

Natural protein engineering: a uniquely salt-tolerant, but not halophilic, α -type carbonic anhydrase from algae proliferating in low- to hyper-saline environments

Umesh K.Bageshwar^{1,2,3}, Lakshmanane Premkumar^{1,3,4}, Irena Gokhman¹, Tatyana Savchenko¹, Joel L.Sussman^{4,5} and Ada Zamir¹

Departments of ¹Biological Chemistry and ⁴Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel and ²Department of Medical Biochemistry and Genetics, Reynolds Building, Texas A & M University, College Station, TX 77843, USA

⁵To whom correspondence should be addressed.
E-mail: joel.sussman@weizmann.ac.il

³These authors contributed equally to this work

***Dunaliella salina* is a unicellular green alga thriving in environments ranging from fresh water to hyper-saline lakes, such as the Dead Sea. An unusual, internally duplicated, 60 kDa α -type carbonic anhydrase (dCA I), located on the surface of this alga, is expected to function over a broad range of salinities. It would therefore differ from other carbonic anhydrases that already lose activity at low salinities and also from halophilic proteins that require high salinities for conformational stability. Enzymatic analyses indeed indicated that dCA I retained activity at salt concentrations ranging from low salt to at least 1.5 M NaCl or KCl for CO₂ hydration, 2.0 M NaCl for esterase activity and 0.5 M for bicarbonate dehydration. Although measurements at higher salinities were constrained by the interference of salt in the respective assayed reactions, activity was noticeable even at 4.0 M NaCl. Comparisons of the internally duplicated dCA I to single-domain derivatives indicated that inter-domain interactions played a decisive role in the stability, activity, salt tolerance and pH responses of dCA I. Hence dCA I is a uniquely salt-tolerant protein, retaining an active conformation over a large range of salinities and, as a Zn metalloenzyme, largely immune to the specific inhibitory effects of anions. Its unique features make dCA I a useful model to understand the physico-chemical basis of halotolerance and protein–salt interactions in general.**

Keywords: carbonic anhydrase activity/*Dunaliella salina* halophilic proteins/protein molecular adaptation/salt-tolerant protein

Introduction

High salinities could be detrimental to the folding, stability and function of proteins (Madern *et al.*, 2000). Extensive studies have been devoted to unraveling the molecular strategies that enable proteins to retain a functional conformation at salinities as high as those encountered in natural habitats of extreme halophilic archaea (Ebel *et al.*, 1999a). These organisms require multimolar salt concentration for growth and achieve osmotic balance against the external salinity by accumulating iso-osmotic levels of mainly potassium salts. Likewise, proteins from halophilic archaea require high salinities to form and maintain an active conformation (Lanyi, 1974;

Madern *et al.*, 2000; Mevarech *et al.*, 2000). Structurally, halophilic proteins are characterized by an excess of acidic over basic amino acid residues as compared with mesophilic counterparts. Crystal structure determinations of several halophilic proteins indicated that a negative surface potential generated by clustering of acidic residues allowed the formation of protective hydrated ion networks (Dym *et al.*, 1994; Frolow *et al.*, 1996; Madern *et al.*, 2000).

In addition to proteins from halophilic archaea, several enzymes of eubacterial (Deutch, 2002; Chantawannakul *et al.*, 2003) and eukaryotic origins (Ahmad *et al.*, 2001; Su and Lee, 2001) were reported to exhibit considerable salt tolerance, i.e. they remain active over relatively broad salinity ranges, without showing obligatory salt dependence. Despite the great interest in the structural and mechanistic aspects that underlie protein salt tolerance, these proteins have not been subjected yet to rigorous analyses.

A potential source of novel salt-tolerant proteins is the unicellular green alga *Dunaliella salina* that is capable of proliferating in salinities as varied as those prevailing in mesophilic all the way to halophilic habitats. Unlike extreme halophiles, the alga maintains a low intracellular ionic level over practically the entire range of salinities and attains osmotic balance by intracellular accumulation of glycerol (Avron, 1986). Consequently, intracellular *Dunaliella* proteins are not exposed to the environmental salinity, in contrast to surface proteins, which are expected to remain active over the broadly varying salinities sustaining algal growth.

A protein fulfilling this expectation is dCA I (*Dunaliella* carbonic anhydrase I, formerly designated Dca, accession number P54212), a salt-inducible 60 kDa protein consisting of two, ~52% identical, tandemly arranged α -type carbonic anhydrase domains (Fisher *et al.*, 1996; Gokhman *et al.*, 1999). Carbonic anhydrases catalyze the reversible hydration of CO₂ and perform essential functions in animals, plants, eukaryotic algae and eubacteria (Burnell, 2000; Fukuzawa *et al.*, 2000; Kozliak *et al.*, 2000; Parkkila, 2000). Its internally duplicated structure distinguishes dCA I from other, single-domain α -type carbonic anhydrases (EC 4.2.1.1) that are ~30 kDa in size and it is the first internally duplicated α -type carbonic anhydrase to be analyzed enzymatically. The plasma membrane (PM) association of dCA I (Fisher *et al.*, 1994, 1996) is attributed to a still unspecified post-translationally added anchor. Based on its function, salt inducibility and extracellular orientation, dCA I was proposed to play a role in enhancing the supply of CO₂ to the cells, especially under CO₂ limiting conditions such as high salinities (Fisher *et al.*, 1994). The expected salt tolerance of dCA I, as compared with the salt sensitivity typically exhibited by α -type carbonic anhydrases, was first supported by assays of a crude protein fraction from *D.salina* including dCA I (Fisher *et al.*, 1996).

That dCA I may belong to a larger set of salt-tolerant carbonic anhydrases in *D.salina* has recently been indicated by

the identification of another extracellular protein consisting of a single α -type carbonic anhydrase domain (Premkumar *et al.*, 2003b). Designated dCA II (*Dunaliella* carbonic anhydrase II), this enzyme is ~50% identical in sequence with each of the dCA I domains, similarly enriched in acidic amino acid residues and exhibits comparable activity to dCA I (Premkumar *et al.*, 2002). Recently, two other cDNA sequences for carbonic anhydrases from *Dunaliella* differing from dCA I and dCA II were reported (AAP33144, AAO83593), but still need to be characterized on the protein level.

Aside from implying global stability in the presence of low to multimolar salt concentrations, the expected salt tolerance of dCA I is of special interest in view of the fact that, like other α -type carbonic anhydrases, this enzyme is a Zn metalloprotein. The active-site Zn is tetrahedrally coordinated to three conserved His residues and a hydroxyl or water moiety (Liljas *et al.*, 1972; Stams and Christianson, 2000). In the CO₂ hydration reaction, the Zn-bound hydroxide reacts with CO₂ to form bicarbonate, a step followed by the regeneration of the Zn-bound hydroxide by intermolecular proton transfer from Zn-bound water, that replaces the released bicarbonate, to a buffer molecule in the solution (Lindskog and Silverman, 2000). Various anions, as well as sulfonamides and other inhibitors, interfere directly with the catalytic process by coordinating with the active-site Zn, thus replacing the catalytically essential, Zn-bound water/hydroxyl as well as the 'deep water' (hydrogen bonded to Zn-bound water/hydroxyl and peptide nitrogen of Thr199) (Liljas *et al.*, 1994). For this reason, α -type carbonic anhydrases are usually inhibited by relatively low concentrations of halides and other salts.

The results of the analyses presented here establish the unique salt/anion tolerance of the enzyme, point to structural elements underlying these responses and provide an intriguing example for the type of enzyme structural modifications integral to the evolution of salt-tolerant organisms.

Materials and methods

Algal cultures

The *Dunaliella salina* strain and growth conditions were essentially as described elsewhere (Ben-Amotz and Avron, 1983; Sadka *et al.*, 1989; Fisher *et al.*, 1994). In addition to the standard components, the medium used for induction of dCA I contained 3.5 M NaCl, 50 mM Tricine-HCl, pH 8.5 and 25 mM Na bicarbonate. Cultures, usually started by inoculating cells from single algal colonies into liquid media, were grown to a density of $\sim 2 \times 10^6$ cells/ml and further propagated by diluting to $(1-2) \times 10^5$ cells/ml in fresh media.

