A Salt-resistant Plasma Membrane Carbonic Anhydrase Is Induced by Salt in Dunaliella salina*

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The mechanisms allowing proliferation of the unicellular green alga Dunaliellia salina in up to saturating NaCl concentrations are only partially understood at present. Previously, the level of a plasma membrane M, 60,000 protein, p60, was found to increase with rising external salinities. Based on cDNA cloning and enzymatic assays, it is now shown that p60 is an internally duplicated carbonic anhydrase, with each repeat homologous to animal and Chlamydomonas reinhardtii carbonic anhydrases, but exceptional in the excess of acidic over basic residues. Increasing salinities, alkaline shift, or removal of bicarbonate induced in D. salina parallel increases in the levels of p60, its mRNA, and external carbonic anhydrase activity. Moreover, purified p60 exhibited carbonic anhydrase activity comparable to other carbonic anhydrases. A p60-enriched soluble preparation showed maximal carbonic anhydrase activity at ~1.0 M NaCl and retained considerable activity at higher salt concentrations. In contrast, a similar preparation from C. reinhardtii was ~90% inhibited in 0.6 M NaCl. These results identified p60 as a structurally novel carbonic anhydrase transcriptionally regulated by CO2 availability and exhibiting halophilic-like characteristics. This enzyme is potentially suited to optimize CO2 uptake by cells growing in hypersaline media.

Mechanisms that enable plants to cope with water and salt stresses are of great current interest. Photosynthetic organisms displaying marked salt tolerance provide potentially useful models to study such mechanisms. The extremely salt-tolerant unicellular green algae of the genus Dunaliella are capable of growth in salinities as different as 0.05 and 5.0 M NaCl, while maintaining a relatively low intracellular sodium concentration (1). Lacking a rigid cell wall, these algae react to increases or decreases in the external salinity by immediate shrinking or swelling, respectively. Subsequent synthesis or elimination of glycerol to an intracellular concentration osmotically balancing the external salinity permits the cells to regain their original volume and resume growth (2, 3). The glycerol-mediated osmotic adjustment is only one of the means enabling algal survival in varying salinities. Other envisaged requirements include, for example, the control of ionic fluxes across the plasma membrane and the optimization of inorganic carbon uptake in the face of the severe limitation in CO2 availability in hypersaline media (4–6).

In an effort to identify components potentially involved in mechanisms enabling algal growth in high salinities, screens were previously conducted to identify proteins preferentially induced in Dunaliella salina growing in high salt. The level of two plasma membrane proteins, p150 (3) and p60 (7), was greatly elevated with increasing salt concentration. Furthermore, following a drastic hyperosmotic shock, the induction of these proteins coincided with growth resumption. These observations suggested that p150 and p60 could be involved in mechanisms assisting algal growth in high salt.

In the present study, cDNA cloning and enzymatic analyses led to the identification of p60 as a unique carbonic anhydrase. Structurally, it constitutes a fusion into a single polypeptide chain of two repeats, each similar to animal carbonic anhydrases. Functionally, it is highly salt-resistant. The unique characteristics of the D. salina carbonic anhydrase potentially enable the enzyme to optimize inorganic carbon utilization in high salinities.

MATERIALS AND METHODS

Algae and Growth Conditions—Algae, the medium composition, and growth conditions were essentially as described (7–9). Unless stated otherwise, the growth medium contained 0.5 M NaCl concentration. Chlamydomonas reinhardtii strain 137 M † (obtained from I. Ohad, Hebrew University, Jerusalem, Israel) was grown as described (10).

Preparation and Screening of a D. salina cDNA Library—D. salina cells grown in 0.5 M NaCl were transferred in two steps to 3.5 M NaCl (hyperosmotic shock), essentially as described (7). Total RNA was prepared from cells harvested 9 h after the shock by extraction with 1 ml/107 cells of Tri Reagent (Molecular Research Center, Inc.; Ref. 11). Poly(A) mRNA was isolated by using the Poly(A) Tract mRNA Isolation System (Promega). Synthesis of cDNA, using 3 μg of poly(A) mRNA, was by the ZAP-cDNA synthesis kit (Stratagene). Cloning in the Uni-ZAP XR vector used the Stratageneoning kit. The original library contained 7 × 106 plaque-forming units. An amplified library was screened with anti-p60 antibodies (7) using the Stratagene picoBlue immunoscreening kit. Positive clones were further processed to rescue the corresponding recombinant plasmids.

DNA Sequencing—Phage-rescued plasmids were subjected to DNA sequence analysis by the dyeoxy sequencing method in the Applied Biosystems 373A DNA sequencer.

