

## An unusual halotolerant $\alpha$ -type carbonic anhydrase from the alga *Dunaliella salina* functionally expressed in *Escherichia coli*

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### Abstract

A 60-kDa, salt-inducible, internally duplicated  $\alpha$ -type carbonic anhydrase (Dca) is associated with the plasma membrane of the extremely salt-tolerant, unicellular, green alga *Dunaliella salina*. Unlike other carbonic anhydrases, Dca remains active over a very broad range of salinities (0–4 M NaCl), thus representing a novel type of extremely halotolerant enzyme. To elucidate the structural principles of halotolerance, structure–function investigations of Dca have been initiated. Such studies require considerable amounts of the enzyme, and hence, large-scale algal cultivation. Furthermore, the purified enzyme is often contaminated with other, co-purifying algal carbonic anhydrases. Expression in heterologous systems offers a means to produce, and subsequently purify, sufficiently large amounts of Dca required for activity and structural studies. Attempts to over-express Dca in the *Escherichia coli* BL21(DE3)pLysS strain, after optimizing various expression parameters, produced soluble, but weakly active protein, composed of fully reduced and variably –S–S– cross-linked chains (each of the Dca repeats contains a pair of cysteine residues, presumably forming a disulfide bond). However, when the *E. coli* Origami B(DE3)pLysS strain was used as a host, a functionally active enzyme with proper disulfide bonds was formed in good yield. Affinity-purified recombinant Dca resembled the native enzyme from *D. salina* in activity and salt tolerance. Hence, this expression system offers a means of pursuing detailed studies of this extraordinary protein using biochemical, biophysical, and crystallographic approaches.

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Most enzymes exhibit catalytic activity only under mild conditions and suffer structural and functional damage when exposed to high temperatures, extreme pH or high salinity. However, enzymes from extremophilic organisms such as extreme halophilic archaea and thermophiles properly fold and/or function optimally only in high salt concentrations [1,2] and elevated temperature [3], respectively. Very few, if any, enzymes tolerate both mild and extreme conditions.

A member of a new class of proteins, referred to as halotolerant, that remain active over an extremely broad range of salinities (from 0 to 4 M NaCl), was identified in our studies of the unicellular green alga *Dunaliella salina* [4]. The unique ability of *D. salina* to proliferate over the entire range of salinities is enabled, among other factors, by an efficient osmo-regulatory mechanism whereby the cells accumulate iso-osmotic levels of glycerol [5]. Therefore, intracellular algal proteins are not challenged by salt, whereas extracellular proteins are expected to have evolved outstanding salt tolerance.

We explored this expectation with the *D. salina*  $\alpha$ -type carbonic anhydrase, duplicated carbonic anhydrase (Dca), a plasma membrane-associated, outwardly oriented enzyme, of ~60 kDa, that catalyzes the reversible hydration of CO<sub>2</sub> [6] and also exhibits esterase activity. The inherently soluble Dca is composed of two tandem

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repeats, designated as N-Dca<sup>2</sup> and C-Dca, that share ~52% sequence identity. Each repeat shows considerable sequence homology with other algal and animal  $\alpha$ -type carbonic anhydrases. However, Dca is outstanding in containing a large excess of acidic over basic amino acid residues and an acidic pI, compared to other  $\alpha$ -type carbonic anhydrases [7].

Since Dca was localized on the cell surface and attributed an important role in providing cells with CO<sub>2</sub>, it was expected to remain functional over nearly the entire range of salinities [4]. Indeed, earlier studies revealed that, unlike mesophilic and halophilic enzymes, Dca does indeed remain active throughout broad concentration ranges of various salts [6] (Bageshwar et al., unpublished).

The elucidation of the structural and mechanistic factors that enable Dca to retain structural stability, solubility, and activity under such broadly varying ionic conditions is a highly challenging task. The understanding of the effect of salt on the nature of protein-solvent and intra- and inter-molecular protein interactions as well as the catalytic process itself is expected to shed light on fundamental issues of protein structure and function. It may also assist in potential biotechnological applications such as engineering enzymes that function in special solvents or over broadly changing salt concentrations [8].