Affinity chromatographic purification of dCA I from *D.salina*

Cells from a 30 l culture were harvested by centrifugation at 2000 g and lysed by re-suspension in 100 ml of 25 mM Tris-SO₄, pH 7.5, 50 mM Na₂SO₄, 100 mM NaClO₄, 5 mM ϵ -caproic acid, 1 mM benzamidine, 0.1% Triton X-100 (Equilibration Buffer 1). The suspension was centrifuged at 186 000 g for 60 min at 4°C and the supernatant, containing ~500 mg of protein, was loaded on to a column (45 \times 2.2 cm i.d.) of agarose-coupled *p*-aminomethylbenzenesulfonamide (PAMBS) (Sigma) (Whitney, 1974) in Equilibration Buffer 1. After washing the column with 400 ml each of Equilibration Buffer 1, 0.2 M NaClO₄ in the same buffer and a similar

solution but without Triton X-100, dCA I was eluted with 0.5 M NaClO₄, 0.1 M Na acetate, pH 5.6, 5 mM ϵ -caproic acid, 1 mM benzamidine (Whitney, 1974). Fractions with $\geq 95\%$ pure dCA I were equilibrated with 25 mM Tris-SO₄, pH 7.5, 50 mM Na₂SO₄, 5 mM ϵ -caproic acid, 1 mM benzamidine and concentrated to ~1.5 ml by using an Amicon spin column (30 kDa cut-off size). The total recovery of pure dCA I was 1–3 mg.

Preparation of a plasma membrane fraction and isolation of a 40 kDa proteolytic product of dCA I (p40)

A 25 l *D.salina* culture was grown and harvested as described above. The cells were re-suspended in 40 ml of iso-osmotic buffer containing 4.5 M glycerol, 5 mM ϵ -caproic acid, 1 mM benzamidine and 10 μ M ZnSO₄ and lysed by adding 160 ml of 10 mM Tris-MOPS [3-(*N*-morpholino)propanesulfonic acid], pH 7.5. The lysate was centrifuged at 8000 g for 10 min at 4°C and the supernatant was centrifuged at 200 000 g for 2 h at 4°C. The plasma membrane-enriched pellets (Azachi *et al.*, 2002) were resuspended in 10 ml of 10 mM Tris-MOPS, pH 7.5, 5 mM ϵ -caproic acid, 1 mM benzamidine, 10 μ M ZnSO₄, 0.2% Triton X-100 (Suspension Buffer) and the suspensions were incubated on ice for 10 min and centrifuged at 200 000 g for 2 h at 4°C. The supernatant, containing 15–25 mg of protein, was loaded on to a DEAE-Sephadex (A-25) column (30 \times 1.8 cm i.d.), pre-equilibrated with Suspension Buffer and the column was washed with 250 ml of the same buffer and eluted with 200 ml of a 0–0.6 M NaCl linear gradient in the same buffer. Aliquots were assayed for carbonic anhydrase activity as described below.

Heterologous expression and purification of dCA I, N-ter and C-ter

Mature dCA I (Fisher *et al.*, 1996) was expressed in the *Escherichia coli* Origami B strain (Novagen) and affinity purified as described previously (Premkumar *et al.*, 2003a,b). The cDNA sequence encoding N-ter (the recombinant N-terminal repeat of dCA I) without the leader peptide (amino acid residues 55–317) was amplified from the full-length cDNA clone by PCR (Fisher *et al.*, 1996). The forward primer (5'-GATTGGAATTCGTGAGTGAGCCTCACG-3') contained an *Eco*RI site and the reverse primer (5'-TGGAAAAGCTTTTAACCCTTGTACTCGT-3') contained a stop-codon and a *Hind*III site. The PCR product was digested with *Eco*RI and *Hind*III and cloned into likewise-digested pET21a vector (Novagen), to create pN21a. In this construct, translation of N-ter was initiated from the T7-tag in the pET21a vector. This construct was further modified by fusion of N-ter to a His₆-tag by replacing the stop codon (TAA) in pN21a by a Gly codon (GGA) using a site-directed mutagenesis procedure (QuikChange, Stratagene, La Jolla, CA) with the entire pN21a as template. The primers used for mutagenesis were 5'-CGAGTACAAGGGTGGAAAGCTTGCGGCCGC-3' and 5'-GCGGCCGCAAGCTTTCCACCCTTGTACTCG-3' and the resulting vector was designated pHtN21a. Expression of N-ter from pN21a or pHtN21a in the *E.coli* Origami B strain was as described previously (Premkumar *et al.*, 2003a). For His₆-tag-based affinity purification of N-ter, a 100 ml culture of transformed *E.coli* was lysed in 25 mM Tris-SO₄, pH 8.0, 15 mM MgSO₄, 0.1% Triton X-100, 0.5 mM ZnSO₄, 1 mM imidazole, 20 μ g/ml DNase I and 200 mM NaCl by repeated freezing and thawing as described previously (Premkumar *et al.*, 2003a,b). The lysate was cleared by centrifugation and mixed with Ni-NTA resin (Qiagen), pre-equilibrated with 25 mM Tris-SO₄, pH 8.0, 1 mM imidazole, 300 mM NaCl.

After transfer to a column, the resin was washed with the same buffer with added 20 mM imidazole and N-ter was eluted by raising the imidazole concentration to 100 mM. N-ter containing fractions were concentrated and the buffer exchanged with 20 mM bis-Tris-Cl, pH 6.5. Further purification of N-ter was achieved by anion-exchange chromatography on HiTrap Q (Amersham-Pharmacia) by elution with a 0–600 mM NaCl linear gradient. The coding sequence for C-ter [the recombinant C-terminal repeat of dCA I including residues 318–589 (Fisher *et al.*, 1996)] was amplified by PCR using 5'-GATTGGAATTCGTGAGTGAACCCAACGA-3' and 5'-GGAAAAGCTTTTAAGCAGCAGCACCGTTATATCCG-3' as forward and reverse primers, respectively. In the forward primer, the coding sequence was 5'-extended by Val and Ser codons, to mimic the N-terminal sequence of the mature dCA I (Fisher *et al.*, 1996). The amplified fragment, digested with *EcoRI* and *HindIII*, was cloned into the likewise-restricted pET-28a or pET21a to form pC28a and pC21a, respectively. Expression of C-ter from pC21a in the *E.coli* Origami B strain or from pC28a in the *E.coli* BL21(DE3)pLysS strain were as described previously (Premkumar *et al.*, 2003a,b). C-ter was purified from *E.coli* lysates by DEAE-Sephadex chromatography essentially as described for the purification of p40 from *D.salina* extracts described above. Purification by affinity chromatography on agarose-coupled PAMBS (Sigma) was essentially as described previously (Premkumar *et al.*, 2003b).

CO₂ hydration assay

Assays were performed essentially as described elsewhere (Booth and Beardall, 1991; Fisher *et al.*, 1996), with some modifications. The assays were performed in a double-walled chamber kept at 0–4°C by circulating ice-cold water and the reaction mixtures, in 2.75 ml, contained 6.5 mM veronal buffer, pH 8.3, 10 μM ZnSO₄ and the indicated concentrations of salts or inhibitors. The reaction, started by adding 250 μl of ice-cold saturated CO₂ in water, was electrochemically monitored by a temperature-compensated pH meter. Activity unit is defined by $[(t_0 - t)/t] \times 10$, where t is the time for the pH to change from 8.3 to 7.3 in the presence of enzyme and t_0 is the corresponding time for non-catalyzed pH change (Booth and Beardall, 1991).

Bicarbonate dehydration assay

Initial rates of bicarbonate dehydration were determined at 25°C at pH from 5.6 to 7.4 and Na bicarbonate concentrations ranging from 2 to 120 mM in an Applied Photophysics stopped-flow apparatus using the changing pH indicator method (Khalifah, 1971). The buffer–indicator pairs used were MES [2-(*N*-morpholino)ethanesulfonic acid] and chlorophenol red for pH 5.6–6.2 and MOPS and *p*-nitrophenol for pH 6.8–7.4 and the reactions were monitored at 574 and 400 nm, respectively. Before adding NaCl, mixtures with different buffers and pHs were adjusted to 50 mM ionic strength with Na₂SO₄. Each data point used to derive kinetic constants was an average of seven independent measurements. Rates of uncatalyzed reactions under the conditions of the assay were subtracted from the rates of the enzyme-catalyzed reaction. Kinetic constants were determined by fitting the data to the Michaelis–Menten equation.

Esterase assay

Steady-state initial rate measurements were carried out at 25°C in a Spectronic Genesys spectrophotometer. The rate of *p*-

nitrophenyl acetate hydrolysis was monitored by following for 3 min the change in absorbance at 348 nm, the isosbestic point for *p*-nitrophenol acetate and *p*-nitrophenolate ion (Verpoorte *et al.*, 1967). Mixtures with 1–3 mM substrate were prepared by diluting a 107 mM acetone solution of *p*-nitrophenyl acetate (Sigma) with water or NaCl solutions to reach the desired final concentrations of substrate and salt in 3% acetone. To 975 μl of this mixture, in a 1 ml cuvette, were added 25 μl of 1 M Tris-SO₄, pH 8.2 and the reaction was immediately initiated by adding 5–10 μl of enzyme solution. Blank runs in the absence of enzyme provided values for non-enzymatic hydrolysis rates that were subtracted from the rates measured in the presence of enzyme.