Northern Blot Analysis—Total RNA was extracted as described above from cells treated as indicated for each experiment. Seventeen μg RNA were analyzed on each lane of the formaldehyde-agarose gel (12). The probe used contained the full-length cDNA for p60. Blot hybridization was as described (13).

Amino Acid Sequence—Purification of p60 was as described (7). The band containing p60 was excised from the preparative gel and transferred to an SDS-containing 10–20% polyacrylamide gradient gel in the presence of 2 μl/lane of the Staphylococcus aureus V8 protease (14). The digestion was continued for 30 min, and the oligopeptide products were separated by electrophoresis and blotted on a polyvinylidiene difluoride membrane (15). The N-terminal amino acid sequences of the two major proteolytic fragments as well as of the undigested p60 were analyzed using the Applied Biosystems model 475A protein microsequencer, equipped with a model 120A on-line high-performance liquid chromatography phenylthiodydantoin amino acid analyzer and a model 900A...
data acquisition and processing unit.

Purification of p60—The procedure used differed from the one described previously (7). The protein was purified from a plasma membrane fraction (16) of cells grown in 3.5 M NaCl, and membranes were solubilized with 0.2% reduced Triton X-100 (Sigma), 1 mM EDTA, and 10 mM MOPS-Tris (pH 7.5) containing protease inhibitors as described (16), using 1 ml of this solution per 2 mg of membrane protein. After 10 min in ice, the solution was centrifuged in a Beckman Optima TL ultracentrifuge at 75,000 rpm for 30 min at 4°C. The p60-containing supernatant was further fractionated by perfusion chromatography (PerSeptive Biosystems, Cambridge, MA) on a Poros PI column eluted with a 0–1.0 M NaCl gradient in 0.1% reduced Triton X-100 and 10 mM MOPS-Tris (pH 7.5). Fractions containing p60 were eluted between 0.65 and 0.85 M NaCl.

Preparation of Cell-free Soluble Carbonic Anhydrase—Cells of C. reinhardtii, grown to a density of $2 \times 10^6$ cells/ml, were collected by centrifugation, washed twice with H2O, followed by two washes with 20 mM KH2PO4, and adjusted to pH 7.4 with KOH; then the periplasmic carbonic anhydrase was partially released by washing with 0.2 M KCl, essentially as described (10). To partially release the surface carbonic anhydrase from D. salina, the cells were grown with continuous air bubbling to a density of $2 \times 10^6$ cells/ml in a medium containing 1 M NaCl and 20 mM Tris-HCl, pH 7.4, without added NaHCO3. The cells were pelleted at 2000 × g for 5 min, washed twice with ice-cold fresh growth medium, and suspended in 2.5 M NaCl. After 10 min in ice, the cells were removed by centrifugation in the microcentrifuge for 1 min at 4°C. The supernatant contained the released carbonic anhydrase.

Carbonic Anhydrase Assays—Carbonic anhydrase activity of D. salina whole cells was assayed essentially as described (17). Cultures grown at different salinities were frequently transferred to fresh medium to maintain the pH at around 8.5. To study the effect of a shift to high pH, cells grown for two generations (to $2 \times 10^5$ cells/ml) in a medium contained 25 mM Na-Hepes, pH 7.2, and 25 mM NaHCO3 were collected by centrifugation, resuspended to the same density in a similar medium but with 25 mM TAPS, pH 9.0, and further grown for the indicated periods. Prior to the assay, suspensions containing the desired number of cells were collected by centrifugation and resuspended in 1 ml of growth medium containing 0.5 M NaCl without NaHCO3 (for CO2-limited cells) or with NaHCO3 and 1.0 M NaCl (for cells grown in different salinities). Assays, with aliquots containing 5 × 109 or 109 cells, indicated that the activity was proportional to the amount of the cells assayed. The cellsuspensions were assayed in reaction mixtures of 2.75 ml, adjusted to contain 0.5 or 1.0 M NaCl, to match the salt concentration in the assayed cell suspensions, and containing 6.5 mM veronal buffer, pH 8.4, and 250 μl of a cold saturated CO2 solution in H2O. The assay, conducted at 4°C, was started by the addition of the CO2 solution. Enzyme activity was calculated from the length of time required for the pH to change from 8.4 to 7.4, taking into account the values for nonenzymatic acidification (17).

Assays of carbonic anhydrase preparations solubilized from whole cells were carried out similarly. The reaction mixtures contained 25 or 50 μl of the soluble preparations, 3.6 mM KCl, and NaCl as indicated. Chromatographically purified p60 was assayed similarly in reaction mixtures containing 1 M NaCl with 20- or 40-μl aliquots of the column-eluted fractions. The level of purified p60 was estimated by SDS-polyacrylamide gel electrophoresis alongside defined amounts of bovine serum albumin and staining.

