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A vacuolar ATPase and pyrophosphatase in *Acetabularia acetabulum*

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Vacuole-rich fractions were isolated from *Acetabularia acetabulum* by Ficoll step gradient centrifugation. The tonoplast-rich vesicles showed ATP-dependent and pyrophosphate-dependent H^+-transport activities. ATP-dependent H^+-transport and ATPase activity were both inhibited by the addition of a specific inhibitor of vacuolar ATPase, bafilomycin B_1. A 66 kDa polypeptide present in the preparation cross-reacted with the anti-IgG fractions against the α and β subunits of *Halobacterium halobium* ATPase and with the antibody against the A subunit (68 kDa subunit) of mung bean vacuolar ATPase. A 56 kDa polypeptide present in the vacuole preparation showed cross-reactivity with the antibody against the B subunit (57 kDa) of mung bean vacuolar ATPase but not with the anti-β subunit of *H. halobium* ATPase. A 73 kDa polypeptide cross-reacted with the antibody against inorganic pyrophosphatase of mung bean vacuoles. These results suggest that vacuolar membrane of *A. acetabulum* equipped energy transducing systems similar to those found in other plant vacuoles.

Introduction

ATPases, which play important roles in energy conversion and utilization in living organisms, are classified into three categories: P, V and F types [1]. Archaebacterial ATPase has been reported to be closely related to the V type and not related to the F type [2–4]. Mukohata et al. proposed that ATPases in archaebacteria be given an independent classification, i.e. A type, in the ATPase family. Evolutionary aspects of the ATPase family have been one of the most current topics in the field. One hypothesis is that common progenotes may have been three different progenitors of ATPase (V, A and F types) [5–7].

Acetabularia acetabulum, a unicellular marine alga, belongs to the Dasycladaceae, and is one of the most ancient eukaryotes. From intensive electrophysiological studies by Gradmann et al. [8,9], it has been proposed that this organism has an electrogenic Cl^- pump in its plasmalemma that maintains a membrane potential around −170 mV in the dark. The purification and reconstitution of a Cl^--translocating ATPase from the organism have been published previously [10,11]. The Cl^--ATPase had catalytic properties attributable to all of the well-known P, V and F types of ATPase, and showed considerable similarity to the F type by immunological and preliminary chemical characterization (unpublished data). It was also necessary to isolate and characterize chloroplast ATPase, since the Cl^--ATPase was similar to the F type. The chloroplast ATPase showed characterstics attributable mainly to the F type, but also possessed several properties of the V type such as susceptibility to nitrate and isothiocyanate (un-

Abbreviations: ATPase, adenosine triphosphatase; PPase, inorganic pyrophosphatase; PP_i, pyrophosphate; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; OVA, ovalbumin; TCS, 3,3′,4′,5-tetrachlorosalicylanilide; HRP, horseradish peroxidase.

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published data). Therefore, vacuolar ATPase present in this organism is of great interest both from structural and evolutional points of view.

In this report, we found H^+-transport activities driven by ATP and PP_i in vacuole-rich fractions from *A. acetabulum*. Immunological and preliminary biochemical properties of the H^+-ATPase and H^+-PPase are presented.

Materials and Methods

Reagents

Pipes, Tris, Tricine, Ficoll 400, valinomycin and EGTA were purchased from Sigma Co. (St. Louis, MO, U.S.A.). DTT was from Biomol GmbH (Ilvesheim, Germany), and PMSF from Merck (Darmstadt, Germany). HRP-conjugated goat anti-rabbit IgG fraction was obtained from Kent Lab. Inc. (Redmond, WA, U.S.A.). A Konica Immunostain HRP kit (Konica Co., Tokyo, Japan) was used for visualization of the reactions with antibodies. Anti-IgG fractions against the α and β subunits of *Halobacterium halobium* ATPase were prepared as described in Ref. 2, antibodies against mung bean vacuolar ATPase as in Ref. 12 and the antibody against mung bean inorganic PPase as in Ref. 13. Other reagents of analytical grade were purchased from Wako Pure Chemicals Inc. (Osaka, Japan).

