogenic fungi which encode orthologues of the yeast enzyme and eventually for designing novel antifungal therapies.

1. Introduction

Saccharomyces cerevisiae, one of the most studied budding yeasts and a widely used model of eukaryotic life forms has a genome comprising 6275 genes condensed into 16 chromosomes, which was completely sequenced in 1996.[1] 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.[2] Subsequently, it has been shown by several groups[3–5] that this protein is a β-carbonic anhydrase (CA, EC 4.2.1.1)[6] 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.[3–6] Although several transcriptional analysis studies involving Nce103 of *S. cerevisiae* have been reported,[2–5] and the CO_2 hydrase activity of the β-CA encoded by the Nce103 gene has been measured by Amoroso et al.,[4] 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.[4] 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[6–10] also has a β-CA encoded by the Nce103 gene (the orthologue 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 it from the *C. albicans* β-CA, which has been denominated in earlier publications as Nce103,[1,7–10] and we shall maintain this nomenclature here too.

In preceding communications[7] we have reported the cloning, purification, kinetic properties and inhibition by simple anions of three β-carbonic anhydrases (CAs, EC 4.2.1.1): from the fungal pathogens *C. albicans* (denominated Nce103), *Cryptococcus neoformans* (denominated Can2) and from the yeast *S. cerevisiae*.[7c] 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.[6,11–14] Yet, CAs other than the α-class are widely distributed in Nature, with the β-CAs being the most abundant such catalyst for the interconversion between carbon dioxide and

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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.[6,7,14] 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.[6,7,14]

In this article, we report a method for the cloning and purification of high enough amounts of scCA 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 sulfonamides, known to interact with most metal centers of such metalloenzymes, but mainly investigated as α-CA inhibitors (CAIs).[6,15,16] 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[4] 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[6,15] (these last enzymes belong to the α-CA class, unlike scCA which is a β-CA). As a second goal, we investigated the interaction of sulfonamides and their isosteres with scCA. Indeed, in this class of CAIs there are at least 30 clinically used drugs presently known,[6] and even if *S. cerevisiae* is not a pathogenic organism, this enzyme type (encoded by the Nce103 gene) is present in pathogenic, related fungi (such as *C. albicans*, *Candida glabrata*, or *C. neoformans* among others).[7–10] As a consequence, inhibition studies of scCA may be used for designing inhibitors with possible applications for designing novel antifungal/anti-yeast therapies.

2. Results and discussion

2.1. scCA Catalytic activity

scCA has been overexpressed in *E. coli* and purified by an original procedure leading to high amounts of pure protein (see Section 4 for details) possessing a good enzyme activity for the physiologic reaction, that is, CO_2 hydration to bicarbonate. Indeed, although scCA has been cloned and purified earlier,[4] its kinetic parameters for the catalyzed physiological reaction, that is, CO_2 hydration to bicarbonate and a proton, are not available in the 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[7] (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 9.4×10^5 s^{-1}, and k_{cat}/K_m of 9.8×10^7 M^{-1} s^{-1}.[17] 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),[16,17] 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 orthologue 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 widespread human isoform hCA I, being only slightly less effective than the most efficient mammalian isozyme, hCA II.[3,6] Furthermore, scCA has an affinity for the clinically used sulfonamide acetazolamide (**AAZ**) 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 from *C. albicans*).

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 *S. cerevisiae*) measured at 20 °C, pH 8.3 in 20 mM Tris buffer and 20 mM $NaClO_4$[17]

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	82.6

Inhibition data with the clinically used sulfonamide acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also provided.
[a] Data from Ref. 7a.
[b] This work.

2.2. scCA Inhibition with sulfonamides/sulfamates

Table 2 shows the scCA inhibition data with a panel of 36 sulfonamides and one sulfamate, some of which are clinically used drugs,[6] such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA** (an orphan drug),[6] topiramate **TPM**, sulpiride **SLP**, indisulam **IND**, zonisamide **ZNS**, celecoxib **CLX**, valdecoxib **VLX**, sulthiame **SLT** and saccharin **SAC**. The simpler derivatives **1–22** were also included in the study as they were the scaffolds most extensively used to design potent or isoform-selective CAIs.[6,16] Data for the inhibition of the dominant human isoforms hCA I and II with these compounds are also included in Table 2, for comparison reasons. The following SAR can be observed from data of Table 2:

(i) A first group of compounds, including **1, 2, 4, 10, 20–22** and **SLT, SAC**, showed ineffective scCA inhibitory activity, with K_Is in the range of 0.976–18.45 μM. These compounds are generally simple benezenesulfonamide derivatives incorporating 2-, 4- and 3,4-substituents of the amino, methyl, carboxy, hydroxymethyl/ethyl or iodo type, the most complicated scaffold being that of sulthiame **SLT**. Saccharin **SAC**, an intramolecularly acylated sulfonamide weakly inhibiting CA I and II,[18] also acts as a very ineffective scCA inhibitor. It should be observed that most of these compounds act as ineffective hCA I inhibitors (micromolar range) and rather effective hCA II ones (nanomolar range, Table 2).