Towards these goals, biochemical/biophysical, and crystallographic studies provide highly valuable tools. Such studies often require large amounts of the highly purified enzyme, the preparation of which involves the growth and processing of large algal cultures. Furthermore, obtaining highly purified preparations of the enzyme is complicated by the presence of co-purifying additional algal carbonic anhydrases (Bageshwar et al., unpublished). These difficulties can be potentially solved by the development of an efficient heterologous expression system for Dca. The obvious advantages of the use of the *Escherichia coli* system for this purpose include the ease of handling and generation of site-directed mutants.

Here, we report on the synthesis of recombinant Dca in *E. coli* and show that halotolerant proteins can be functionally over-expressed, purified, and stored similar to mesophilic proteins. The ability to produce recombinant Dca will aid in uncovering the structural principles that underlie the high degree of salt tolerance of these proteins and potentially open up new vistas in biotechnological applications.

## Materials and methods

### *Strains, plasmids, biochemicals, and chemicals*

The *E. coli* hosts BL21(DE3)pLysS [(F<sup>-</sup> *ompT hsdS<sub>B</sub>* (r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>) *gal dcm* (DE3) pLysS (Cm<sup>R</sup>)] and Origami B(DE3)pLysS [(F<sup>-</sup> *ompT hsdS<sub>B</sub>* (r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>) *lacYI gor522::Tn10* (Tc<sup>R</sup>) *trxB::kan* (DE3) pLysS (Cm<sup>R</sup>)], and the expression vectors pET28c and pET21c were obtained from Novagen (Madison, WI). Taq DNA polymerase (Taqzol) was purchased from Talron (Israel). Restriction enzymes were obtained from Biolabs (England). Electrophoretically purified human erythrocyte carbonic anhydrase (hCA-I) was obtained from Sigma. Antibiotics, IPTG, agarose-coupled p-aminomethyl benzene sulfonamide, and pNPA were from Sigma Chemicals. EDTA-free protease inhibitor cocktails were from Roche Molecular Biochemicals and Calbiochem. Luria-Bertani (LB) plates with appropriate antibiotics and 2× LB and 2YT growth media were obtained from the Bacteriology Services (Weizmann Institute) and prepared as described [9]. All other reagents were of analytical grade.

### *Expression vector design and PCR conditions*

The cDNA sequence encoding amino acid residues 55–589 of Dca (U53811) (the sequence encoding the initiation codon and leader peptide [6] was omitted) was amplified from the full-length cDNA clone by PCR. The forward (5'-GATTGGAATTCGGCTTGTGAGTGAGCCTCACG-3') and reverse (5'-GGAAAA GCTTTTAAGCAGCAGCACCGTTATATCCG-3') primers used for PCR amplification were designed to include *Eco*RI and *Hind*III restriction sites, respectively, and to permit directed, in-frame cloning into the expression vectors pET28c and pET21c. In addition to the nucleotides upstream to the restriction sites, the forward primer contained five nucleotides (GGCTT) downstream to the *Eco*RI site to facilitate successful PCR amplification. The conditions used for PCR amplification were 95 °C, 1 min (melting); 42 °C, 2 min (annealing); and 72 °C, 3 min (extension) for 4 cycles, followed by 0.5 °C raise/cycle in annealing temperature for another 11 cycles, followed by 12 additional cycles with annealing temperature of 52 °C. The PCR product was digested with *Eco*RI and *Hind*III and the digested fragment of 1619 bp was cloned into pET28c or pET21c vectors, double-digested with *Eco*RI and *Hind*III, to create the expression vectors p28c60 and p21c60, respectively. The insert was fused in-frame to upstream vector sequences containing a translation initiation site, followed by sequences encoding His-tag and T7-tag in p28c60, or only a T7-tag in p21c60 (see Novagen catalogue for sequences).