Results

Purified dCA I and its truncated derivatives, isolated from *D.salina* or heterologously expressed in *E.coli*, were analyzed for the effects of salts and inhibitors on the physiologically significant carbonic anhydrase activities of CO₂ hydration and bicarbonate dehydration and the accompanying esterase activity. Human carbonic anhydrase isozyme I (hCA I) (Sigma), the enzyme closest in sequence to dCA I (37% identity), was assayed as a representative mesophilic α-type carbonic anhydrase. The maximum salt concentrations tested in the respective assays were kept below levels that compromised the accuracy of the measurements.

CO₂ hydration activity

The effect of salt on dCA I activity was first determined in assays of CO₂ hydration activity in the presence of Li⁺, Na⁺, K⁺ and Mg²⁺ chlorides or the potassium salts of Br⁻ and I⁻. A comparison of various cations at up to 1.5 M (Figure 1A) revealed little effect or even enhancement of activity at the lower range of salt concentrations. At salt concentrations >1.0 M, the activity gradually declined to levels close to those measured in the absence of salt. Quantitatively, the Li⁺ salt exerted the least activation and highest inhibition whereas salts of Na⁺, K⁺ and Mg²⁺, albeit at different concentrations, enhanced the activity by nearly 2-fold. A comparison of different potassium halides (Figure 1B) indicated an enhancement of activity at relatively low concentrations of Cl⁻ and Br⁻ but not I⁻ salts. At higher concentrations, Br⁻ was more inhibitory than Cl⁻ and I⁻ was inhibitory already at 0.1 mM, but the inhibition was not meaningfully increased up to 100 mM. These patterns of response can be understood as reflecting activation by cations such as Na⁺, K⁺ or Mg²⁺ superimposed on moderate inhibitory effects of anions such as Cl⁻ or Br⁻. Thus, the interplay between stimulatory and inhibitory effects resulted in the maintenance of the enzymatic activity within relatively narrow limits over widely varied concentrations of naturally abundant salts.

Parallel assays of hCA I (Figure 1A and B) indicated that the enzyme was strongly inhibited at 0.1 M salt (5 mM for KI) and showed only residual or no activity at higher salt concentrations. These results generally match previous reports (Franchi *et al.*, 2003).

The unusual responses of dCA I to salts, as compared with hCA I, prompted us to examine the effects of several potent carbonic anhydrase inhibitors, i.e. N₃⁻, ClO₄⁻, PAMBS and acetazolamide, on the activity of both dCA I and hCA I (Figure 1C). The results indicated that dCA I resembled hCA I in sensitivity to sulfonamides and ClO₄⁻ but dCA I was distinctly more resistant to N₃⁻ than hCA I. To evaluate these

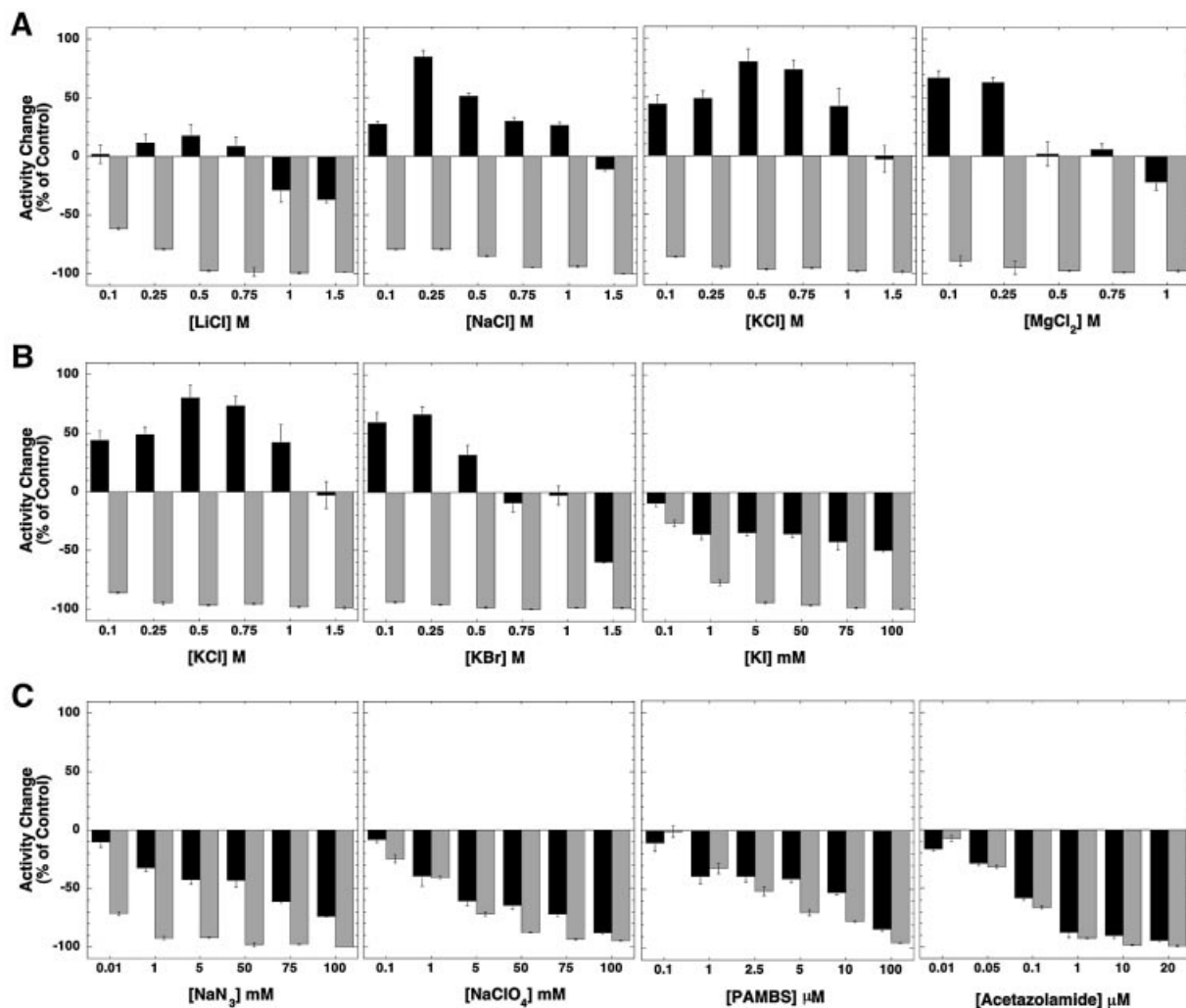


Fig. 1. Effect of salts and inhibitors on the CO₂ hydration activity of dCA I and hCA I. Affinity-purified dCA I (12 nM) and hCA I (3 nM) were assayed for CO₂ hydration activity in the presence of the indicated concentrations of salts or inhibitors as described in Materials and methods. The activities measured are depicted as percentage change in activity (activation/inhibition) relative to the control without added salts or inhibitors (all mixtures contained 6.5 mM veronal buffer, 10 μM ZnSO₄, as described in Materials and methods). (A) Activity measured in the presence of chloride salts of Li⁺, Na⁺, K⁺ and Mg²⁺; (B) activity measured in the presence of potassium salts of Cl⁻, Br⁻ and I⁻; (C) activity measured in the presence of the carbonic anhydrase inhibitors azide, perchlorate, PAMBS and acetazolamide. Black columns, dCA I; gray columns, hCA I.

data in a broader context, the inhibitory effects of salts and inhibitors on the CO₂ hydration activity of dCA I and hCA I were expressed as apparent I_{50} values (Carlier *et al.*, 1999) and compared with corresponding values (reported as such or converted from K_i) for human carbonic anhydrase II (hCA II) and the periplasmic carbonic anhydrase(s) (cCA) from the unicellular fresh water alga *Chlamydomonas reinhardtii* (Table I). The compiled data indicate that despite the large variation in inhibitor sensitivity shown by the different α -type carbonic anhydrases, dCA I stands out with its apparently low affinity for Cl⁻ and Br⁻ and its relatively low sensitivity to I⁻ and N₃⁻. On the other hand, the sensitivity of dCA I to the sulfonamides and ClO₄⁻ stays within the range exhibited by the other carbonic anhydrases included in the comparison.