(ii) A rather large number of the investigated sulfonamides, such as **3, 5–9, 11–14, 16, 17, 19** and **VLX**, showed medium potency inhibitory activity against scCA, with K_Is in the range of 154–654 nM (Table 2). Again these compounds are rather heterogeneous from the chemical point of view, including 4-substituted benzenesulfonamides (**3, 5–9**) incorporating aminoalkyl/carboxy-alkyl moieties or halogenosulfanilamides; benzene-1,3-disulfonamides (**11** and **12**); simple heterocyclic sulfonamides (**13** and **14**); sulfanilyl-sulfonamides (**16** and **17**); the 5-aryl-substituted-1,3,4-thiadiazole-2-sulfonamide **19**, as well as the complicated scaffold present in valdecoxib **VLX**. As for the preceding derivatives, these compounds generally act as weak hCA I inhibitors and more effective hCA II inhibitors (Table 2).

(iii) Most of the clinically used drugs, **AAZ-CLX** and **15** (aminobenzolamide), showed good scCA inhibitory activity, with K_Is in the range of 82.6–133 nM (Table 2). The most effective inhibitors in this subclass were acetazolamide **AAZ** and ethoxzolamide **EZA** (K_Is < 100 nM) whereas all other sulfonamides/sulfamate showed a rather compact behavior, with inhibition constants around 100–130 nM. This is a clear example of the fact that the β-class enzyme investigated

here is also susceptible to inhibition with sulfonamides and sulfamates, similarly to the α-CAs from mammals, although generally these compounds possess lower affinity for scCA as compared to hCA II (and sometimes also hCA I). Indeed, many of the clinically used drugs examined here act as low nanomolar inhibitors of hCA II (K_Is in the range of 3–40 nM, and some of them also efficiently inhibit hCA I, with K_Is in the range of 15–50 nM, (Table 2)). SAR is thus very difficult to interpret for the inhibition of scCA with these compounds, as no X-ray crystal structures of the enzyme, alone or in adducts with inhibitors, are available. However, our data prove that many sulfonamide/sulfamate scaffolds, incorporating aromatic, heterocyclic or sugar moieties can be used for designing efficient β-CA inhibitors, in particular those targeting scCA.

(iv) Only one very efficient scCA inhibitor has been detected in this study, compound **18**, which showed a K_I of 15.1 nM against this enzyme, being at the same time a less efficient hCA II (33 nM) and hCA I (109 nM) inhibitor. This pyrimidinyl-substituted sulfanilamide has a unique scaffold among the 37 compounds investigated here, which probably explains its unexpected scCA inhibitory activity, but at the same time, its potency is of relevance for studying inhibition of this enzyme in vivo, in physiologic studies aimed to better understand the roles of scCA in vivo.

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) orthologue 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.[1,7–9]

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 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. 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 needs the assistance of additional amino acids. The pair Asp108–Arg110 probably has this activation function, as it is conserved in all β-CAs.[1,2,6,19] As a consequence, the catalytic water molecule is activated both by the metal ion (as in metalloproteases[20] and α-CAs[1,21]), but also by an aspartic acid residue, as in aspartic proteases.[22] 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.

3. Conclusion

We investigated the catalytic activity and inhibition of the β-CAs from the yeast *S. cerevisiae* (encoded by the Nce103 gene) with a series of sulfonamides and one sulfamate, some of which are clinically used drugs. Simple benzenesulfonamides substituted in 2-, 4- and 3,4-positions of the benzene ring with amino, alkyl, halogeno and hydroxyalkyl moieties were weak scCA inhibitors with K_Is in the range of 0.976–18.45 μM. Better inhibition (K_Is in the range of 154–654 nM) was observed for benzenesulfonamides incorporating aminoalkyl/carboxy-alkyl moieties or halogenosulfanilamides; benzene-1,3-disulfonamides; simple heterocyclic sulfonamides and sulfanilyl-sulfonamides. The clinically used drugs generally showed effective scCA inhibitory activity, with K_Is in the range of 82.6–133 nM. The best inhibitor (K_I of 15.1 nM) was 4-(2-amino-pyrimidin-4-yl)-benzenesulfonamide. These inhibitors may be useful to better understand the physiological role of β-CAs in yeast and some pathogenic fungi which encode orthologues of the yeast enzyme, and eventually for designing novel antifungal therapies.