<sup>2</sup> Abbreviations used: pNPA, p-nitrophenyl acetate; CA, carbonic anhydrase; Amp, ampicillin; Kan, kanamycin; CAP, chloramphenicol; Tet, tetracycline; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; N-Dca, N-terminal of Dca; C-Dca, C-terminal of Dca; rDca, recombinant Dca.

### Small-scale expression

The *E. coli* host strain BL21(DE3)pLysS or Origami B(DE3)pLysS was transformed with p28c60 or p21c60, respectively. Transformants of the BL21(DE3)pLysS strain were selected on LB plates supplemented with 50 µg/mL Kan and 34 µg/mL CAP. Origami B pLysS transformants were selected on LB plates supplemented with 50 µg/mL Amp, 15 µg/mL Kan, 12.5 µg/mL Tet, and 34 µg/mL CAP. Well-grown single colonies from the selection plate were inoculated into culture media containing appropriate antibiotics and incubated overnight at 37 °C in an orbital shaker (225 rpm). Ten mL of this culture was added to 1 L fresh culture medium and the culture was grown in an orbital shaker at 37 °C, for 2–2.5 h, to  $A_{600}$  of 0.6. Thereafter, expression of Dca was induced by adding IPTG to 1 mM concentration and incubation was continued for 10 h at 25 °C. The bacterial cells were collected by centrifugation for 10 min at 5000g and stored at –80 °C until use. The 2× LB medium with 50 µg/mL Kan and 34 µg/mL CAP, or 2YT medium with 50 µg/mL carbenicillin and 34 µg/mL CAP, was used to grow BL21(DE3)pLysS or Origami B(DE3)pLysS, respectively.

### Large-scale expression

For large-scale expression, cultures were grown in a 12 L fermentor (BioFlo 2000 or New Brunswick Scientific, USA MODEL 19). The speed of the agitator shaft was maintained at 300–350 rpm and aeration was supplied at a constant rate of 10 standard liters per minute. The growth medium and antibiotics used were as described for the flask cultures. In general, 100–120 mL starter culture was added to 10–12 L culture medium in the fermentor. The fermentor was first operated at 37 °C until the bacterial culture reached  $A_{600}$  of 0.5 (~4–5 h), the temperature was then lowered to 25 °C and Dca expression was induced by adding IPTG to 1 mM final concentration and incubation for 10–12 h before harvesting the cells.

### Single-step affinity-purification of Dca

Approximately 10 g pelleted *E. coli* cells were suspended in 40 mL lysis buffer containing 25 mM Tris–SO<sub>4</sub>, pH 7.5, 2 mM EDTA, 0.1% Triton X-100, and 1 mL EDTA-free protease cocktail. Cells were lysed by repeated freezing in liquid nitrogen and thawing in an ice-cold water bath. After the first cycle of freezing and thawing, DNase I, MgSO<sub>4</sub>, and ZnSO<sub>4</sub> were added to the bacterial lysate to final concentrations of 20 µg/mL, 15 mM, and 0.5 mM, respectively. The lysate was spun at 178,000g for 1 h in an ultracentrifuge (Beckman, Ti60) and the resulting supernatant was subjected to affinity-purification.

The supernatant was loaded onto a 10-mL affinity column (agarose-coupled aminomethyl benzene sulfonamide), which was pre-equilibrated with equilibration buffer (50 mM Na<sub>2</sub>SO<sub>4</sub>, 25 mM Tris–SO<sub>4</sub>, pH 7.5). The column was initially washed with 300 mL wash buffer I (50 mM Na<sub>2</sub>SO<sub>4</sub>, 200 mM NaClO<sub>4</sub>, and 25 mM Tris–SO<sub>4</sub>, pH 7.5) and then with 50 mL equilibration buffer. Non-specifically bound proteins were removed further with 300 mL wash buffer II (300 mM Na<sub>2</sub>SO<sub>4</sub>, 25 mM Tris–SO<sub>4</sub>, pH 7.5), followed by 50 mL of 100 mM sodium acetate, pH 6.0, and subsequently with 250 mL of 200 mM Na<sub>2</sub>SO<sub>4</sub>, 100 mM sodium acetate, pH 6.0. The bound Dca was eluted with elution buffer (200 mM NaClO<sub>4</sub>, 100 mM sodium acetate, pH 5.6), as described [10]. All buffers employed in the purification contained 0.5 mM ZnSO<sub>4</sub> and complete EDTA-free protease cocktail from Roche Molecular Biochemicals (1 tablet per 50 mL). The eluted fractions containing Dca were extensively dialyzed against 25 mM Tris–SO<sub>4</sub> (pH 8.8) and 10 µM ZnSO<sub>4</sub> and concentrated using Centricon spin columns (YM-30, Amicon). The entire purification procedure was carried out at 4 °C.