RESULTS

Full-length cDNA Cloning and Sequence Determination of p60—A cDNA expression library was constructed in the λ Zap vector using D. salina poly(A)1 mRNA isolated 9 h after the cells were subjected to a hyperosmotic shock. Based on the time course of p60 accumulation (7), cells at this stage were likely to be enriched in the corresponding mRNA. The cDNA library was screened with anti-p60 polyclonal antibodies. Several cross-reacting phages were isolated and found to contain identical 2.2-kilobase cDNA inserts. Sequence determination of the cloned cDNA indicated that it included a 1.767-kilobase open reading frame along side defined amounts of bovine serum albumin and staining.

FIG. 1. Nucleotide and deduced amino acid sequence of the cDNA for p60. Underlined, amino acid sequences matching sequences determined for intact p60 and two proteolytic products.
on its 5'-end by 84 base pairs and on its 3'-end by 364 base pairs attached to a poly(A) tail (Fig. 1).

To confirm that this clone represented the cDNA for p60, partial amino acid sequences were determined for the purified protein. These sequences included the 20 N-terminal amino acids of the intact protein as well as the N-terminal sequences of Mr 8,000 and Mr 13,000 oligopeptides generated by digestion of p60 with protease V8. As shown (Fig. 1), the cDNA includes coding sequences matching these amino acid sequences.

The assignment of the ATG starting at nucleotide 85 as the initiator codon (Fig. 1) was based on the following considerations: (i) it is the only in-frame ATG codon found upstream to the codons specifying the N-terminal sequence of the purified p60; (ii) it is flanked by nucleotides conforming to ANATGGGC/C, the core consensus sequence for translation initiation in plants and animals (18, 19).

Sequence Homology to Carbonic Anhydrases—Separate representation and alignment of the N and C halves of the deduced p60 sequence (Fig. 2) shows that the protein sequence is comprised of an internal duplication. Each of the repeats is homologous to animal carbonic anhydrases isozymes I, II, and III (represented in the alignment by the human enzymes) as well as to nearly identical, periplasmic carbonic anhydrases from C. reinhardtii. The Chlamydomonas enzymes are synthesized as precursors that are cleaved posttranslationally to yield large and small subunits, pairs of which assemble to form a tetrameric carbonic anhydrase cross-linked by disulfide bridges (20–22). Discounting the leader peptide, the N and C halves of the Dunaliella protein are 52% identical and, counting conserved replacements, 70% similar. Among the residues retained in all of the sequences compared are the zinc-liganding His-94, His-96, and His-119 (residue numbers according to the aligned human Cah1 enzyme) and the residues forming the hydrogen-bond network to zinc-bound solvent molecules; circled residues, cysteines involved in disulfide bond formation (22).

According to the cDNA sequence, the mature p60, of Mr 58,719, is extended by a 54-amino acid leader peptide. In comparison, the C. reinhardtii carbonic anhydrases are synthesized with leader peptides of 20 amino acids (21, 23). Common to the leader peptides of the Dunaliella and C. reinhardtii proteins is the presence of one or two arginine residues two to three residues from the initiator methionine, followed by 15–17 hydrophobic residues. In addition to the removal of the leader peptide, the C. reinhardtii precursors are further cleaved at Ala-305 and Leu-340 (Fig. 2). Of the three polypeptides formed, those containing the N- and C-terminal ends form the large and small subunits, respectively. The lost middle oligopeptide has no counterpart in either repeat in the Dunaliella protein.

A comparison of the amino acid sequences present in the mature forms of the various enzymes reveals two gross differences between the algal and human sequences. Both repeats of the Dunaliella enzyme and each of the two C. reinhardtii enzymes include insertions of varying lengths at positions N-terminal to residues 74 and 227 (numbering as in the aligned human Cah1). Most other gaps/inserts in the aligned sequences do not appear to be related to the taxonomic origin of the enzymes.

A striking difference between the Dunaliella sequence and sequences of carbonic anhydrases from C. reinhardtii and a variety of animal sources is the ~2-fold higher ratio of acidic...
over basic amino acid residues, resulting in a striking difference between the predicted isoelectric points of the p60 repeats and the other carbonic anhydrases included in the comparison (Table I). An isoelectric point of 4.6 was previously determined for p60 by isoelectric focusing (7).

Hydropathy plots (data not shown) indicated that Dca is largely hydrophilic throughout its length and contains no sequence matching a potential membrane-spanning domain.

