

The Plasma Membrane ATPase of *Dunaliella parva*

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Plasma membrane Mg^{2+} , Ca^{2+} ATPases were isolated from *Dunaliella parva* by differential centrifugation and subsequent sucrose gradient centrifugation and analyzed for their properties with special emphasis on ecophysiological requirements of this extremely salt-tolerant alga. Most properties (V_{max} - and K_M -values, substrate specificity, vanadate and DES sensitivity, resistance against ouabain) indicate that the ATPases of the plasma membrane of *D. parva* are basically of the same type as that found in the plasma membrane of other algae or higher plants. However, some interesting deviations from the normal characteristics of plasma membrane ATPases of plants were observed for the *Dunaliella* ATPases. These modifications partially may reflect adaptations of the ATPase and/or the microenvironment of the ATPase to the highly saline environment of this alga: 1) The plasma membrane ATPase of *D. parva* requires unusually high concentrations of divalent cations (up to 100 mM Mg^{2+} or Ca^{2+}) for maximal activity. Both cations can substitute for each other. 2) The plasma membrane ATPase of *D. parva* is extremely resistant against salt. It was stimulated by NaCl or KCl at concentrations up to 800 mM, whereas at higher salt concentrations the enzyme was inhibited. However, about 2.5 M NaCl was required for half-maximal inhibition of ATPase activity. 3) The ATPase was inhibited by inhibitors of anion transport such as SITS and DIDS, which suggests direct or indirect involvement of ATPase in anion transport. The possible functions of the plasma membrane ATPases are discussed with special emphasis on problems related to the hypersaline environment of this alga.

Introduction

The plasma membrane of the extremely halotolerant unicellular green alga *Dunaliella parva* must withstand molar concentration gradients of both salts and glycerol, in opposite directions: It has to prevent a deleterious influx of salt into the cell and *vice versa* an extensive leakage of intracellular glycerol into the medium. This implies an unusually low permeability of the plasma membrane in combination with effective ion pumping capacity. The permeability properties of the plasma membrane of *Dunaliella* cells have been investigated by *in vivo* measurements ([1], and references therein), but for the analysis of active transport, *e.g.* the investigation of ATPases, the isolation of plasma membranes from intact cells is required [2]. For various reasons the isolation of such vesicles from *Dunaliella* cells is more difficult than the corresponding isolation from cells of higher

plants. The identification of a membrane species isolated from a cell homogenate containing a large number of different types of membranes requires a suitable marker enzyme [3, 4]. The only known marker enzyme for the plasma membrane of plant cells is the glucan synthetase II [5, 6]. Since *Dunaliella* lacks a rigid cell wall, this enzyme is missing in this alga. Alternatively plasma membranes can be specifically labeled by artificial markers before fractionation. However, enzyme-linked labeling of plasma membrane (for example iodination of membrane proteins by lactoperoxidase [7] or labeling of glycoproteins with NaB^3H by galactose oxidase [8]) is not possible with *Dunaliella*, because the high salt concentrations of the culture medium inhibit enzyme activities. Chemical labeling of plasma membranes with external markers on the other side often requires unphysiological reaction conditions (low pH, high temperature, organic solvents etc.). Because of these difficulties it was only recently that attempts were successful to identify and analyze the plasma membrane fraction of *Dunaliella* homogenates [9–16]. In this study we isolated plasma membranes from *D. parva* homogenates and characterized the ATPase present in this membrane. It turned out that the plasma membrane ATPase of this alga has prop-

Abbreviations: Chl, chlorophyll; CCCP, carbonylcyanide-*m*-chlorophenylhydrozone; DCCD, N' , N' -dicyclohexylcarbodiimide; DES, diethylstilbestrol; DIDS, 4,4'-diisothiocyanostilben-2,2'-sulfonic acid; DTT, dithiothreitol; SITS, 4-acetoamido-diisothiocyanostilben-2,2'-disulfonic acid.

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erties different from that of higher plant cells. In part these differences can be attributed to adaptations to the high salinities in the environment of this alga.

Materials and Methods

Plant material

Dunaliella parva was cultured as described previously [17] with the exception that 6 l Erlenmeyer flasks were used as culture vessels. Algae were adapted to a salinity of 1.5 M NaCl. 24 l of algae (density $1-2 \times 10^6$ cells per ml) were harvested by centrifugation ($700 \times g$, 20 min, 22 °C) and washed once in fresh culture medium. *Osmotic rupture* of cells was carried out by adding 40–50 ml of a hypo-osmotic medium (5 mM Tris/Mes buffer, pH 7.6, 1 mM DTT) to the algal pellet at 0 °C. Subsequently, 15 ml aliquots of the resulting homogenate were passed twice through a precooled Yeda press (N_2 , 8 MPa), which caused further fragmentation.

Isolation of plasmalemma vesicles

Differential centrifugation. Algal homogenates were subject of differential centrifugations at 4 °C, starting with a centrifugation at $2000 \times g$ (10 min) (pellet 1), which was followed by centrifugation at $15,000 \times g$ (30 min) (pellet 2), at $50,000 \times g$ (60 min) (pellet 3) and finally at $170,000 \times g$ (90 min) (pellet 4). Pellets were resuspended in ice cold ATPase buffer (25 ml Tris/Mes, pH 7.6, 2 mM DTT). Electron-microscopic analysis of the pellets demonstrated that during the differential centrifugation vesicle size became considerable smaller and that vesicle population became more homogenous (data not shown). Lamellar membranes (thylakoids) sedimented in pellet 2, but were essentially absent in pellet 4 (less than 1% of the total).

Sucrose gradient centrifugation. Pellet 4 from the differential centrifugation was layered onto a discontinuous sucrose gradient (35 ml tubes, containing from bottom to top (density in g per ml in brackets): 4 ml 48% sucrose (1.22); 4 ml 41% (1.19); 5 ml 38% (1.17); 5 ml 34% (1.15); 3 ml 30% (1.13); 4 ml 26% (1.11); 4 ml 18% (1.07)). The equilibrium centrifugation was carried out using a vertical rotor (TV 850) and a Kontron TG 65 ultracentrifuge ($100,000 \times g$, 3 h, 4 °C). The gradient was separated into 1 ml fractions which were kept at 4 °C in the dark for further treatment.