CO₂ hydration activity of truncated forms of dCA I

To address the possible contribution of the tandemly repeated structure to the outstanding salt tolerance of dCA I, as reflected in CO₂ hydration activity, the full-length protein was compared with truncated variants including only a single complete α -type carbonic anhydrase domain (cf. Figure 2).

Table I. Comparison of inhibitory effects of anionic and sulfonamide inhibitors on dCA I and other α -type carbonic anhydrases

Inhibitor	I_{50}			
	dCA I	hCA I	cCA	hCA II
Acetazolamide (μM)	97×10^{-3}	86×10^{-3}	7.8×10^{-3a}	40×10^{-3b}
PAMBS (μM)	5.8	~2.5	ND ^c	ND
ClO ₄ ⁻ (μM)	2.2×10^3	1.3×10^3	69 ^a	1.3×10^{3b}
N ₃ ⁻ (μM)	$\sim 5.0 \times 10^4$	1.2 ^b	39 ^a	1.5×10^{3b}
I ⁻ (mM)	100	0.3	2.5 ^a	26 ^b
Br ⁻ (mM)	$>1.0 \times 10^3$	4 ^b	ND	63 ^b
Cl ⁻ (mM)	$>1.5 \times 10^3$	6 ^b	56 ^a	200 ^b

Assays of CO₂ hydration activity of dCA I and hCA I (at 12 nM and 3 nM, respectively) were performed as described in Materials and methods. I_{50} , concentration of inhibitor causing 50% inhibition. Some of the quoted values of I_{50} were obtained by using the equation $I_{50} = K_i + [E_0]/2$ (K_i , inhibitor dissociation constant; $[E_0]$, initial enzyme concentration) as described (Murakami and Sly, 1987), assuming non-competitive inhibition. All data other than those indicated in the following footnotes were determined in the present study.

^aFrom Husic (1990).

^bFrom Vullo *et al.* (2003).

^cND, not determined.

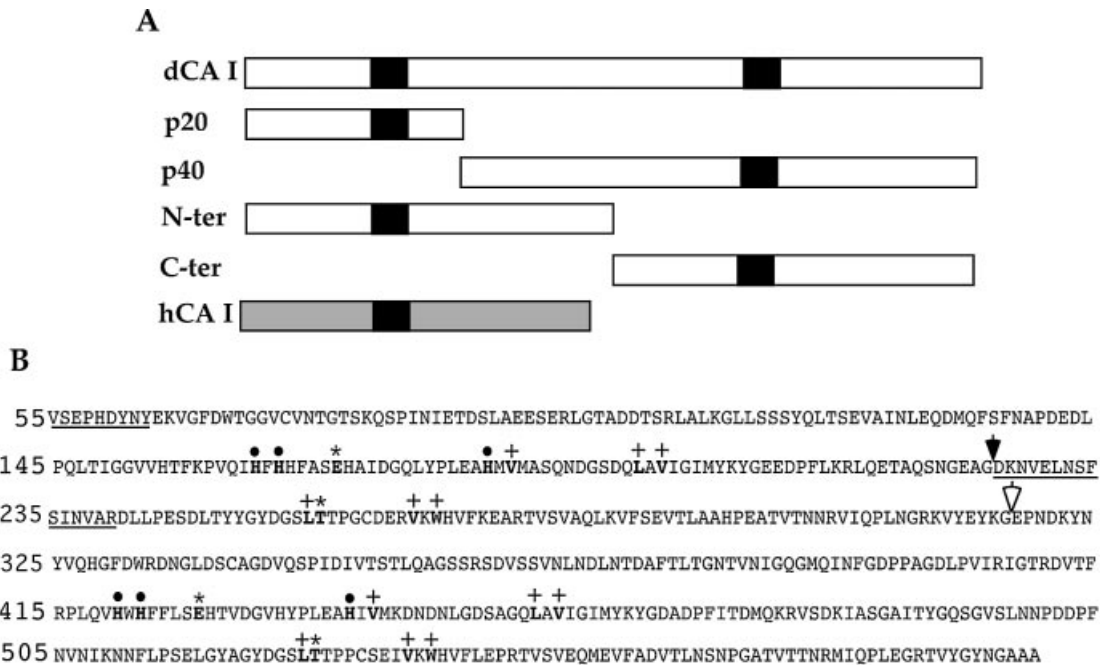


Fig. 2. Description of dCA I and its truncated derivatives. (A) Schematic diagram of mature dCA I [after removal of its leader peptide (Fisher *et al.*, 1996)], its cleavage products p20 and p40, recombinant N-ter and C-ter and hCA I. The regions containing the Zn-liganding His residues are depicted as black boxes. (B) Amino acid sequence of mature dCA I. Filled arrow, proteolytic cleavage site separating dCA I into p20 and p40; underlined, N-terminal amino sequences of isolated p20 and p40; empty arrow between Gly317 and Glu318, separation between N- and C-ter domains of dCA I; bold, conserved residues forming the active site; dots, Zn-coordinating residues; asterisks, active site residues involved in hydrogen bonding; plus signs, residues forming the substrate-binding pocket.

Polypeptides of ~40 and ~20 kDa (p40 and p20, respectively) cross-reacting with anti-dCA I antibodies were detected as minor components of affinity-purified dCA I from *D.salina* (Figures 2 and 3A). Partial amino acid sequencing of gel-eluted proteins identified p20 as the N-terminal fragment of the mature dCA I and p40 as the remaining part of the protein, with Asp226 as its N-terminal residue (Figure 2). The two polypeptides apparently arose by a single proteolytic cleavage of dCA I. Whereas p20 does not include the complete set of amino acid residues essential for enzymatic activity (Merz, 1990), p40 extends over the entire length of the C-terminal domain and can be considered as a single-repeat variant of dCA I (Figure 2).

Isolation of a p40-enriched fraction, free of p20 and intact dCA I, was achieved by ion-exchange chromatography of a solubilized plasma membrane fraction. Early-eluted p40 fractions, apparently free of dCA I and p20, were assayed for CO₂ hydration (Figure 3A) and found to exhibit a specific activity of 490 units/mg protein, amounting to ~10% of the value determined for dCA I. The comparison of the effect of increasing concentrations of NaCl on the activity of p40 and dCA I (Figure 3B) indicates that p40 is salt tolerant (apparent $I_{50} \approx 1.0$ M), although to a lesser degree than that exhibited by the full-length dCA I (apparent $I_{50} > 1.5$ M).

Enzymatic activity and salt responses were also studied with N-ter and C-ter, separately expressed in *E.coli* (Figures 2 and 4A). The recombinant C-ter was first purified by ion-exchange chromatography and at later times by affinity chromatography on immobilized PAMBS. As N-ter failed to bind to the carbonic anhydrase-specific affinity resin, it was fused to a His₆-tag, which permitted its purification on an Ni-NTA column. The recombinant C-ter exhibited a specific activity of

450 units/mg protein, closely resembling the value determined for p40, whereas the homogeneously purified N-ter showed no detectable CO₂ hydration activity (data not shown).

Assays of CO₂ hydration activity of C-ter in the presence of several salts (Figure 4B) showed that it resembled dCA I in responses to LiCl and KI but displayed a lower extent of activation and a higher degree of inhibition with NaCl or KCl. Nevertheless, like p40, C-ter exhibited considerable salt tolerance.

Bicarbonate dehydration activity of dCA I and C-ter

Initial rates of bicarbonate dehydration by dCA I were determined in a stopped-flow spectrometer in assay mixtures containing 2–120 mM Na bicarbonate as substrate. The data fitted the Michaelis–Menten equation for one but not two types of substrate-binding site, suggesting that dCA I harbored two highly similar catalytic sites or that only one of the two carbonic anhydrase domains in dCA I was responsible for the observed activity.

Apparent k_{cat} values were determined at pHs from 6.2 to 7.4 in reaction mixtures with 0 or 0.1 M NaCl adjusted with Na₂SO₄ to 0.05 or 0.15 M ionic strength as described in Materials and methods (Figure 5). The pH dependence curves at the two ionic strengths were characterized by a rise below pH 6.5 and two inflection points corresponding to pK_as of ~6.5 and 6.8–7.0. A marked increase in k_{cat} at 0.15 M compared with 0.05 M ionic strength was most pronounced at acidic pHs but persisted up to neutral pH. The increase in k_{cat} with ionic strength differentiated dCA I from enzymes such as hCA II and human carbonic anhydrase IV (hCA IV) where similar increases in ionic strength were reported to have no effect on k_{cat} in a similar pH range (Pocker and Miao, 1987; Baird *et al.*, 1997).