### Electrometric assay for carbonic anhydrase activity

Carbonic anhydrase activity was determined as described [6], with some modifications. The assay was performed in the presence or absence of NaCl in a double-walled chamber with circulation of ice-cold water containing a total reaction volume of 2.75 mL. The enzyme-catalyzed reaction mixtures contained 2 µg Dca in 25 mM Tris–SO<sub>4</sub>, pH 8.3, and the reaction was initiated by adding 0.3 mL ice-cold CO<sub>2</sub>-saturated water. The enzyme activity was determined by monitoring the time required for the pH of the assay solution to change from pH 8.3 to 7.3. The pH was recorded using a pH meter interfaced to a PC. CA activity is expressed in Wilbur–Anderson (WA) units per milligram of protein and was calculated using the formula  $[(t_0/t) - 1] \times 10/\text{protein (mg)}$ , where  $t_0$  and  $t$  represent the times required for the pH to change from 8.3 to 7.3 in a buffer control (non-catalyzed) and enzyme catalyzed reaction, respectively.

### Esterase activity

The esterase activity of Dca was spectrophotometrically assayed in the presence or absence of NaCl as described [11]. The 1-mL assay mixtures contained 1 mM *p*NPA, 3% acetone, and 25 mM Tris–SO<sub>4</sub>, pH 8.0, and the reaction was started by the addition of 1.6 nmole Dca. The hydrolysis of *p*NPA into *p*NP was monitored at 25 °C by following the increase in absorbance at 348 nm. Background values for non-catalyzed ester hydrolysis were subtracted.

### Analytical procedures

SDS-PAGE [12] and immunoblot analysis under reducing and non-reducing conditions (i.e., in the presence or absence of DTT, respectively) were performed using a Bio-Rad small electrophoresis unit. Samples were prepared for electrophoresis by diluting in SDS-PAGE sample buffer (60 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, and 0.002% (w/v) bromophenol blue) and boiling for 5 min in the presence of 50 mM DTT (reducing conditions) or in the absence of DTT (non-reducing conditions). Samples were then subjected to electrophoresis on 10% polyacrylamide gels in the presence of SDS. Protein bands were visualized by staining with Coomassie blue. Immunoblot analysis was carried out, as described [6]. The blots were probed with rabbit anti-Dca polyclonal primary antibodies and horseradish peroxidase-conjugated rabbit IgG as secondary antibodies (at 1:7500 and 1:5000 dilutions, respectively).

### Results and discussion

#### Expression and purification of Dca

Our early attempts to express Dca in *E. coli* BL21(DE3)pLysS cells yielded mostly an insoluble recombinant protein. To increase the yield of soluble, active enzyme, we screened the effects of different parameters such as growth medium (M9 minimal medium, LB, or 2× LB), IPTG concentrations (0.1–2 mM), growth temperature (25–37 °C), cell disruption methods (sonication, freezing, and thawing in the presence or absence of detergents such as Triton X-100 or *N*-lauryl sarcosine and lysozyme) (data not shown). Optimal conditions for the synthesis of soluble Dca, chosen on the basis of these experiments, are presented in Materials and methods.