Analytical methods

Phosphate was analyzed by the method of Ames [18]. For all other analytical methods see Kaaden and Gimmler [16]. The activity of ATPases (EC 3.6.1.X) was followed by the release of phosphate. Samples contained vesicles corresponding to 20 µg of protein and were incubated for 20 min at 37 °C. The standard reaction medium contained 25 mM Tris/Mes buffer (pH 7.6), 2 mM DTT and 1 mM ATP. For concentrations of monovalent and divalent cations consult tables and figures.

External markers. 100 ml of algae corresponding to 10^{10} cells were incubated with 2 ml of 4 mM fluorescamin, rapidly mixed and immediately centrifuged ($700 \times g$, 15 min). The pellet was homogenized and treated as described above. Aliquots of the sucrose gradient were suspended in 2 ml of water and analyzed for fluorescence at 475 nm (actinic light 390 nm) in a fluorimeter (Aminco SPD-500). Fluorescamin reacts with primary amino groups of amino acids and proteins forming a fluorescent derivate, but decomposes in aqueous media to a non-fluorescent compound with a half-time of few seconds [19]. Provided the uptake into the cell is slow in comparison to decomposition, it should exclusively react with the proteins of the plasma membrane. For NaB³H-labeling of the plasma membrane [20] 50 ml of algae (3×10^{10} cells) were incubated for 5 min with 0.5 ml sodiumperiodate (final concentration 1 mM) at 0 °C. Then oxidation of sialic acids of the glycoproteins were stopped by the addition of 10 mM glycerol. Algae were centrifuged and the pellet washed twice with fresh culture medium. Subsequently, algae were incubated for 2 min at 0 °C with 75 MBq NaB³H and centrifuged again. Then algae were washed five times by centrifugation with fresh culture medium. Finally cells were homogenized and treated as described above. Aliquots of the sucrose gradient were counted for radioactivity.

Results

Isolation of plasma membrane vesicles

After osmotic rupture, Yeda press treatment of *Dunaliella* cells and subsequent *differential centrifugation* of the homogenate, a microsomal fraction (pellet 4) was obtained which is devoid of thylakoids and mitochondrial membranes, but contains high activities of pyrophosphatase, acid phosphatase (sup-

posed marker enzymes for vacuoles), latent IDPase (marker for Golgi vesicles) and of Antimycin A-insensitive NADPH-dependent cytochrome *c*-reductase (marker enzyme for the endoplasmic reticulum) (not shown, but compare ref. [16]). Pellet 4 was also enriched in ATPase activity [16] and it was therefore that this pellet was analyzed by *sucrose gradient centrifugation* (Fig. 1). Five major protein peaks were observed in the gradient. The protein peak at fraction 30 (density 1.17–1.19 g cm⁻³) coincided with chlorophyll (the marker for thylakoids). However, it must be kept in mind that this contamination reflects less than 1% of the total chlorophyll content of the cell. The two protein peaks at fraction 13 and 17 corresponded to peaks in RNA and reflect ribosomes. The DNA peak at fractions 7–8 corresponds partially, but not completely with the major protein peak of the gradient. Cytochrome *c*-oxidase (marker

enzyme of mitochondrial membranes) exhibits a peak at fractions 28–29 (density 1.17–1.18 g cm⁻³). Latent IDPase demonstrates a relatively broad distribution at the top of the gradient (fractions 1–10), with maximal activities in fractions 6–7. Antimycin A-resistant cytochrome *c*-reductase, a marker enzyme of the endoplasmic reticulum, exhibits a distinct peak at fraction 5. Fraction 5 was also enriched in pyrophosphatase. Acid and alkaline phosphatases are found in decreasing activities in fractions 1–7. Three distinct ATPase peaks are observed in the sucrose gradient (Fig. 2). Since different ATPase species require different experimental conditions for optimal activity, only the positions of the peaks but not peak heights are of importance. The small peak close to the top of the gradient can be assigned to the ATPase of the endoplasmic reticulum the properties of which were analyzed in a recent paper [16]. The