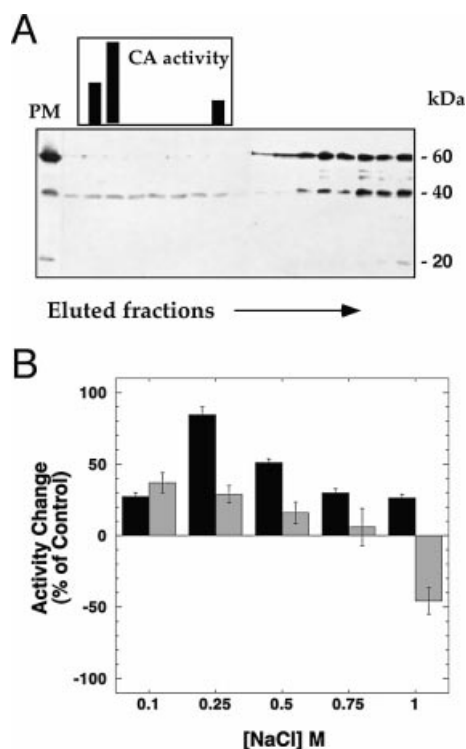


Fig. 3. Isolation and salt tolerance of p40. (A) Isolation of p40: cells grown in 3.5 M NaCl were fractionated by differential centrifugation and the fraction containing plasma membrane was resolved by ion-exchange chromatography as described in Materials and methods. Eluted fractions were analyzed by SDS-PAGE and immunoblotting with anti-dCA I antibodies (bottom panel) and 0.2 ml of early-eluting fractions enriched in p40 (as indicated by Coomassie Brilliant Blue staining; not shown) were assayed for CO₂ hydration activity (shown in arbitrary units on the upper panel). (B) Effect of salt on the CO₂ hydration activity of p40: partially purified p40 (29 nM) was assayed for CO₂ hydration activity in the presence of the indicated concentrations of NaCl in mixtures as described in the caption to Figure 1. The activities measured (gray bars) are depicted, alongside corresponding activities of dCA I (black bars), as percentage change in activity relative to controls without added salt.

To test the effect of higher salinities on the steady-state kinetic constants of both dCA I and C-ter, assays were conducted with up to 0.5 M NaCl, at pH 6.8 (Table II). At pH <6.8, a high signal-to-noise ratio at salinities above 0.15 M NaCl interfered with the accuracy of the readings, especially at low substrate concentrations. The results for dCA I indicated only small increases in k_{cat} and K_{m} and a practically unchanged K_{enz} ($k_{\text{cat}}/K_{\text{m}}$) at up to 0.5 M NaCl. An entirely different response was exhibited by C-ter, where the rate of the reaction in the absence of salt did not level off even at Na bicarbonate concentrations as high as 120 mM. However, in the presence of 0.5 M NaCl, lower bicarbonate concentrations were sufficient to saturate the rate of the reaction thus permitting to determine K_{m} and k_{cat} values that were 2–3- and ~2-fold higher, respectively, relative to the corresponding constants for the full-length dCA I.

A similar analysis of hCA I indicated that 0.1 M NaCl was sufficient to bring about a 4-fold increase in K_{m} whereas k_{cat} remained unchanged under these conditions.

For the same reasons as specified above, additional assays of dCA I and C-ter at variable pHs and salinities were conducted with a single substrate concentration (50 mM Na bicarbonate).

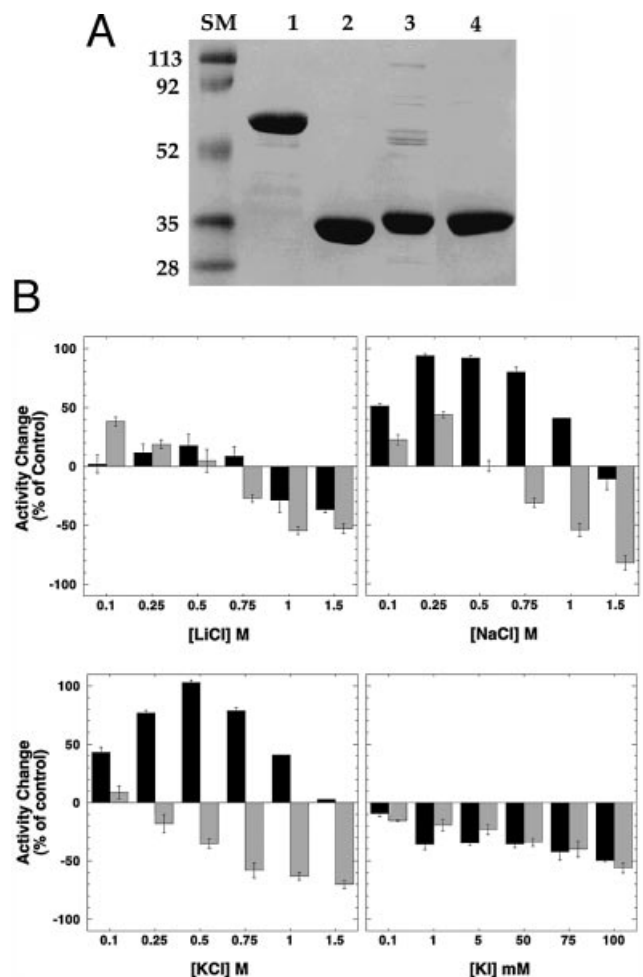


Fig. 4. Affinity purification of recombinant dCA I, N-ter and C-ter and the effect of salt on CO₂ hydration activity of C-ter. (A) dCA I, N-ter and C-ter were expressed in *E. coli* and purified as described in Materials and methods. Recombinant proteins were resolved on 10% SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. SM, size markers in kDa; lane 1, PAMBS-based affinity purification of recombinant dCA I; lane 2, PAMBS-based affinity purification of C-ter; lane 3, Ni-NTA-based affinity purification of His-tagged N-ter; lane 4, HiTrap Q fractionation of purified His-tagged N-ter. (B) CO₂ hydration activities of C-ter assayed in mixtures as described in Figure 1 in the presence of different concentrations of the indicated salts (gray bars) are depicted alongside corresponding activities for dCA I (black bars) as percentage change in activity relative to controls without added salts.

Table II. Effect of salt on the steady-state kinetic constants of bicarbonate dehydration activity of dCA I, C-ter and hCA I

Enzyme	[NaCl] (M)	K_{m} (mM)	k_{cat} [(ms) ⁻¹]	k_{enz} [(M μs) ⁻¹]
dCA I	0	37.3 ± 7.1	39.4 ± 3.8	1.0 ± 0.2
	0.1	37.1 ± 4.6	41.6 ± 2.2	1.1 ± 0.2
	0.5	45.0 ± 7.3	45.0 ± 4.1	1.0 ± 0.2
C-ter	0	>120	ND ^a	ND
	0.5	118.0 ± 29.7	81.1 ± 12.3	0.7 ± 0.2
hCA I	0	22.4 ± 3.9	31.8 ± 1.9	1.4 ± 0.3
	0.1	89.8 ± 22.2	30.0 ± 4.4	0.3 ± 0.1

Initial rates of bicarbonate dehydration activity were measured in 50 mM MOPS, pH 6.8 and the indicated concentrations of NaCl, as described in Materials and methods. Na bicarbonate concentrations were 5–90 mM in assays of dCA I and hCA I and 5–120 mM in assays of C-ter. The enzyme concentrations used were 0.22, 1.8 and 0.28 μM for dCA I, C-ter and hCA I, respectively. The kinetic constants were derived by fitting the data to the Michaelis–Menten equation.

^aND, not determined.

The results (Table III; Figure 6), expressed as $V_0/[E]_0$, indicated a parallel decline in the activities of dCA I and C-ter with a rise in pH from 5.6 to 7.4. Within this pH range, C-ter displayed 5–8% of the activity of dCA I. Similar analyses of N-ter (data not shown) showed that it exhibited at most ~2% of dCA I activity and it was not analyzed further. The activity of dCA I was practically unaffected at ≤ 0.25 M NaCl and at most ~40% reduced at 0.5 M NaCl. In comparison, the responses of C-ter were markedly more sensitive to salt and pH variations. The bar diagram in Figure 6 shows the effects of salt at each pH normalized to the corresponding activity in the absence of salt. The moderate effects of pH changes on the salt responses of dCA I contrast with the large fluctuations exhibited by C-ter, including inhibition at pH 5.6, salt concentration-dependent inhibitory or stimulatory responses at pH 6.2 and 6.8 and stimulatory effects at pH 7.4.