The crude extract of BL21(DE3)pLysS cells expressing Dca was applied to the affinity column and the purification was followed by Western blot analysis of differentially eluted fractions. A protein fraction that eluted with 200 mM NaClO<sub>4</sub> at pH 5.6 contained an immuno-reactive protein band of ~64 kDa, as expected for the 60-kDa Dca moiety fused to the 4-kDa N-terminal His- and T7-tag sequences (see Materials and methods). However, protein staining of the same gel showed, in addition to Dca, another major band of ~117 kDa (Fig. 1). Altered elution conditions from the affinity column or employing a Ni-NTA column (an affinity-matrix used to purify 'His-tagged' proteins) to remove the high molecular weight protein were unsuccessful. Subsequent analysis of the 117-kDa protein by peptide mapping identified it as β-galactosidase of host cell origin (data not shown) that accumulated during the

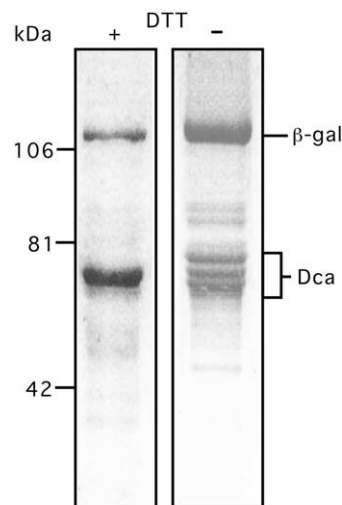


Fig. 1. Analysis of disulfide bond formation in affinity-purified Dca expressed in BL21(DE3)pLysS. Enzyme solutions pretreated with DTT (+) or without (-) pretreatment were analyzed by SDS-PAGE (10%, w/v), run under reduced and non-reduced conditions, respectively, and stained with Coomassie blue, as described in Materials and methods. Positions of β-galactosidase and different forms of Dca are indicated. Band identity was corroborated by immunoblot analyses (data not shown).

prolonged induction of Dca and bound non-specifically to the column resin.

Affinity chromatography on the affinity column yielded a 64-fold purified recombinant Dca, as compared to the crude *E. coli* extract (Table 1). However, the carbonic anhydrase activity of the purified enzyme was only ~400 U/mg protein, amounting to only ~10% of the activity of the native Dca isolated from *D. salina* (Bageshwar et al., unpublished).

Among several possibilities considered to account for the lower activity of the recombinant protein, we focused on incomplete disulfide bond formation, owing to the high intracellular redox potential in *E. coli* [13]. The existence of two intra-repeat disulfide bonds in Dca was inferred from the sequence alignment of each of the Dca repeats with extracellular human carbonic anhydrase isozymes (Fig. 2A and B) that indicated the conservation of two cysteine residues engaged in disulfide bond formation [14–16]. Indeed, SDS-PAGE and Western blot analysis under reducing and non-reducing conditions (Fig. 1) revealed that the recombinant Dca consisted of a mixture of molecules with partially and fully formed disulfide bonds. In line with this observation, the carbonic anhydrase activity of the recombinant Dca, after complete reduction with 10 mM DTT, showed ~5% (194 U/mg protein) of the activity of native enzyme in comparison to ~10% (338 U/mg protein) of the non-reduced recombinant enzyme. These results strongly suggested that the two pairs of disulfide bonds, formed between Cys residues at the N- and C-termini of each of the Dca repeats, were essential for maximal stability and activity (Fig. 2C).

Table 1  
Purification of recombinant Dca expressed in *E. coli*

<i>E. coli</i> strains	Total protein (mg)	Total activity (U)	Sp. activity (U/mg)	Relative yield	Purification fold
<i>BL21(DE3) pLysS</i>					
Crude extract	342	2075 <sup>a</sup>	6.1 <sup>c</sup>	100	1
Affinity-purified	4	1570 <sup>a</sup>	393 <sup>c</sup>	76	64.4
<i>Origami B(DE3) pLysS</i>					
Crude extract	1595	2696 <sup>b</sup>	1.7 <sup>d</sup>	100	1
Affinity-purified	15	2415 <sup>b</sup>	161 <sup>d</sup>	89.6	95.3

<sup>a</sup> CA activity.

<sup>b</sup> Esterase activity.