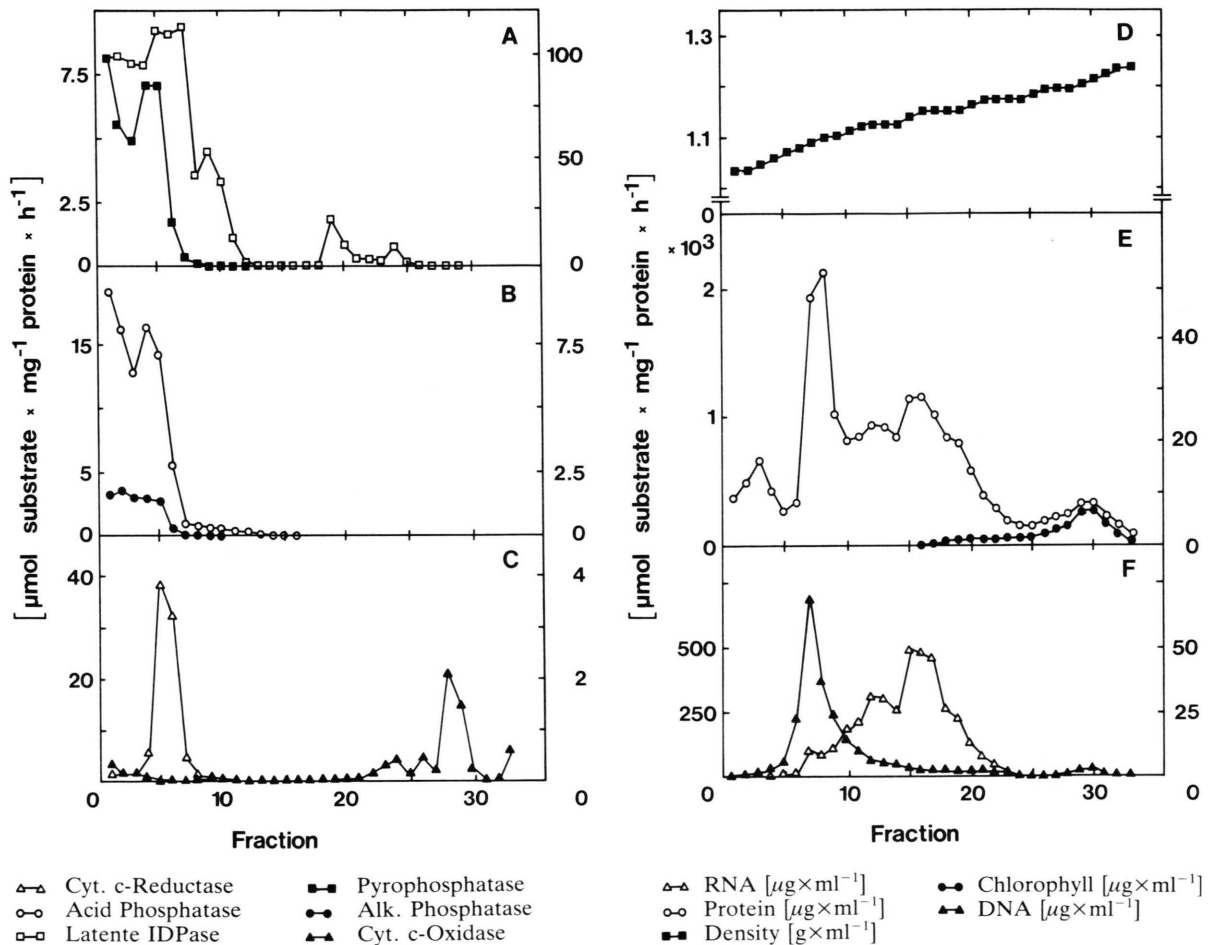


Fig. 1. Sucrose density gradient of *D. parva* membranes (pellet 4 of the differential centrifugation). A–C: Distribution of marker enzymes, D: density of the gradient, E–F: distribution of chlorophyll, protein and nucleic acids.

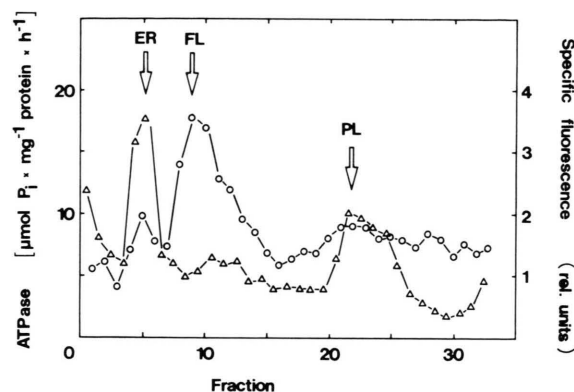


Fig. 2. Sucrose density gradient of *D. parva* membranes (compare Fig. 1). (Δ) Specific fluorescence of fluorescamin bound to membrane proteins before fragmentation. (\circ) ATPase activity (50 mM MgCl_2 , 50 mM CaCl_2 , 2 mM ATP). ER = endoplasmic reticulum, FL = flagella, PL = plasma membrane.

second peak represents the ATPase of the flagella [11]. The membranes of the broad peak between fractions 21 and 25 have a density of about $1.16\text{--}1.17 \text{ g cm}^{-3}$. This value matches corresponding data for plasma membranes of other plants [2] and especially those published for the plasma membrane of *D. salina* [12]. Polyacrylamide gel electrophoresis revealed that membranes of these fractions have a protein pattern significantly different from that of thylakoids, endoplasmic reticulum and flagellar membranes [11]. Thus if the membrane species of fraction 21–25 are not identical with thylakoids, mitochondrial membranes, Golgi vesicles, vesicles of the ER, flagellar membranes and since a tonoplast is missing in this alga (if present tonoplast vesicles would occur in the gradient at densities of $1.10\text{--}1.13 \text{ g cm}^{-3}$, [2]), we have to conclude that this fraction represents the plasma membrane. This conclusion is confirmed by the density data. Since a positive identification is preferable to identification by exclusion, attempts were carried out to identify the plasma membrane fraction by means of external markers. Cells were incubated for less than a minute with fluorescamin. After separation of membrane species by sucrose density centrifugation, two peaks of specific fluorescence were observed (Fig. 2): One peak can be assigned to the ER, the other one (fraction 21–24) coincides with the third ATPase peak. This confirms the view that the latter fractions represent the plasma membrane fraction. The

occurrence of two fluorescent peaks instead of only one is obviously due to the fact that the permeability coefficient of the plasma membrane for the lipophilic fluorescamin is too high in comparison to its half-time of decomposition. After its entry into the cells fluorescamin seems to bind also to the proteins of the ER which forms a layer between the plasma membrane and the big cup-shaped chloroplast of this alga. Nevertheless, the fraction that contains during the differential centrifugation the majority of thylakoids (pellet 2) exhibits a much lower specific labeling with fluorescamin than the plasma membrane (not shown). In another approach sialic acids (mainly N-acetyl-neuraminic acids) of the glycoproteins of the plasma membrane of *D. parva* cells were oxidized with periodate and subsequently reduced with NaB^3H . The distribution of the label in the sucrose gradient was similar to that of fluorescamin binding (not shown). Sheffer and Avron [12] used sulforhodamin B as a probe to identify the plasma membrane of *Dunaliella salina* and found results which support the view that the fraction under investigation is the plasma membrane fraction. We conclude that fractions 21–24 represent the plasma membrane of *D. parva*. We refer in the following to these pooled fractions as plasma membrane vesicles and to the associated ATPase as plasma membrane ATPase.