4. Experimental

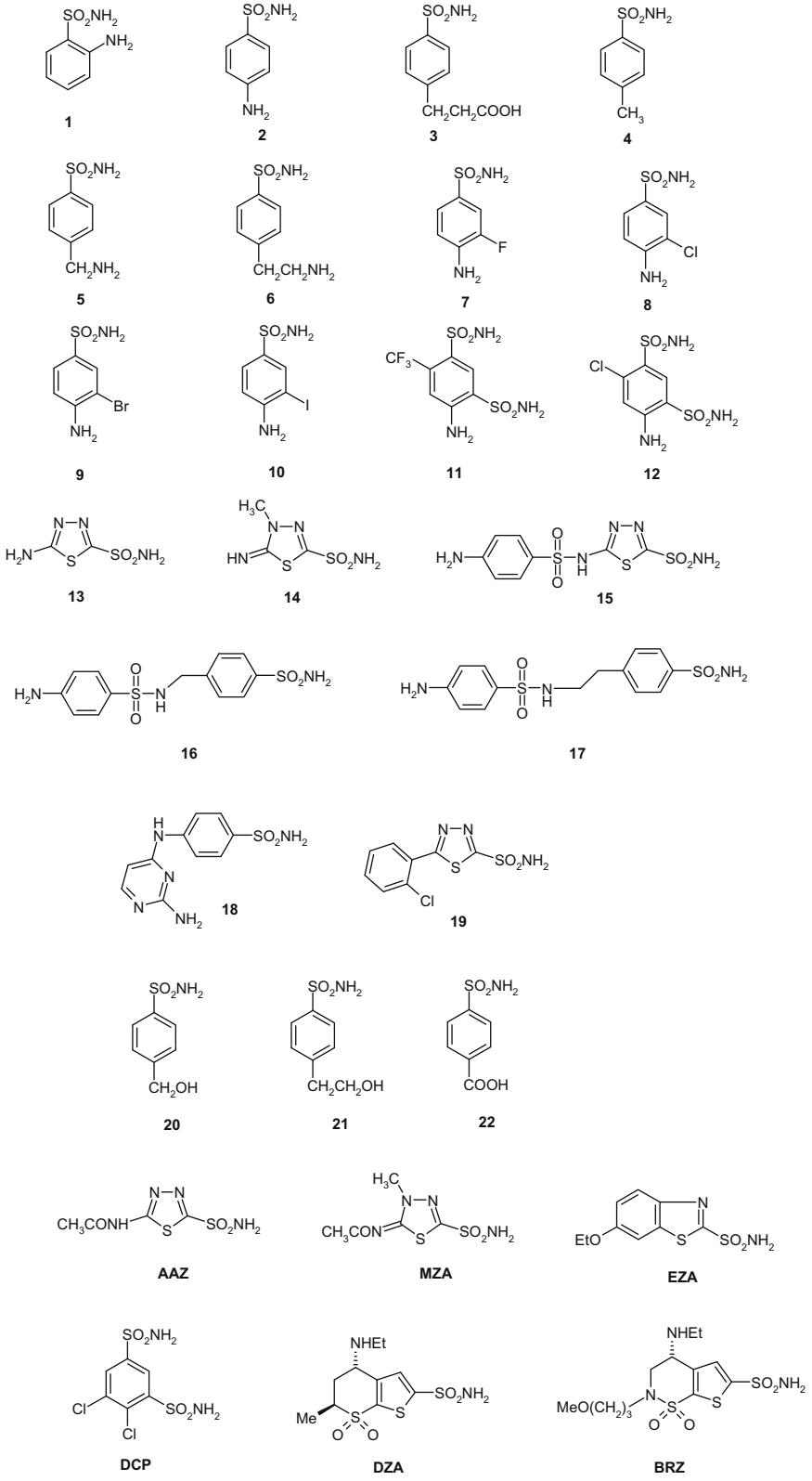
4.1. Cloning and purification of scCA

The haploid yeast strain CEN.PK2–1C (MATa; ura3-52; trp1-289; leu2-3_112; his3Δ1; MAL2-8^C; SUC2) was kindly provided by Dr. K.-D. Entian (Frankfurt, Germany). The *E. coli* strain DH5α (SupE44Δ lacU169 (Φ80 LacZ ΔM15) hsdR17recA1 endA1 gyrA96 thr-1 rl A1) was used for cloning and strain BL21 (DE3) (*E. coli* B F— dcm ompT hsdS(rB—mB–) gal λ(DE3) was used for overexpression of the Nce103 gene product. Yeast cells were grown for overnight at 30 °C in YPD medium made as described by Johnston.[23] *E. coli*

Table 2
hCA I, II, and scCA inhibition data with sulfonamides **1–22** and **15** clinically used derivatives **AAZ–SAC**. Data of isoforms hCA I and II are from Ref. 12

Inhibitor	K_I[a] (nM)		
	hCA I[b]	hCA II[b]	scCA[c]
1	45,400	295	12,300
2	25,000	240	18,500
3	6690	495	165
4	78,500	320	16,100
5	25,000	170	433
6	21,000	160	163
7	8300	60	389
8	9800	110	457
9	6500	40	248
10	6000	70	976
11	5800	63	223
12	8400	75	169
13	8600	60	447
14	9300	19	360
15	6	2	124
16	164	46	166