Correlations between p60 Accumulation and Carbonic Anhydrase Activity—Protein level and enzyme activity of whole cells were compared under several sets of conditions. High salinities were previously shown to induce p60 accumulation. Analysis of exponentially growing cells in standard media with 0.5, 1.0, or 2.0 M NaCl (Fig. 3A) indicated a parallel increase in p60 level and surface carbonic anhydrase activity with rising salinity.

Analyses were also conducted under two sets of conditions limiting the availability of CO$_2$. The equilibrium concentration of CO$_2$ is markedly decreased at high pH (4). As illustrated in Fig. 3B, a shift from pH 7.2 to pH 9.0 of cells grown in a medium containing 0.5 M NaCl with NaHCO$_3$ induced a large increase in carbonic anhydrase activity, which closely paralleled a rise in the level of p60.

CO$_2$ availability was also limited by eliminating bicarbonate from the medium. Cells grown in 0.5 M NaCl and 25 mM bicarbonate at pH 7.2 were transferred to a similar medium without added bicarbonate. A parallel increase in enzyme activity and p60 protein was observed with time following the transfer (Fig. 3C).

Carbonic Anhydrase Activity of Purified p60—The protein was purified from a plasma membrane fraction by perfusion chromatography on an anion-exchange column. The p60 was eluted in a peak that was preceded by, and partially overlapped with, the elution peak of two closely migrating M$_r$ ~50,000 proteins (Fig. 4). Carbonic anhydrase assays clearly indicated that the activity profile paralleled the elution profile of p60 and not that of the M$_r$ ~50,000 proteins (Fig. 4). Moreover, activity was exhibited by fractions containing practically no other protein except for p60. Based on these as well as other assays of purified p60, the specific activity of the enzyme was estimated to be between 40,000 and 60,000 units/mg of protein. In comparison, the specific activity determined for a periplasmic carbonic anhydrase from C. reinhardtii was 20,000–25,000 units/mg of protein (24).

Transcript Analysis—The gene encoding p60 was designated dca (duplicated carbonic anhydrase). To investigate the mode of dca regulation, the cells analyzed for carbonic anhydrase and

| Enzyme          | Fraction of total residues | pl   |
|----------------|--------------------------|------|
|                | Asp + Glu | Arg + Lys |      |
| p60/Dca-N      | 14.9     | 7.6     | 4.6  |
| p60/Dca-C      | 12.1     | 5.9     | 4.4  |
| cah1 chire     | 9.7      | 8.0     | 7.2  |
| cah1 human     | 10.4     | 9.6     | 7.1  |
| cah2 human     | 12.3     | 12.0    | 7.5  |
| cah3 human     | 12.0     | 11.5    | 7.3  |
| cah1 mouse     | 9.2      | 8.7     | 7.0  |
| cah1 rabbit    | 9.8      | 10.2    | 8.5  |
| cah1 bovine    | 10.1     | 10.5    | 8.9  |
| cah2 rat       | 10.8     | 10.4    | 7.4  |

p60 (Fig. 3) were also analyzed for dca mRNA by Northern blot hybridization. The analyses (Fig. 5) revealed a single transcript hybridizing to the dca cDNA probe that migrated between the 28 S and 18 S rRNAs. In cells grown continuously in different salinities at pH 8.5 (Fig. 5A), the transcript level increased in proportion to the external salinity. A shift from pH 7.2 to pH 9.0, or removal of bicarbonate from a medium buffered at pH 7.2, resulted in the induction of dca transcript accumulation (Fig. 5B).
hybridization with the boxes, is attributable to a difference in the pH of the respective cultures. Upper analyzed in Fig. 3 Fig. 4

bridization with a standard rRNA probe; dca sition of rRNA markers and a

treatments. In Methods.“

tained by Northern blot hybridization as described under “Materials and Methods.”

We followed the time course of dca mRNA accumulation under the two sets of CO2-limiting conditions (data not shown), and the data indicated a considerable increase in transcript level by 2 h after transfer to induction conditions, with maximal levels attained at 8–10 h after transfer. Maximal accumulation of transcript preceded maximal accumulation of the protein.

Effect of Salt on the Activity of Surface Carbonic Anhydrases in D. salina and C. reinhardtii—Based on the localization of p60/Dca to the cell surface by immunoelectron microscopy (7) and the demonstration of its activity in whole cells, this external carbonic anhydrase is expected to be accessible to the medium. For Dca to remain active in the hypersaline media typical for D. salina, the enzyme should exhibit exceptional salt tolerance.