Properties of the plasma membrane ATPase

Divalent cations. The plasma membrane ATPase of *D. parva* requires unusual high concentrations of Mg^{2+} and Ca^{2+} for full activity independent on the nature of the accompanying anion (Fig. 3). ATPase activity in the absence of Ca^{2+} and Mg^{2+} can be completely suppressed by EDTA or EGTA, indicating a certain contamination of the fractions with these cations. Other divalent cations cannot substitute Mg^{2+} or Ca^{2+} (Table 1). Highest ATPase activities are observed when a mixture of both Mg^{2+} and Ca^{2+} is added, for example 50 mM of each cation (Fig. 3A).

K_M -values and substrate specificity. The plasma membrane ATPase of *D. parva* exhibited typical Michaelis-Menten-kinetics in respects to its substrate ATP (Fig. 4). K_M - and V_{max} -values depend very much on the concentration of divalent cations applied, but are in the same order of magnitude as corresponding data from other ATPases [2] (Table III): Both parameters increase with increasing cation concentrations. Conversely also the " K_m " of the

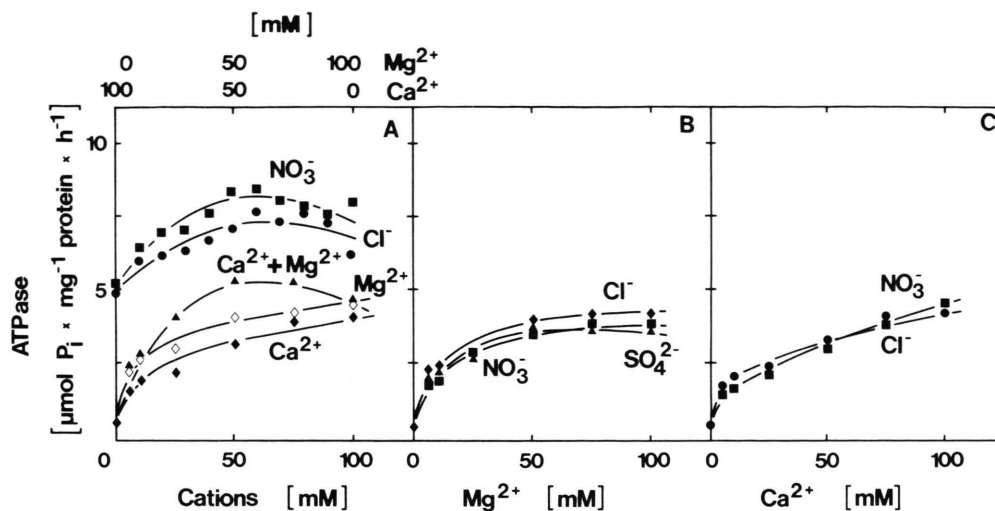


Fig. 3. Plasmalemma ATPase activity as function of divalent cations (1 mM ATP). Average of three independent experiments. A: Lower three curves (refer to lower *x*-axis): Cations were applied as chlorides. Equal concentrations of Ca²⁺ and Mg²⁺ were applied were indicated. Upper two curves (refer to upper *x*-axis): Different ratios of Mg²⁺ and Ca²⁺ were applied either in the form of nitrate or chloride. B, C: Different Mg²⁺ (B) or Ca²⁺ (C) salts were applied.

Table I. The effect of divalent cations (50 mM, added as chlorides) on plasma membrane ATPase. Average of three independent experiments. The reaction medium contained 1 mM ATP.

Cation	ATPase activity [%]
—	10
Mg ²⁺	100
Ca ²⁺	86
Mn ²⁺	40
Zn ²⁺	19
Co ²⁺	6
Cu ²⁺	0

ATPase for Mg²⁺ depends on the ATP concentration (Table IV): Increasing concentrations of ATP induced increasing “*K_M*”-values for Mg²⁺. The plasma membrane ATPase of *D. parva* exhibits substrate specificity (Table II). Specificity is more pronounced in the presence of Ca²⁺. In the presence of Mg²⁺ ATP can be replaced partially by CTP and GTP, whereas the Ca²⁺-ATPase is widely dependent on ATP.

pH-profile. The plasma membrane ATPase of *D. parva* operates optimally at pH values between 7 and 7.6, but the pH dependency is rather broad (Fig. 5). The latter is in agreement with corresponding results for the ATPase of *D. salina* [12], but is differ-

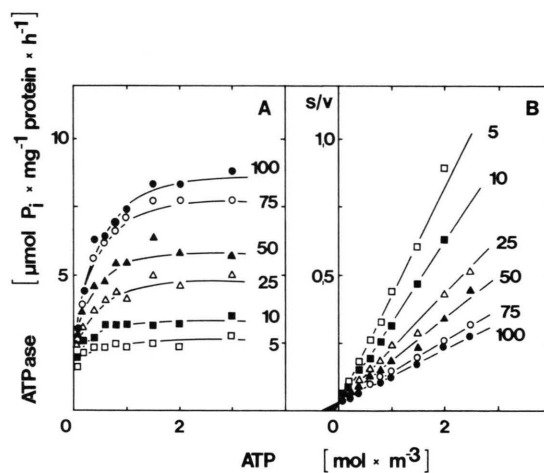


Fig. 4. The effect of ATP on plasma membrane ATPase as affected by Mg²⁺ (number in brackets = mM concentrations). Average of two independent experiments (A). B: ATP concentration over ATPase activity as function of ATP concentration (Hanes plot). In this plot the intercept of lines with the *x*-axis yield $-K_M$, whereas the intercept with the *y*-axis yield K_M/V_{max} .

ent from the pH optimum of 6.5 reported for ATPases from higher plant cells [2]. However, some ATPases of halophytes and marine algae exhibit also pH optima at slightly alkaline pH values [22–24].