17	185	50	154
18	109	33	15.1
19	690	12	565
20	55	80	8970
21	21,000	125	7540
22	23,000	133	14,500
AAZ	250	12	82.6
MZA	50	14	119
EZA	25	8	98.4
DCP	1200	38	103
DZA	50,000	9	110
BRZ	45,000	3	114
BZA	15	9	111
TPM	250	10	110
SLP	12,000	40	124
IND	31	15	133
ZNS	56	35	106
CLX	50,000	21	108
VLX	54,000	43	654
SLT	374	9	1020
SAC	18,540	5950	12,500

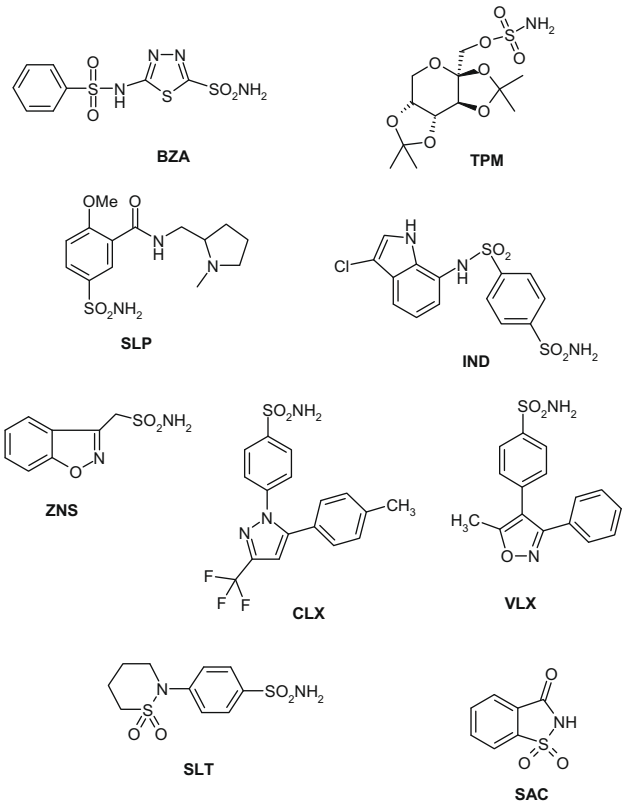
[a] Errors in the range of 5–10% of the shown data, from three different assays.
[b] Human recombinant isozymes, stopped flow CO_2 hydrase assay method, pH 7.5, 20 mM Tris–HCl buffer.[17]
[c] Yeast recombinant enzymes, at 20 °C, pH 8.3 in 20 mM Tris buffer and 20 mM $NaClO_4$.[17]



strains were grown in LB medium at 37 °C enriched with 10 μg/ml ampicillin.

4.1.1. Cloning NCE103 gene by PCR based strategies

Yeast genomic DNA was isolated using the Johnston's procedure.[23] The Nce103 gene was amplified from genomic DNA by PCR based strategies using the following oligonucleotides; NCE103ORF-for (5′-AGGATCCATGAGCGCTACCGAA-3′) and NCE103 ORF-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.