<sup>c</sup> Enzyme units are defined as Wilbur–Anderson (WA) units.

<sup>d</sup> One enzyme unit is defined as formation of nmole *p*NP per minute.

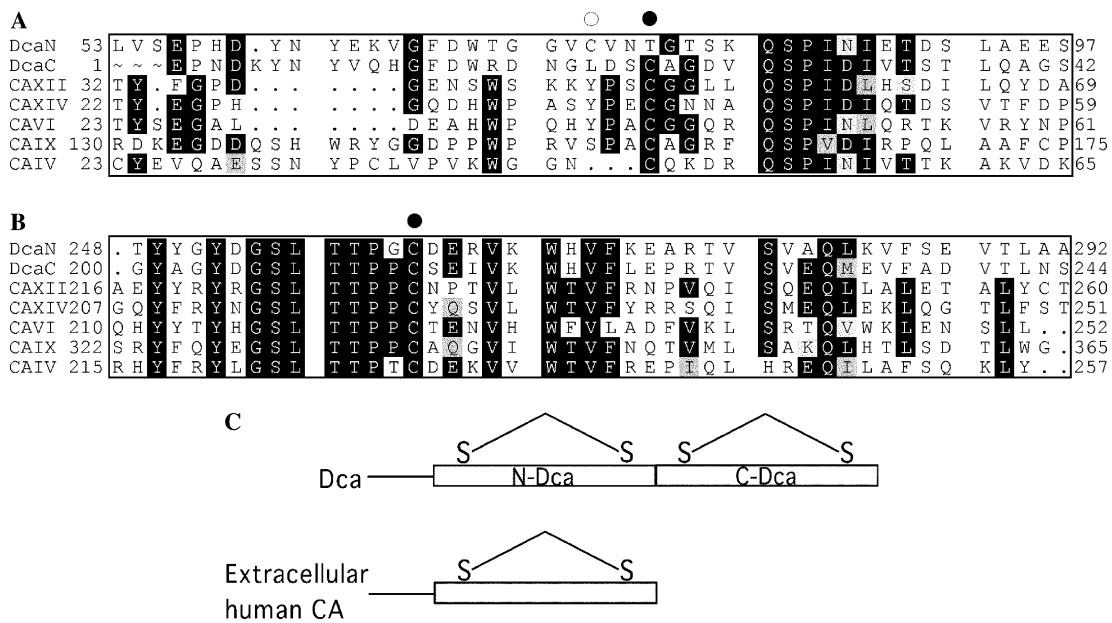


Fig. 2. Multiple amino acid sequence alignment of regions flanking conserved cysteines in extracellular human carbonic anhydrase isozymes IV, VI, IX, XII, and XIV and corresponding regions in N-Dca (DcaN) and C-Dca (DcaC). N-terminal sequences (A) and C-terminal sequences (B) were aligned using Pileup (GCG 10, Wisconsin package). Identities, black blocks; similarities, gray blocks; conserved Cys residues participating in disulfide bond formation, filled circles; Cys residue probably involved in disulfide bond formation in N-Dca, empty circle. (C) Schematic representation of disulfide bond(s) in extracellular human isozymes and Dca.

Based on this premise, we chose to try another *E. coli* expression host, *Origami B(DE3)pLysS*. In this host, mutations in *trx*B (thioredoxin) and *gor* (glutathione reductase) interfere with the cytoplasmic reduction pathway [13], thus facilitating the formation of disulfide bonds in proteins. The strain is also deleted for *lacY* (lac permease), a mutation that enables better control of the induction step and eliminates contamination with  $\beta$ -galactosidase.

The use of the *Origami* host made it necessary to insert the Dca cDNA in the compatible expression vector, pET21c. The use of this vector allowed one to express the inserted cDNA from an N-terminally fused T7-tag, but without a His-tag, which could potentially interfere with crystallization of the recombinant protein. The Dca was expressed initially in <2 L cultures grown

in flasks. Later, the process was scaled up to a 10–12 L fermentor, as described in Materials and methods. Growth in the fermentor increased biomass production to 4–5 g wet cells/L, amounting to a 2–3-fold enhancement compared to the flask cultures (1.5–2 g wet cells/L). Moreover, it was observed that Dca was relatively better expressed in the fermentor-grown culture compared to flask culture (data not shown).