Table II. Substrate specificity of plasma membrane ATPase. The reaction medium contained 50 mM of cations and 1 mM of various substrates. Average of two independent experiments.

Substrate	ATPase activity [%]		
	Mg ²⁺	Ca ²⁺	Mg ²⁺ + Ca ²⁺
ATP	100	100	100
ITP	42	23	22
UTP	57	37	27
GTP	29	23	16
CTP	68	37	36
ADP	25	9	16
IDP	31	0	9
UDP	37	14	9
GDP	46	9	24
CDP	42	0	6
AMP	6	4	3
Pyrophosphate	34	12	22

Table III. The effect of Mg²⁺ and Ca²⁺ on K_M - and V_{max} -values of plasma membrane ATPase. Average of two independent experiments.

Cation	[mM]	K_M [mM]	V_{max} [$\mu\text{mol P mg}^{-1} \text{ protein h}^{-1}$]
Mg ²⁺	5	0.05	2.7
	10	0.07	3.3
	25	0.10	4.3
	50	0.15	6.1
	75	0.18	7.6
	100	0.23	8.5
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Ca ²⁺	10	0.04	1.4
	50	0.13	5.1
	100	0.18	6.8

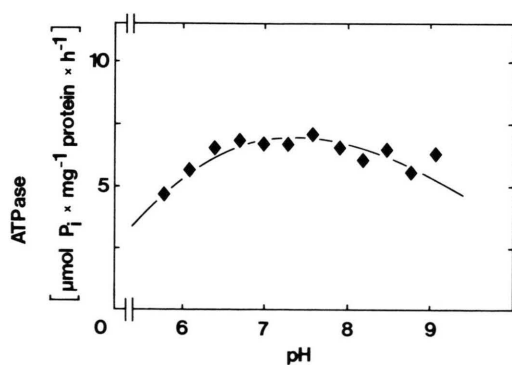


Fig. 5. pH-profile of plasma membrane ATPase. The reaction medium contained 50 mM Mg(NO₃)₂ and 2 mM ATP.

Monovalent cations and anions. The plasma membrane ATPase of *D. parva* is extremely insensitive to NaCl or KCl (Fig. 6A): With up to 800 mM of NaCl or KCl, a significant stimulation of the ATPase activity was observed. Above 1 M a continuous inhibition did take place. Half-maximal inhibitions was approached at about 2.4 M of these salts. Similar effects were observed with LiCl, RbCl and CsCl (Fig. 6B). The largest stimulations are observed with NH₄Cl or KCl and smallest with LiCl or NaCl (Table V). NaCl and NaNO₃ cause a stimulation of the ATPase, followed by inhibitions at higher salt concentrations (Fig. 7). Stimulation by nitrate was larger than by chloride. Na₂SO₄ is inhibitory from the beginning. The degree of stimulation of ATPase by nitrate and chloride was dependent on the Mg²⁺ concentration: Maximal stimulations were observed between 10 and 25 mM Mg²⁺ (not shown). In Fig. 8A ATPase activity as influenced by ATP concentrations is plotted as

Table IV. The effect of ATP on " K_M "-values for divalent cations of the plasma membrane ATPase. Average of two independent experiments.

ATP [mM]	" K_M " for Mg ²⁺ [mM]	" K_M " for Ca ²⁺ [mM]
0.20	—	19
0.25	6	—
0.30	—	24
0.40	—	26
0.50	9	28
0.75	10	36
1.00	12	—
1.50	13	—
2.00	14	—
2.50	13	—

Table V. Stimulation of plasma membrane ATPase by 600 mM of various chlorides (compare Fig. 6). The sequence of stimulation by alkali cations is in agreement with corresponding data for other plasma membrane ATPase [2].

Salts	Stimulation of ATPase activity (control = 100%)
LiCl	118
NaCl	139
KCl	188
RbCl	165
CsCl	143
NH ₄ Cl	212

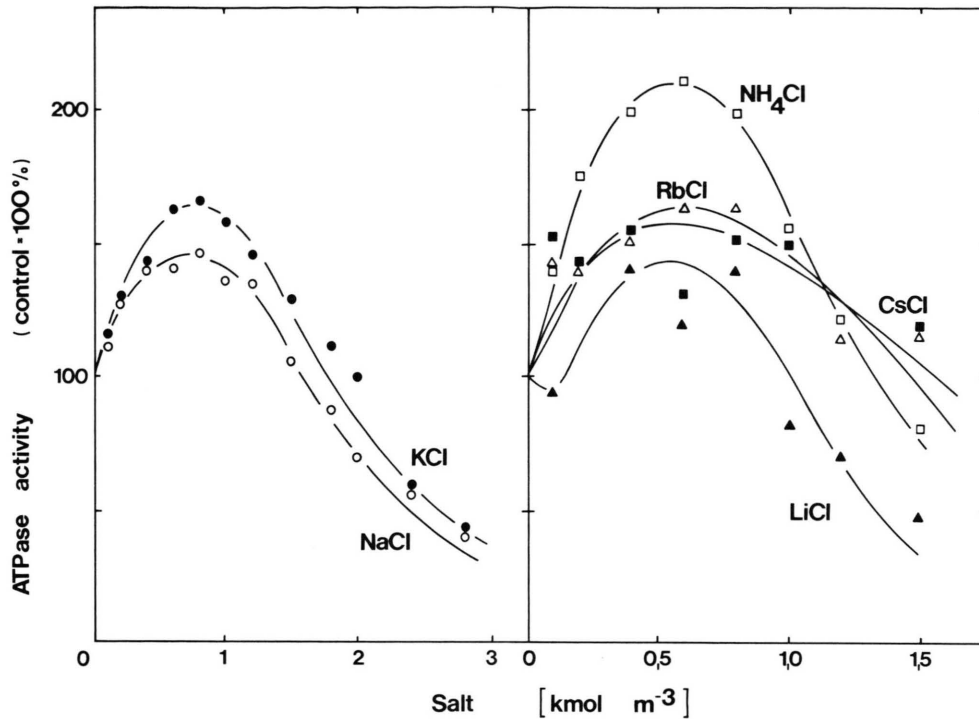


Fig. 6. The effect of NaCl, KCl (A) and of NH₄Cl, RbCl, CsCl and LiCl (B) on the plasma membrane ATPase (B). The reaction medium contained 25 mM MgCl₂ and 1 mM ATP.