4.1.2. Overexpression and purification of Nce103 gene product, scCA

Nce103 was overexpressed in a pET21a(+)expression vector containing T7 promoter region. After transformation of *E. coli* BL21 (DE3), overexpression of scCA was initiated by addition of 1 mM IPTG for 14 h at 30 °C. To purify the protein, *E. coli* cells were collected by centrifugation at 3000 rpm for 10 min at 4 °C. The pellet was washed with buffer (50 mM Tris–HCl, pH 7.6) and pellet was resuspended in lysis buffer (20 mM Tris/0.5 mM EDTA/0.5 mM EGTA/pH 8.7). 100 μl of 100 mM PMSF (1 mM final concentration) and 250 μl of a 10 mg/ml solution of lysozyme were added and the pellet was thawed at room temperature. After 30 min 1 ml of the 3.0% protamine sulfate solution was added to the cell lysate and centrifuged. The proteins in clear supernatant were precipitated by addition of $(NH_4)_2SO_4$. The pellet was suspended in small volume of 50 mM Tris–SO_4 buffer (pH 7.4) and the obtained solution was applied to a Sephadex G-100 Gel Filtration Chromatography column and proteins were eluted and screened by SDS–PAGE.

4.2. CA kinetic and inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[17] 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,[7] and represent the mean from at least three different determinations. Sulfonamides **1–22** and **AAZ**-**SAC** were either prepared as reported earlier by this group,[12,15,16,21] or were commercially available reagents from Sigma–Aldrich, and Merck.

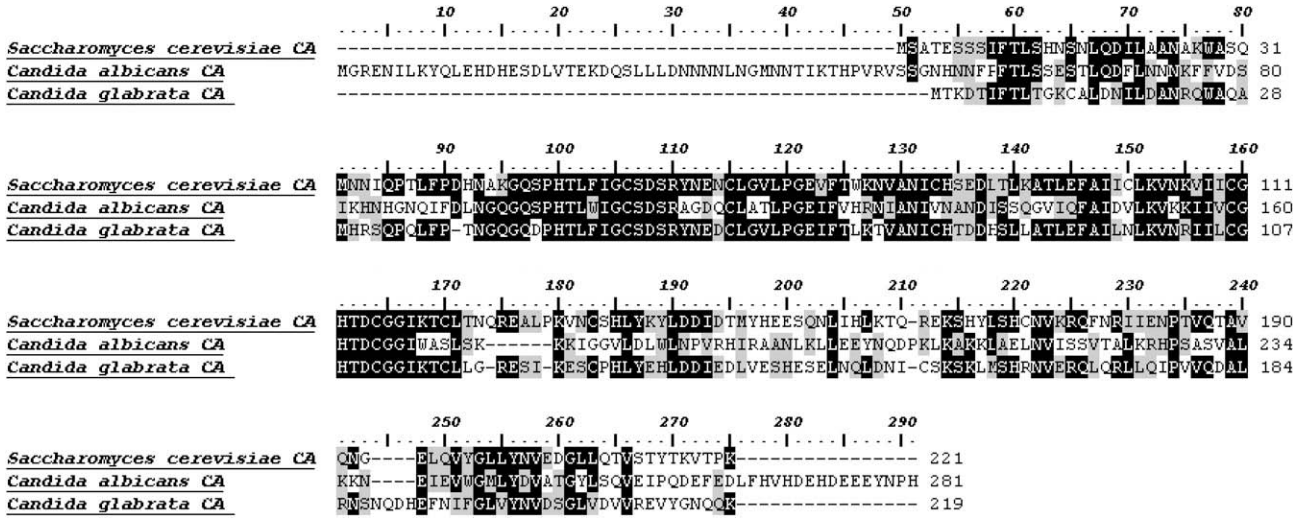


Figure 1. Alignment of scCA, Nce103 (from *C. albicans*) and Nce103 (from. *C. glabrata*) amino acid sequences. The three zinc ligands are conserved in all these three enzymes (Cys106, His161 and Cys164) whereas the other conserved/semiconserved amino acid residues between the three β-CAs are evidenced by black boxes. The two residues Asp108, Arg110, thought to be involved in the β-CA catalytic cycle[1] are also conserved in the three enzymes (the numbering system used here corresponds to the Nce103 of *C. albicans* amino acid sequence).[7–10]

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