The affinity-purified Dca appeared >90% pure on Coomassie-stained gels (Table 1, Fig. 3). SDS-PAGE under reducing and non-reducing conditions showed a single band. Carbonic anhydrase assays revealed that the activity of the recombinant protein was comparable to that of native Dca. In general, 1 g wet bacterial cell pellets yielded 1 mg of pure Dca. The bacterial cell pellets could be frozen and stored at

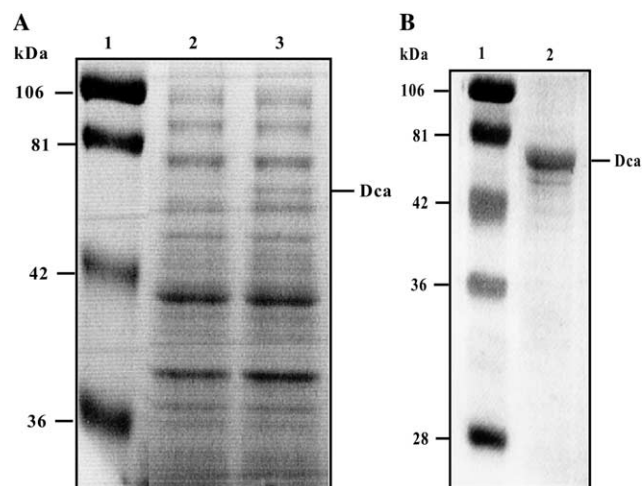


Fig. 3. Expression of Dca in the Origami B(DE3)pLysS strain and electrophoretic profile of purified recombinant Dca. Analysis by non-reducing SDS-PAGE and Coomassie staining of (A) lane 1, molecular-mass markers; lane 2, crude extract of Origami cells transformed with pET21c (control vector); lane 3, crude extract of Origami cells transformed with pET21c60, induced with 1mM IPTG for 10h at 25°C. (B) Affinity-purified Dca from Origami B(DE3)pLysS. Lane 1, molecular-mass markers; lane 2, Dca affinity-purified on an affinity column, as described in Materials and methods.

–80°C for several months without apparent loss in enzyme activity.

#### Concentration and storage

The purified Dca, dialyzed against 25 mM Tris-SO<sub>4</sub>, pH 7.5, could not be concentrated to over 0.7 mg/mL without noticeable precipitation. To attain higher con-

centrations of the protein, as required for crystallization, concentration of the Dca solution was attempted using centricon spin columns (YM-30, Amicon) spun with different buffers and varying ionic strengths and pHs. Each trial was started with 0.5 mL of 0.65 mg/mL Dca and the overall amount and concentration of Dca were determined after 30 min centrifugation at 1500 g (data not shown). Using 25 mM Tris-SO<sub>4</sub>, pH 8.8, as the buffer permitted one to concentrate Dca to over 9 mg/mL and the solution remained fully soluble and active for at least 2 months of storage at 4 or –20°C. It is noteworthy that the presence of salt (0–4 M NaCl) during Dca storage had no effect on the enzyme stability and activity.

#### Effect of salt on enzyme activity

The first indication for the outstanding salt-tolerance of Dca was provided by a comparison of the effect of salt on the carbonic anhydrase activity of a Dca-enriched crude fraction from *D. salina* with a crude fraction of periplasmic carbonic anhydrase from *Chlamydomonas reinhardtii*, a freshwater microalga [4]. The activity of Dca was not only retained in up to 2.3 M NaCl, but was actually stimulated by salt, whereas the *C. reinhardtii* enzyme was ~65% inhibited at 0.3 M salt. More recently, the salt tolerance of Dca was reaffirmed in analyses of homogeneously purified Dca as compared with the human hCA-I (Bageshwar et al., unpublished). The latter enzyme was chosen as a representative mesophilic carbonic anhydrase, since it was found to be the closest in sequence to Dca in pairwise sequence alignments (Bestfit, GCG package). Assays of carbonic anhydrase and