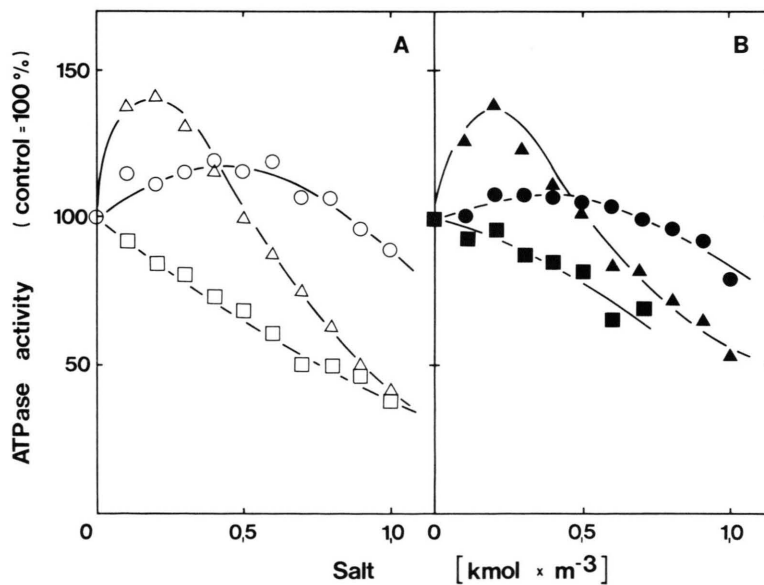


Fig. 7. Plasma membrane ATPase activity as affected by different anions. The reaction medium contained 50 mM MgCl₂ and 1 mM ATP. A: sodium salts; B: potassium salts; triangles: nitrate; circles: chlorides; squares: sulfate.

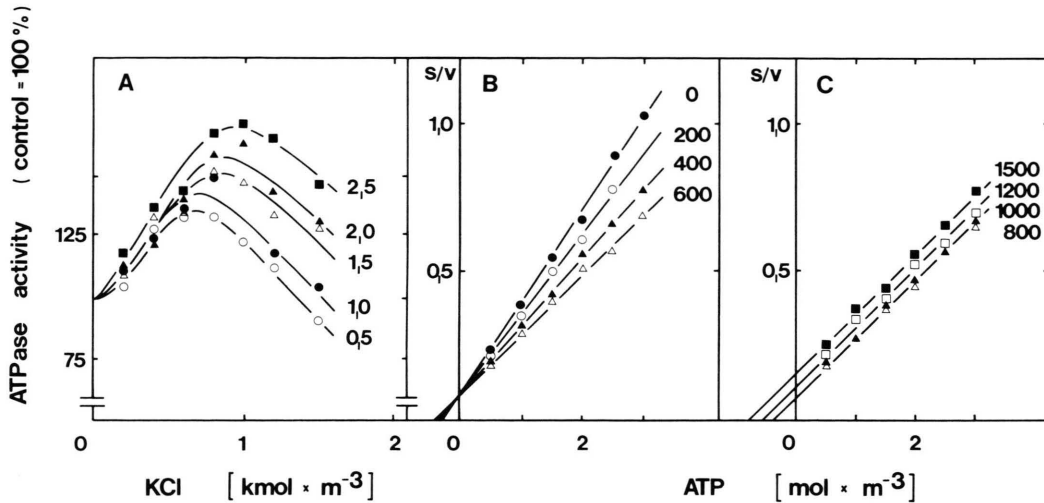


Fig. 8. Plasma membrane ATPase activity as function of KCl and ATP (mm concentrations in parenthesis) concentration (A). The reaction medium contained 50 mM MgCl_2 . Average of two independent experiments. In B and C ATP concentrations over ATPase activity are plotted as function of ATP concentration (Hanes plot, compare Fig. 4, KCl concentrations in mM in parenthesis). B: Plot for the stimulating range of KCl concentrations. C: Plot for the inhibiting range of KCl concentrations.

function of KCl concentrations and in Fig. 8B and C these primary data are replotted according to Hanes (ATP concentrations/rate of ATPase activity *versus* ATP concentration). For the stimulating range of KCl concentrations a bundle of lines is obtained with a common intercept on the y-axis. This is indicative according to Hanes for a reaction with two substrates ("ping-pong mechanism") (Fig. 8B). Although K_M -values do not change with increasing salt concentrations, V_{\max} -values increase (Table VI). For the inhibitory range of KCl concentrations the lines of the Hanes plot (Fig. 8C) run parallel indicating a competitive inhibition. Increasing inhibitions by KCl can be overcome by increasing ATP concentrations. V_{\max} remains unaffected, whereas K_m increases with increasing KCl concentration (Table VI).

Inhibitor studies. Sodium azide and sodium molybdate do not affect the ATPase which indicates that this plasma membrane fraction is not contaminated by mitochondrial membranes or acid phosphatase (Fig. 9A). The insensitivity of the plasma membrane ATPase against ouabain demonstrates that this ATPase is different from the Na^+/K^+ -ATPase in animal cells. However, the ATPase is inhibited by DES and *ortho*-vanadate. Half-maximal inhibition of ATPase by vanadate occurs at 5×10^{-7} M. The inhibition is non-competitive (not shown). The ATPase is surpris-

Table VI. K_M -values for ATP and V_{\max} -values of the plasma membrane ATPase as influenced by KCl. Average of two independent experiments.