Esterase activity

The esterase activity of α -type carbonic anhydrases shares several basic characteristics with reversible CO_2 hydration activity, although some disparities have also been documented (Pocker and Stone, 1967; Verpoorte *et al.*, 1967; Baird *et al.*, 1997; Elleby *et al.*, 1999). Preliminary experiments demonstrated that dCA I possessed esterase activity and indicated

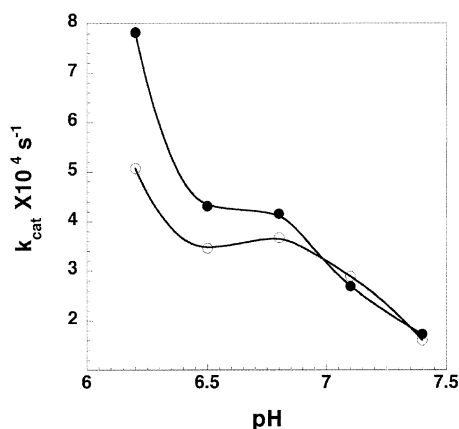


Fig. 5. pH dependence of k_{cat} for bicarbonate dehydration by dCA I. Bicarbonate dehydration activity was assayed at ionic strengths of 0.05 M (adjusted for different buffers and indicators with Na_2SO_4) and 0.15 M (with added 0.1 M NaCl) at pH 5.6–7.4, as described in Materials and methods. The kinetic data were fitted to the Michaelis–Menten equation for a single substrate-binding site per enzyme molecule and the derived k_{cat} constants were plotted against pH (open circles, 0.05 M ionic strength; filled circles, 0.15 M ionic strength).

that, similarly to hCA I, it preferred *p*-nitrophenyl acetate over *o*-nitrophenyl acetate as a substrate (data not shown). Assays under the same conditions for N-ter and C-ter at up to ~ 3 μM protein did not reveal any esterase activity.

The pH dependence curve for the catalysis of *p*-nitrophenyl acetate hydrolysis by dCA I corresponded to a $\text{p}K_{\text{a}}$ of 7.3 (data not shown), similar to the value reported for hCA I, suggesting that the same ionizable group(s) may be involved in catalysis by the two enzymes (Pocker and Stone, 1967).

As pointed out in earlier studies (Verpoorte *et al.*, 1967), the low water solubility of the substrate made it difficult to reach concentrations high enough to approach K_{m} values. However, kinetic data with 1–3 mM substrate at 0–2.0 M NaCl, obtained from slopes of Lineweaver–Burk plots, permitted the determination of k_{enz} values for dCA I under these conditions (Table IV). The results indicated that k_{enz} for dCA I increased with salt up to ~ 2 -fold at 2.0 M NaCl. In comparison, the k_{enz} for hCA I was $\sim 80\%$ reduced at 0.1 M NaCl, in line with the reported inhibitory effects of anions on esterase activity of several α -type carbonic anhydrases (Pocker and Stone, 1967; Thorslund and Lindskog, 1967; Maren and Couto, 1979).

Discussion

The results demonstrate the unique status of dCA I as an exceptionally salt-tolerant member of a family of enzymes typically susceptible to salt inhibition. Although indirect effects of salt on the rate of uncatalyzed background reactions and substrate solubility imposed limits on the salt concentrations that could be accurately tested in the assays, activity could be detected up to 4.0 M NaCl (data not shown).

As manifested in CO_2 hydration assays, dCA I differed drastically from hCA I and other mesophilic counterparts in remaining active in the presence of molar concentrations of Cl^- and Br^- and in showing a lowered sensitivity to I^- and N_3^- . However, the fact that dCA I was inhibited similarly to hCA I by ClO_4^- and sulfonamides suggests a similarity in the affinity of these inhibitors to the two enzymes. The differential responses of dCA I to the two classes of inhibitor may be related to their different modes of interaction with the protein as suggested by structure determinations of protein–inhibitor complexes. Thus, Br^- or N_3^- complexed with hCA II and I^- complexed with hCA I were coordinated to the Zn in a distorted tetrahedral geometry (Jonsson *et al.*, 1993; Nair and Christianson, 1993; Liljas *et al.*, 1994), whereas sulfonamides coordinated to the Zn in a regular tetrahedral configuration stabilized by H-bonding to the $\text{O}\gamma 1$ of Thr199 (Liljas *et al.*, 1994). Still, these differences cannot account for the apparently

Table III. Effect of salt on bicarbonate dehydration activity of dCA I and C-ter at various pHs

[NaCl] (M)	Bicarbonate dehydration activity ($V_0/[E]_0$)							
	pH 5.6		pH 6.2		pH 6.8		pH 7.4	
	dCA I	C-ter	dCA I	C-ter	dCA I	C-ter	dCA I	C-ter
0	43.4 \pm 1.4	2.9 \pm 0.2	29.0 \pm 1.0	1.5 \pm 0.1	16.3 \pm 0.4	0.9 \pm 0.02	9.7 \pm 0.5	0.7 \pm 0.02
0.1	43.0 \pm 1.9	1.4 \pm 0.2	23.4 \pm 0.7	2.0 \pm 0.1	17.1 \pm 0.6	1.5 \pm 0.03	8.5 \pm 0.3	1.0 \pm 0.03
0.25	44.4 \pm 1.2	1.8 \pm 0.1	25.2 \pm 1.1	1.0 \pm 0.1	17.5 \pm 0.7	1.5 \pm 0.03	5.7 \pm 0.2	1.0 \pm 0.02
0.5	27.6 \pm 0.8	1.1 \pm 0.1	18.7 \pm 0.7	0.5 \pm 0.1	15.0 \pm 0.5	0.8 \pm 0.03	7.0 \pm 0.3	0.9 \pm 0.05

Bicarbonate dehydration activity was measured as described in Materials and methods with 50 mM Na bicarbonate at the indicated pHs and NaCl concentrations. The enzyme concentrations were 0.36 and 1.52 μM for dCA I and C-ter, respectively. Activity [ms^{-1}] is expressed as $V_0/[E]_0$, where V_0 is the steady state initial rate of the reaction and $[E]_0$ is the enzyme molar concentration. (The data in this table are presented in a normalized form in Figure 6.)

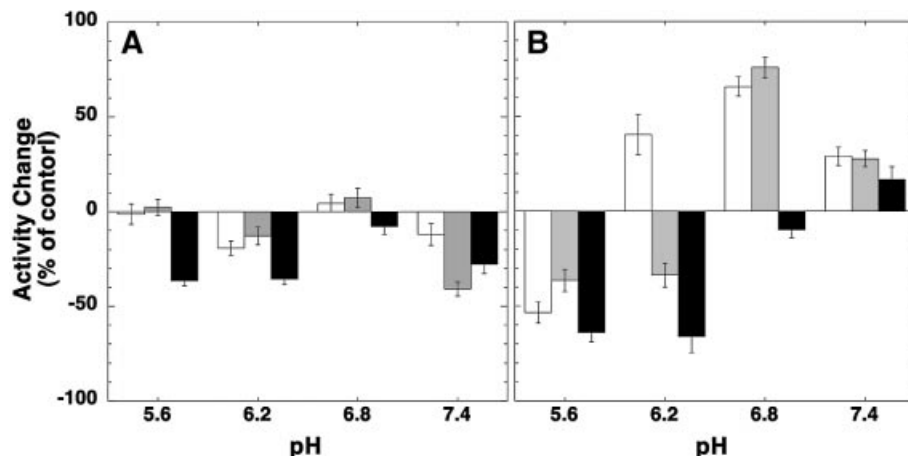


Fig. 6. Effect of salt on the bicarbonate dehydration activity of dCA I and C-ter at various pHs. Bicarbonate dehydration activity of (A) dCA I (0.36 μM) and (B) C-ter (1.52 μM) was assayed at different pHs with the indicated concentrations of NaCl as described in Materials and methods. For each pH, the effects of the three salinities tested are depicted as percentage change relative to the activity in the absence of NaCl. Empty columns, 0.1 M NaCl; gray columns, 0.25 M NaCl; black columns, 0.5 M NaCl.

Table IV. Effect of salt on the steady-state kinetic constants of esterase activity of dCA I and hCA I

Enzyme	[NaCl] (M)	k_{enz} [10^{-6} (ms) $^{-1}$]	K_{enz} (% of control ^a)
dCA I	0	113.1 \pm 2.5	100.0
	0.4	144.2 \pm 4.4	127.5 \pm 4.8
	1	151.8 \pm 4.8	134.2 \pm 5.2
	2	244.9 \pm 10.4	216.5 \pm 10.4
hCA I	0	497.8 \pm 7.5	100.0
	0.1	119.5 \pm 2.1	24.0 \pm 0.6

Esterase activity was assayed as described in Materials and methods. The enzyme concentrations used were 1.66 and 0.54 μM for dCA I and hCA I, respectively. The K_{enz} values were derived from the slope of the Lineweaver–Burk plot.