To evaluate the effect of salt on Dca activity, cell-free preparations of the enzyme were assayed in the presence of increasing salt concentrations. The relative activities at different salinities were compared to those of the periplasmic carbonic anhydrase(s) from C. reinhardtii, an alga incapable of growth in high salinities.

Soluble preparations highly enriched in the D. salina and C. reinhardtii surface carbonic anhydrases were prepared by salt treatments. In D. salina, transfer from 1.0 to 2.5 M NaCl released approximately 10% of the surface carbonic anhydrase activity into the medium without affecting cell viability. SDS-polyacrylamide gel electrophoresis, staining, and immunoblotting showed that p60 was a major protein in the released fraction.

Carbonic anhydrase assays (Fig. 6) indicated that the solubilized D. salina enzyme exhibited maximal activity around 1.0 M NaCl and retained over 60% of this activity at 0.05 or 2.3 M NaCl, the lowest and highest salt concentrations tested. In contrast, the solubilized carbonic anhydrase from C. reinhardtii is progressively inhibited by salt. At 0.6 M NaCl, the activity level drops by ~90% of that observed at 0.05 M salt. Inhibition of purified surface carbonic anhydrase from C. reinhardtii by NaCl was reported previously (25). Thus, the carbonic anhydrase of D. salina differs from the enzyme from C. reinhardtii in exhibiting a broad tolerance for salt.

**DISCUSSION**

The data presented here indicate that p60, the plasma membrane protein previously identified in D. salina grown in high salinities, is a structurally unique, highly salt-resistant form of carbonic anhydrase. A relationship between salinity adaptation and an increase in carbonic anhydrase activity was reported for D. tertiolecta (26). Furthermore, a salt-sensitive mutant of D. tertiolecta was apparently defective in surface carbonic anhydrase (27), indicating that the availability of CO2 limited the growth rate of Dunaliella at high salinities. In D. salina, high salt concentrations were found to increase the affinity of D. salina for CO2 in photosynthesis, a response attributed to the induction of surface carbonic anhydrase activity (17). That the induction of Dca in high salinities is primarily due to CO2 limitation is also supported by our previous observation that transfer to a medium where NaCl was replaced by an iso-osmotic concentration of glycerol only weakly induced p60 accumulation (7). Because CO2 is the major species of inorganic carbon entering the algal cells, the surface carbonic anhydrase can enhance carbon uptake by the conversion of bicarbonate into CO2 (28–30).

The antibodies raised against p60 cross-reacted with a similarly sized protein in D. bardawil (7), a distinctly different Dunaliella strain (8). In both Dunaliella strains, the antibodies bound mainly to a single protein. Moreover, genomic analyses did not indicate the presence of additional dca-like genes in D. salina. Nonetheless, the possibility that other carbonic anhydrases and their corresponding genes remained undetected in the analyses performed cannot be ruled out. The prokaryotic-like carbonic anhydrases of chloroplasts (31) show little structural similarity to the mammalian or algal enzymes (32) and would remain undetected in our analyses.

Under nondenaturing conditions, the internally duplicated Dca migrates electrophoretically as a Mw ~60,000 protein (data not shown). In comparison, the two nearly identical periplasmic carbonic anhydrases from C. reinhardtii (Fig. 2) are heterotetramers of Mw ~75,000 consisting of two large and two small subunits (20). Dca resembles the C. reinhardtii enzymes in encompassing the equivalent of four subunits, except that they are fused to each other in a single polypeptide chain. The fused form of the D. salina enzyme apparently does not rely on stabilization by disulfide bonds similar to those present in the C. reinhardtii external carbonic anhydrases.

The essentially “duplicated” configuration of the carbonic anhydrases from Dunaliella and C. reinhardtii might optimize the catalytic activity of the surface carbonic anhydrases or might facilitate the uptake of CO2 by the cells. Dca differs,
however, drastically from the C. reinhardtii surface carbonic anhydrase(s) in its resistance to salt. Dca does not exhibit a sharp salt optimum and is thus fit to act extracellularly within the wide range of salinities in which D. salina is able to proliferate. Activation by salt, albeit at a lower concentration range than observed for Dca, has been reported previously for certain anhydrase-related enzymes. D. tertiolecta with its hypersaline growth media.

It is intriguing to note that Dunaliella has evolved enzymes adapted to act optimally under the unique constraints of its intracellular composition and extracellular salinity. Internally, Dunaliella accumulates high levels of glycerol, osmotically compensating for the external salinity. Correspondingly, the chloroplast ATP synthase of D. bardawil was found to require glycerol for maximal activity, whereas the halophilic-like character of Dca makes it suitable to act at the interface of the cells with their hypersaline growth media.

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