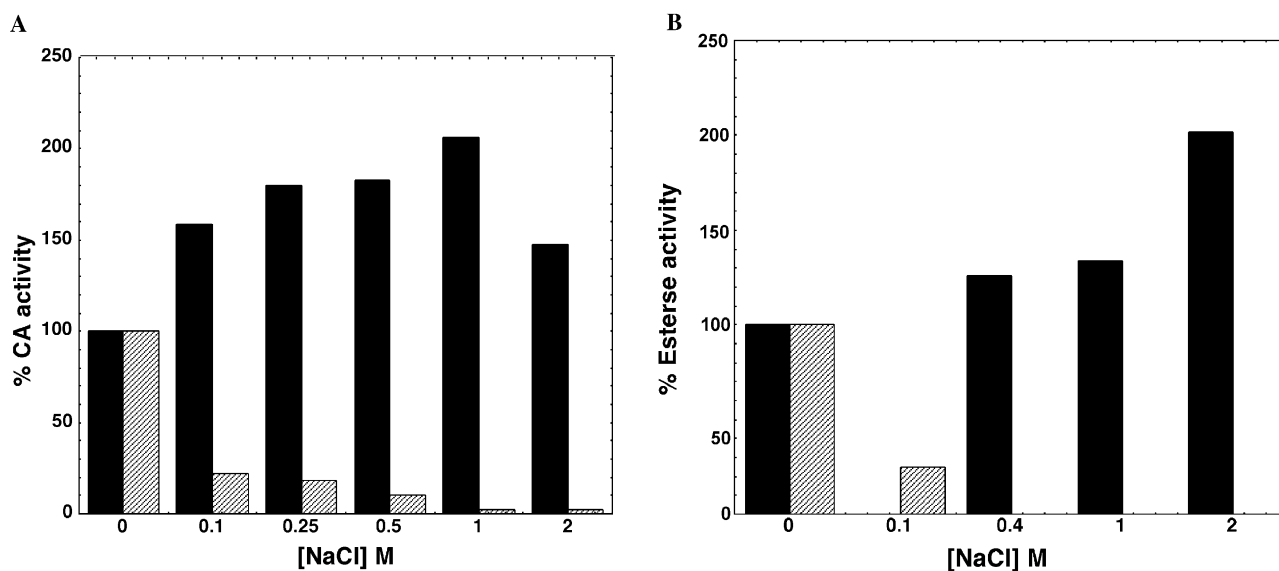


Fig. 4. Comparison of carbonic anhydrase (A) and esterase (B) activities of rDca (black bars) and human CA I (striped bars) in increasing NaCl concentrations. Esterase activity of hCA-I was very low at NaCl concentrations above 0.1 M. Assay conditions were as described in Materials and methods.

accompanying esterase activities re-established the outstanding salt-tolerance of Dca. In comparison, hCA-I was strongly inhibited already at 0.1 M NaCl.

To demonstrate that the recombinant Dca resembled the native protein in salt tolerance, carbonic anhydrase and esterase activity assays were performed in the presence of increasing NaCl concentrations (Fig. 4). The recombinant Dca not only withstood increasing salinities but was stimulated up to 2-fold in both activities. A similar stimulation of carbonic anhydrase activity was observed with the native Dca from *D. salina* [6] (Bageshwar et al., unpublished). In contrast, human CA I (hCA-I) was already inhibited at low salinities. Although salinities higher than 2 M NaCl decreased the solubility of CO<sub>2</sub> and *p*NPA, the respective substrates for carbonic anhydrase and esterase activities, enzymatic activities were manifested in up to 4 M NaCl (data not shown).

The production of large quantities of active, stable recombinant Dca paves the way for detailed biochemical, biophysical, and structural studies, including crystal structure determination, towards the understanding of how this protein achieves its extraordinary salt tolerance.

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