^aActivity in the absence of salt.

exceptional low affinity of the enzyme to halide and azide anions.

Aside from its apparent low affinity for anions, dCA I is up to 2-fold stimulated in CO_2 hydration activity by cations such as Na^+ , K^+ or Mg^{2+} . Although activation of various α -type carbonic anhydrases by a host of organic compounds, including amines and amino acids, has been widely documented (Supuran and Scozzafava, 2000), cation activation as observed for dCA I appears to have no parallel in hCA I and other, homologous enzymes. Other possible manifestations of cation activation of dCA I include the rise in k_{enz} for esterase activity with increasing NaCl concentrations [for effects of salt on esterase activity of other carbonic anhydrases, see elsewhere (Pocker and Stone, 1967; Thorslund and Lindskog, 1967; Steiner and Lindskog, 1972; Maren and Couto, 1979)] and the increase in turnover number for bicarbonate dehydration with ionic strength.

The pH dependence curve of k_{cat} is consistent with $\text{p}K_{\text{a}}$ s of 6.5 and 6.8–7, originating in still unidentified ionizable groups. In other α -type carbonic anhydrases, $\text{p}K_{\text{a}}$ s were attributed to Zn-bound water and the proton-shuttling residue His64, present in hCA II, hCA IV and murine carbonic anhydrase VII (mCA VII) (Earnhardt *et al.*, 1998). Another proposal, perhaps relevant to dCA I, ascribes one of two $\text{p}K_{\text{a}}$ s observed for the

k_{cat} for CO_2 hydration activity of mCA VII to perturbed ionization of Zn-bound water by electrostatic interactions with nearby groups (Earnhardt *et al.*, 1998). However, according to sequence alignments, dCA I does not include His64 or other residues so far implicated in proton shuttling in other enzymes, e.g. His200 in hCA I and Tyr131 in human carbonic anhydrase V (hCA V) (Baird *et al.*, 1997; Qian *et al.*, 1999).

The degree and mode of anion inhibition of various α -type carbonic anhydrases have been shown to be pH dependent (Pocker and Stone, 1967; Baird *et al.*, 1997). This dependence has been linked to the ionization of the Zn-bound water and taken to reflect a higher affinity of anions to water-bound compared with hydroxyl-bound Zn (Baird *et al.*, 1997). Thus, the K_i for sulfate inhibition of bicarbonate dehydration activity of hCA IV gradually rose to reach a 5-fold increase as the pH was shifted from 5.5 to 7.0 (Baird *et al.*, 1997). In comparison, the bicarbonate dehydration activity of dCA I showed only moderate or no response to salt at pH 5.6–7.4 (cf. Table III). This behavior suggests that ionizable groups unique for dCA I may modulate long-range interactions so as to generate a ‘buffering’ capacity that maintains the salt responses of dCA I within relatively narrow limits over a broad pH range.

The unique properties of dCA I can be partly attributed to its two-domain structure. Multiple sequence alignments indicated the conservation in both N- and C-terminal domains of the Zn-coordinating residues His94, His96 and His119, the hydrophobic residues in the substrate-association pocket Val121, Leu141, Val143, Leu198, Val207 and Trp209 and the residues involved in the active-site hydrogen-bonded network involving Glu106 and Thr199 (cf. Figure 2). The kinetic analysis was compatible with the notion that dCA I harbored only a single type of substrate-binding site. Alternatively, the results may suggest that despite their sequence similarity, only one of the two domains is catalytically active. That this site might be localized to the C-terminal domain is supported by analyses of truncated derivatives of dCA I showing that C-ter, but not N-ter, bound to the PAMBS affinity column and, similarly to p40, was enzymatically active. Still, the reduced activity of the single-domain derivatives relative to dCA I points to the large contribution of inter-domain interactions to the enzymatic efficiency.

The salt responses of CO₂ hydration activity catalyzed by C-ter and p40 largely recapitulated the responses of dCA I, albeit to a lower extent, indicating that essential elements required for salt tolerance are localized, although not fully expressed, in the C-terminal domain. The apparent enhancement of substrate affinity (lowering of K_m for bicarbonate) by salt observed in bicarbonate dehydration assays of C-ter, but not of dCA I, most likely reflected the stabilization by salt of C-ter, perhaps mimicking interactions of the C-terminal with the N-terminal domain within dCA I. Similar interactions may also be invoked to explain the differential effects of pH on the salt responses of C-ter and dCA I as expressed in bicarbonate dehydration activity. Overall, these results indicate that inter-domain interactions play a decisive role in the salt responses of dCA I.

Attempts to elucidate these interactions further via the reconstitution of a native-like enzyme by mixing N-ter and C-ter have so far been unsuccessful (data not shown). However, stable inter-domain interactions were indicated by observations with proteolytically cleaved dCA I. The p20 and p40 sub-fragments of dCA I (Figure 2) partly co-fractionated on both affinity and ion-exchange columns (cf. Figure 3 and unpublished data). Furthermore, purified recombinant dCA I, with six extra amino acids inserted between the N- and C-terminal repeats, was spontaneously cleaved into sub-fragments similar in size to p20 and p40 and exhibited the same activity and salt tolerance as the uncleaved dCA I (data not shown). These results strongly support the existence of stable interactions between the N- and C-terminal domains within the structure of dCA I.

Another structural distinction of dCA I, potentially linked to salt tolerance, lies in its biased amino acid composition (Fisher *et al.*, 1996), reminiscent of proteins from extreme halophilic archaea (Mevarech *et al.*, 2000; Fukuchi *et al.*, 2003) but atypical for most other α -type carbonic anhydrases (Gokhman *et al.*, 1999). Like halophilic proteins, the amino acid composition of dCA I is characterized by a decreased proportion of basic amino acid residues, particularly Lys, while the contents of Asp and Glu remain largely unchanged.

The most outstanding characteristic of halophilic enzymes is their obligatory requirement for multimolar concentrations of KCl or NaCl for proper folding and conformational stability (Lanyi, 1974; Eisenberg *et al.*, 1992; Madern *et al.*, 2000; Mevarech *et al.*, 2000). The effects of various salts on the conformational stability of the best-characterized halophilic enzyme, malate dehydrogenase from *Haloarcula marismortui*, was interpreted as reflecting the superposition of independent contributions of cations and anions, partly in accordance with the Hofmeister series, which classifies ion-protein interactions according to their effect on solubility and stability (Ebel *et al.*, 1999b). Proposed mechanisms to account for the obligatory salt requirement of halophilic proteins include, among others, the formation of hydrated cation networks on clusters of negative charges and ion binding to specific sites on the folded structure (Dym *et al.*, 1994; Frolow *et al.*, 1996; Madern *et al.*, 2000).

Electrostatic potential calculations, based on homology modeling of the C-terminal repeat of dCA I, indicated a highly negative surface potential (data not shown) contrasting with other α -type carbonic anhydrases. Similarly to halophilic proteins, a hydrated cation network may promote the global stability of the protein at high salinities. However, dCA I does not exhibit an obligatory salt requirement for proper folding

and activity. In further support of the latter conclusion, we observed that dCA I, unfolded in 8.0 M urea, could be refolded to a fully active form in the absence of salt. Circular dichroism spectra of dCA I taken in the presence of 0–3.0 M NaCl did not indicate any conformational changes in dCA I throughout this salinity range (unpublished data).

In conclusion, the present analyses provide clear evidence that dCA I is a uniquely salt-tolerant protein, i.e. retains an active conformation over a broad range of salinities and, as a Zn metalloenzyme, largely avoids the specific inhibitory effects of anions. A detailed elucidation of the structural features underlying the outstanding salt responses of dCA I as well as of dCA II, currently under way, will contribute to the fundamental understanding of halotolerance and its implied applications.

Acknowledgements

This work was supported by Minerva Foundation (Germany), Magnet program (Israel Ministry of Industry and Commerce), NBT (Eilat, Israel) and Nikken-Sohonsa Corporation (Gifu, Japan). J.L.S. is the Morton and Gladys Pickman Professor of Structural Biology.