KCl [mM]	K_M [mM]	V_{\max} [$\mu\text{mol P mg}^{-1} \text{protein h}^{-1}$]
0	0.29	3.2
200	0.32	3.7
400	0.30	4.1
600	0.32	4.5
800	0.41	5.1
1000	0.58	5.5
1200	0.69	5.4
1500	0.74	5.0

ingly resistant against DCCD, independent on the time of incubation (Fig. 9B). DCCD is believed to be indicative for the involvement of a H^+ -ATPase [2, 25]. Thus DCCD inhibits for example the ATPase of the thylakoids isolated from *D. parva* or the photosynthesis of intact *Dunaliella* cells (not shown). Ion pumps are expected to produce an electrochemical gradient across the membranes. The degradation of such gradients by ionophores should result in a stimulation of ATPase activity. CCCP (protonophore), nigericin (H^+/K^+ -exchanger), and valinomycin (K^+ -ionophore) or combinations of these compounds in concentrations between 10^{-7} and

10^{-5} M did not cause significant stimulations higher than 20% of the control rate. SITS and DIDS are effective inhibitors of anion transport [26]. Both inhibitors strongly suppress plasma membrane ATPase of *D. parva* cells in the presence of either chloride or nitrate (Fig. 9C). DIDS is more inhibitory than SITS.

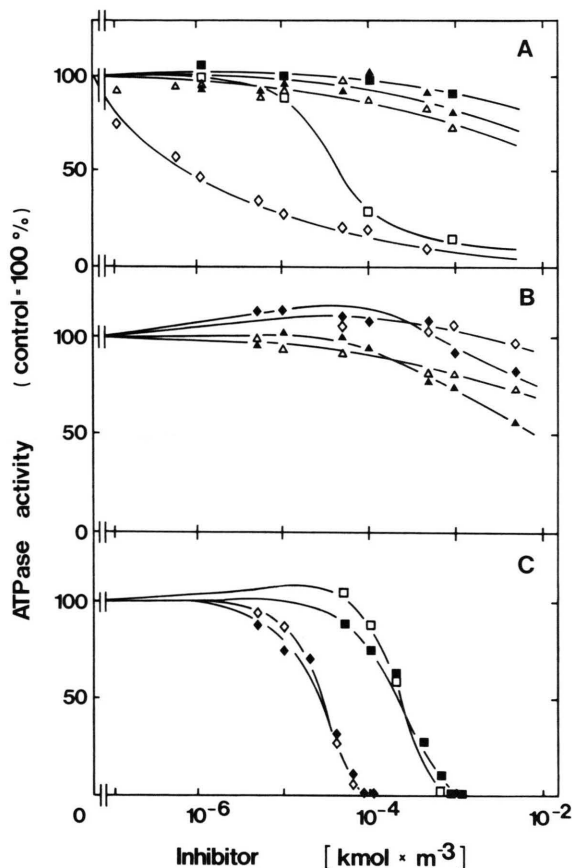


Fig. 9. The effect of inhibitors on plasma membrane ATPase activity. Average of two independent experiments. A: NaNO_3 (▲); sodium molybdate (△); DES (□); ouabain (■); *ortho*-vanadate (◇). The reaction medium contained 25 mM MgCl_2 and 1 mM ATP. B: DCCD. Preincubation time 30 (diamonds) or 60 min (triangles). Open symbols: 10 mM MgCl_2 , 300 mM NaCl, 300 mM KCl, 2 mM ATP. Closed symbols: 50 mM $\text{Mg}(\text{NO}_3)_2$, 100 mM NaNO_3 , 100 mM KNO_3 , 2 mM ATP. C: DIDS (◇); SITS (□). The reaction was carried out in the presence of 50 mM MgCl_2 and 50 mM CaCl_2 and 1 mM ATP (open symbols) or in the presence of 50 mM $\text{Mg}(\text{NO}_3)_2$, 50 mM $\text{Ca}(\text{NO}_3)_2$ and 1 mM ATP (closed symbols).

Discussion

The plasma membrane ATPase of *D. parva* isolated in this study exhibits properties which are partially similar and partially completely different in comparison to properties of the corresponding enzyme from other plant sources. Properties which match properties of ATPases from higher plants, algae and fungi (V_{\max} -values, K_M -values, substrate specificity, inhibition by vanadate and DES, non-inhibition by ouabain) do not need further discussion [2, 27]. However, interesting differences to other ATPases are the dependency on divalent cations, its high salt resistance, the stimulatory effect of chloride and nitrate, the sensitivity against anion transport inhibitors and its insensitivity against DCCD and ionophores. Part of these differences may reflect adaptations to the saline environment of this alga.

Divalent cations. Most plasma membrane ATPases require Mg^{2+} as cofactor, because a MgATP complex is thought to serve as substrate for the ATPase. Optimal $\text{Mg}^{2+}/\text{ATP}$ concentrations ratios vary between 0.5 and 1.0 [28, 29]. High concentrations of free Mg^{2+} usually inhibit ATPase activities, because Mg_2ATP complexes are formed which are not recognized as substrate [2, 30, 31]. The plasma membrane ATPase of *D. parva* does not fit into this pattern. Increasing concentrations of Mg^{2+} up to 100 mM cause a stimulation of the ATPase activity. Even more surprising is that Ca^{2+} can substitute for Mg^{2+} . Normally Ca^{2+} can be an efficient cofactor of ATPases from mitochondria and thylakoids, but not of plasma membrane ATPases [2, 27]. The latter ATPases are inhibited already at moderate Ca^{2+} concentrations. However, the observed requirement of *D. parva* ATPase for Mg^{2+} and/or Ca^{2+} does not necessarily reflect exclusively the interaction of the divalent cations with catalytic sites of the enzyme. We assume, disregarding that MgATP complexation may be influenced by the ionic strength of the reaction medium, that high concentrations of Mg^{2+} and Ca^{2+} are required in the microenvironment of the ATPase, e.g. for the maintenance of membrane structure and membrane stability [32, 33]. Thus these cations may exert indirect effects on the ATPase activity, which partially may overlap the primary effects on the catalytic sites. Salts affect also the fluidity of *Dunaliella* membranes [33]. *D. parva* cells are well adapted to high external concentrations of divalent cations and contain high intracellular concentra-

tions of Mg^{2+} and Ca^{2+} [16, 21], although intracellular free activities are not known. It is noteworthy in this respect, that the ER-ATPase of this algae requires only millimolar concentrations of Mg^{2+} and/or Ca^{2+} and that higher concentrations are inhibitory [16]. *In situ* only the plasma membrane, but not the ER is exposed to the high external concentrations of divalent cations. Thus the different requirement of divalent cations of the plasma membrane ATPase and the ER-ATPase may reflect an adaptation to this situation. Beside this difference both ATPases have in common, that Ca^{2+} can serve as an efficient cofactor.