References

- Ahmad,A., Akhtar,M.S. and Bhakuni,V. (2001) *Biochemistry*, **40**, 1945–1955.
- Avron,M. (1986) *Trends Biochem. Sci.*, **11**, 5–6.
- Azachi,M., Sadka,A., Fisher,M., Goldshlag,P., Gokhman,I. and Zamir,A. (2002) *Plant Physiol.*, **129**, 1320–1329.
- Baird,T.T., Waheed,A., Okuyama,T., Sly,W.S. and Fierke,C.A. (1997) *Biochemistry*, **36**, 2669–2678.
- Ben-Amotz,A. and Avron,M. (1983) *Annu. Rev. Microbiol.*, **37**, 95–119.
- Booth,W.A. and Beardall,J. (1991) *Phycologia*, **30**, 220–225.
- Burnell,J.N. (2000) In Chegwiddden,W.R., Carter,N.D. and Edwards,Y.H. (eds), *The Carbonic Anhydrases – New Horizons*. Birkhauser Verlag, Basel, pp. 501–518.
- Carlier,P.R., Han,Y.F., Chow,E.S., Li,C.P., Wang,H., Lieu,T.X., Wong,H.S. and Pang,Y.P. (1999) *Bioorg. Med. Chem.*, **7**, 351–357.
- Chantawannakul,P., Yoshimune,K., Shirakihara,Y., Shiratori,A., Wakayama,M. and Moriguchi,M. (2003) *Acta Crystallogr. D*, **59**, 566–568.
- Deutch,C.E. (2002) *Lett. Appl. Microbiol.*, **35**, 78–84.
- Dym,O., Mevarech,M. and Sussman,J.L. (1994) *Science*, **267**, 1344–1346.
- Earnhardt,J.N., Qian,M., Tu,C., Lakkis,M.M., Bergenhem,N.C., Laipis,P.J., Tashian,R.E. and Silverman,D.N. (1998) *Biochemistry*, **37**, 10837–10845.
- Ebel,C., Faou,P., Franzetti,B., Kernel,B., Madern,D., Pascu,M., Pfister,C., Richard,S. and Zaccari,G. (1999a) In Oren,A. (ed.), *Microbiology and Biogeochemistry of Hypersaline Environments*. CRC Press, Boca Raton, FL, pp. 227–237.
- Ebel,C., Faou,P., Kernel,B. and Zaccari,G. (1999b) *Biochemistry*, **38**, 9039–9047.
- Eisenberg,H., Mevarech,M. and Zaccari,G. (1992) *Adv. Protein Chem.*, **43**, 1–62.
- Elleby,B., Sioblom,B. and Lindskog,S. (1999) *Eur. J. Biochem.*, **262**, 516–521.
- Fisher,M., Pick,U. and Zamir,A. (1994) *Plant Physiol.*, **106**, 1359–1365.
- Fisher,M., Gokhman,I., Pick,U. and Zamir,A. (1996) *J. Biol. Chem.*, **271**, 17718–17723.
- Franchi,M., Vullo,D., Gallori,E., Antel,J., Wurl,M., Scozzafava,A. and Supuran,C.T. (2003) *Bioorg. Med. Chem. Lett.*, **13**, 2857–2861.
- Frolow,F., Harel,M., Sussman,J.L., Mevarech,M. and Shoham,M. (1996) *Nat. Struct. Biol.*, **3**, 452–458.
- Fukuchi,S., Yoshimune,K., Wakayama,M., Moriguchi,M. and Nishikawa,K. (2003) *J. Mol. Biol.*, **327**, 347–357.
- Fukuzawa,H., Tsuzuki,M. and Miyachi,S. (2000) In Chegwiddden,W.R., Carter,N.D. and Edwards,Y.H. (eds), *The Carbonic Anhydrases – New Horizons*. Birkhauser Verlag, Basel, pp. 535–546.
- Gokhman,I., Fisher,M., Pick,U. and Zamir,A. (1999) In Oren,A. (ed.), *Microbiology and Biogeochemistry of Hypersaline Environments*. CRC Press, Boca Raton, FL, pp. 203–213.
- Husic,H.D. (1990) *Can. J. Bot.*, **69**, 1079–1087.
- Jonsson,B.M., Hakansson,K. and Liljas,A. (1993) *FEBS Lett.*, **322**, 186–190.
- Khalifah,R.G. (1971) *J. Biol. Chem.*, **246**, 2561–2573.
- Kozliak,E.I., Guilloton,M.B., Fuchs,J.A. and Anderson,P.M. (2000) In

- Chegwidden,W.R., Carter,N.D. and Edwards,Y.H. (eds), *The Carbonic Anhydrases – New Horizons*. Birkhauser Verlag, Basel, pp. 547–565.
- Lanyi,J. (1974) *Bacteriol. Rev.*, **38**, 272–290.
- Liljas,A., Kannan,K.K., Bergsten,P.C., Waara,I., Fridborg,K., Strandberg,B., Carlbom,U., Jarup,L., Lovgren,S., Petef,M. (1972) *Nat. New Biol.*, **235**, 131–137.
- Liljas,A., Hakansson,K., Jonsson,B.H. and Xue,Y. (1994) *Eur. J. Biochem.*, **219**, 1–10.
- Lindskog,S. and Silverman,D.N. (2000) In Chegwidden,W.R., Carter,N.D. and Edwards,Y.H. (eds), *The Carbonic Anhydrases – New Horizons*. Birkhauser Verlag, Basel, pp. 175–195.
- Madern,D., Ebel,C. and Zaccari,G. (2000) *Extremophiles*, **4**, 91–98.
- Maren,T. and Couto,E. (1979) *Arch. Biochem. Biophys.*, **196**, 501–510.
- Merz,K. (1990) *J. Mol. Biol.*, **214**, 397–412.
- Mevarech,M., Frolow,F. and Gloss,L. (2000) *Biophys. Chem.*, **86**, 155–164.
- Murakami,H. and Sly,W.S. (1987) *J. Biol. Chem.*, **262**, 1382–1388.
- Nair,S.K. and Christianson,D.W. (1993) *Eur. J. Biochem.*, **213**, 507–515.
- Parkkila,S. (2000) In Chegwidden,W.R., Carter,N.D. and Edwards,Y.H. (eds), *The Carbonic Anhydrases – New Horizons*. Birkhauser Verlag, Basel, pp. 79–93.
- Pocker,Y. and Miao,C.H. (1987) *Biochemistry*, **26**, 8481–8486.
- Pocker,Y. and Stone,J.T. (1967) *Biochemistry*, **6**, 668–678.
- Premkumar,L., Greenblatt,H.M., Sussman,J.L., Bageshwar,U., Savchenko,T., Gokhman,I. and Zamir,A. (2002) *Acta Crystallogr. A*, **58**, C307.
- Premkumar,L., Bageshwar,U.K., Gokhman,I., Zamir,A. and Sussman,J.L. (2003a) *Protein Expr. Purif.*, **28**, 151–157.
- Premkumar,L., Greenblatt,H.M., Bageshwar,U.K., Savchenko,T., Gokhman,I., Zamir,A. and Sussman,J.L. (2003b) *Acta Crystallogr. D*, **59**, 1084–1086.
- Qian,M., Earnhardt,J.N., Wadhwa,N.R., Tu,C., Laipis,P.J. and Silverman,D.N. (1999) *Biochim. Biophys. Acta*, **1434**, 1–5.
- Sadka,A., Lers,A., Zamir,A. and Avron,M. (1989) *FEBS Lett.*, **244**, 93–98.
- Stams,T. and Christianson,D.W. (2000) In Chegwidden,W.R., Carter,N.D. and Edwards,Y.H. (eds), *The Carbonic Anhydrases – New Horizons*. Birkhauser Verlag, Basel, pp. 159–174.
- Steiner,H. and Lindskog,S. (1972) *FEBS Lett.*, **24**, 85–88.
- Su,N.W. and Lee,M.H. (2001) *J. Ind. Microbiol. Biotechnol.*, **26**, 253–258.
- Supuran,C.T. and Scozzafava,A. (2000) In Chegwidden,W.R., Carter,N.D. and Edwards,Y.H. (eds), *The Carbonic Anhydrases – New Horizons*. Birkhauser Verlag, Basel, pp. 197–219.
- Thorslund,A. and Lindskog,S. (1967) *Eur. J. Biochem.*, **3**, 117–123.
- Verpoorte,J.A., Mehta,S. and Edsall,J.T. (1967) *J. Biol. Chem.*, **242**, 4221–4229.
- Vullo,D., Franchi,M., Gallori,E., Pastorek,J., Scozzafava,A., Pastorekova,S. and Supuran,C.T. (2003) *J. Enzyme Inhib. Med. Chem.*, **18**, 403–406.
- Whitney,P.L. (1974) *Anal. Biochem.*, **57**, 467–476.

Received December 30, 2003; accepted January 27, 2004

Edited by Dan Tawfik