Monovalent cations. Stimulation of plasma membrane ATPases by alkali cations as it can be shown also with the ATPase of *D. parva*, were believed for a long time to be indicative for the involvement of K^+ or Na^+ transport in the ATPase reaction [2, 27, 35]. However, there is little direct experimental evidence from transport studies for this view. In this study it could be shown that a large contribution of the stimulatory effect of NaCl and KCl originates from the accompanying anions. NaCl and $NaNO_3$ stimulate the ATPase reaction, but not sodium sulfate. It is not known at present whether this effect is due to a direct effect of anions on the ATPase or whether this reflects the involvement anion transport. Permeant anions like chloride or nitrate are expected to dissipate ATP-induced membrane potentials and thereby should stimulate ATPase. Impermeant anions such as sulfate are expected to increase the membrane potential and thereby should inhibit ATPase. Of large physiological importance is the high salt resistance of the plasma membrane ATPase of *D. parva*, which under *in vivo* conditions is exposed on the external side to salt saturations up to saturation. Half-maximal inhibitions of ATPase activities are observed at NaCl and KCl concentrations around 2.5 M NaCl. The corresponding value for the ER-ATPase of this algae, which is exposed only to the low internal salt concentrations is about 100 mM.

Possible function of the plasma membrane ATPase. In this paper only the hydrolytic activity of the plasma membrane ATPase was investigated, but no transport studies were carried out. This limits discussion of possible ATPase functions. From many studies with intact *Dunaliella* cells the presence of a H^+ -exporting ATPase in the plasma membrane was postulated, but the experimental evidence from *in*

vivo studies is rather poor [36–38]. All attempts to demonstrate with isolated plasma membrane ATPases of this alga ATP-dependent H^+ translocations failed so far (this paper, not shown, Pick, personal communication). Also the results with the inhibitor DCCD, which is believed to inhibit all H^+ -translocating ATPases [25], are ambiguous. In our experiments DCCD always failed to inhibit ATPase activity, although applied at various reaction conditions and incubation times. Uptake into the cells and efficiency of DCCD within the cells were ensured by demonstrating its inhibitory effects on photosynthesis of intact *D. parva* cells. Pick *et al.* [15] did observe an inhibitory effect of 0.1 mM DCCD with the plasma membrane ATPase of *D. salina* and concluded from that the presence of a normal H^+ -ATPase. We conclude from our data that the accessibility of DCCD to the DCCD-binding site within the plasma membrane ATPase of *D. parva* is poor. We do not take DCCD resistance as direct evidence against a H^+ -translocating ATPase. The missing effects of ionophores on the ATPase activities in our experiments unfortunately also do not support the presence of a H^+ -ATPase in the plasmalemma. A lack of ionophore stimulation theoretically could be explained by the presence of highly permeable vesicles. In view of the stimulation of the ATPase by alkali cations and chloride or nitrate it is very unlikely that a large portion of vesicle populations consists of unsealed membranes.

Our kinetic studies suggest that the plasma membrane ATPase of *D. parva* is activated by two substrates, one being ATP and the other unknown. The strong inhibition of ATPase by the inhibitors of anion transport, DIDS and SITS, imply the involvement of anion transport in the ATPase reaction. This view is supported by the chloride and nitrate stimulation of the ATPase. It cannot be decided as yet whether such an anion transport is coupled directly to the ATPase or indirectly *via* an associated H^+ /anion symporter. Recently evidence for coupling of a Na^+/H^+ antiporter to the plasma membrane ATPase of *D. salina* was presented [14, 15]. Both observations do not exclude each other. However, for a final conclusion transport studies are required. It will be of special interest to look at ATP-mediated translocations of chloride and bicarbonate.

Comparison with other ATPases of *Dunaliella parva*. Different localization of ATPases within the cells by no means is equivalent to the existence of differ-

ent types of ATPases. However, the properties of the plasma membrane ATPase are described in this paper are completely different from that of the ER-ATPase of this alga [16]. This refers especially to the difference in the requirement of divalent cations, the difference in salt resistance and the different stimulation by anions. However, the properties of the plasma membrane ATPase are very similar to the properties of the flagellar ATPase of *D. parva* [11].

Comparison with higher plant ATPases. Higher plant cells basically contain beside the coupling factors of the chloroplasts and the mitochondria two types of electrogenic, H^+ -translocating ATPases [2]. 1) The vanadate sensitive plasma membrane ATPase, which is stimulated by alkali cations, whereas anions have little effect. 2) The vanadate insensitive tonoplast ATPase, which is strongly inhibited by

SITS, DIDS and nitrate and stimulated by chloride, whereas alkali cations have little effect. This investigation shows that the plasma membrane ATPase of *D. parva* does not fit exactly into this pattern. It exhibits properties of both types. The sensitivity to vanadate, SITS and DIDS and the stimulatory effects of alkali cations and of both chloride and nitrate indicates a different type of ATPase. But in spite of the missing inhibition by DCCD and missing stimulation by ionophores we do not suggest that the plasma membrane ATPase of *D. parva* is different to higher plant ATPase in regard to H^+ pumping.

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