Isolation of vacuole-rich fraction from Acetabularia acetabulum

Vacuole-rich fractions were, in principle, isolated according to the method described in Ref. 14. Axenic cells (3 to 5 cm in length, 58 g wet weight) were cut into small pieces, suspended in 100 ml of a buffer consisting of 50 mM Pipes-Tris (pH 8), 0.55 M sorbitol, 0.5 mM $MgSO_4$, 2 mM DTT and 25 μM PMSF. The suspension was stirred at room temperature for 5 min and filtered through four layers of cheesecloth. The cell debris retained in the cheesecloth was re-extracted twice with 100 ml of the above buffer. The filtrates were combined and centrifuged at $5000 \times g$ for 2 min to remove most of the chloroplasts. The supernatant was then centrifuged at $11000 \times g$ for 20 min and the pellets were suspended in approximately 18 ml of the above buffer containing 1 mM EGTA and 0.3 M sorbitol (buffer A) instead of 0.55 M sorbitol. About 3 ml of the suspension was layered onto a Ficoll step gradient consisting of 2.5 ml each of 2.5 and 7% (w/v) Ficoll in buffer A, and 1.5 ml each of 10 and 15% (w/v) Ficoll in buffer A. The six gradient tubes were centrifgued at $6000 \times g$ for 35 min in a swinging bucket rotor. The fraction in the 2.5% Ficoll (F_2), at the Ficoll interphases 2.5 and 7% (F_3) and the 7 and 10% (F_4) were collected. The fractions were diluted with 1 or 2 volumes of buffer A and centrifuged at $28000 \times g$ for 20 min. The pellets were resuspended in 0.5 ml of buffer A and then diluted with 5 ml of a hypotonic buffer consisting of 50 mM Tricine-KOH (pH 8), 1 mM EGTA and 1 mM DTT. After centrifugation at $28000 \times g$ for 20 min, the pellets were resuspended in 5 ml of the hypotonic buffer and centrifgued again. The pellets were resuspended in 0.5 ml of the hypotonic buffer and used for experiments.

Measurement of H^+-transport activity

For H^+-transport activity measurements fractions F_2 and F_3 were combined, washed with the hypotonic buffer without DTT in the end, and sonicated for 5 min in a bath-type sonicator to form tonoplast-rich vesicles. ATP-dependent H^+-transport activity was measured using the method described by Moriyama and Nelson [15]. The reaction mixture contained 20 mM Tricine-KOH buffer (pH 8), 0.1 M KCl, 2 mM $MgCl_2$, 1 μg/ml valinomycin, 2 μM acridine orange, and tonoplast-rich vesicles (approx. 15 μg protein) with a final volume of 0.4 ml. After addition of 2.5 mM ATP, fluorescence quenching of acridine orange (excitation 492 nm, emission 540 nm) was measured at 30 °C in a shimadzu RF-500LC fluorescence spectromonitor (Kyoto, Japan).

PP_i-dependent H^+-transport activity was measured in the same manner as described above, except that 1 mM PP_i instead of ATP was added to the reaction mixture.

Assay of ATPase and PPase activities

ATPase activity in vacuole-rich fraction was assayed, in principle, as described previously [10]. The reaction mixture consisted of 25 mM Pipes-Tris buffer (pH 8.0), 0.25 M sorbitol, 3 mM each of ATP and $MgSO_4$ and enzyme (2.5 to 10 μl) with a final volume of 0.1 ml. PPase activity was measured in the presence of azide, sodium molybdate and KNO_3 as described in Ref. 16. The reaction mixture consisted of 25 mM Pipes-Tris (pH 7.5), 0.5 mM azide, 0.5 mM sodium molybdate, 5 mM ammonium chloride, 50 mM potassium nitrate, 3 mM $MgSO_4$ and enzyme (2.5 to 10 μl) with a final volume of 0.1 ml. The reaction was conducted at 37 °C for 1 min. Inorganic phosphate released by enzymic reaction was assayed by the method of Lanzetta et al. [17].

SDS-PAGE and immunoblotting

SDS-PAGE was performed on mini-gels and immunoblotting was also carried out as described previously [10]. Binding of antibody was detected by colorimetric reaction (basically with 4-chloronaphthol) of the HRP conjugated to the secondary antibody.

Protein determination

Protein concentration of samples was determined by the method of Heil and Zillig [18] using BSA as the standard.

Results and Discussion

Identification of ATP-dependent H^+-transport activities

ATPase and PPase activity in the F_2 to F_4 fractions are summarized in Table I. The specific activity of ATPase in fraction F_4 was low when compared with the activities in fraction F_2 and F_3. The distribution of PPase activity paralleled that of ATPase activity. In addition, the ATPase activity in the F_4 fraction was inhibited by 70% in the presence of azide, a characteristic of chloroplast/mitochondrial ATPases. The effects of anions both on the ATPase and PPase activities in the combined F_2/F_3 fraction are summarized in Table II. At the concentrations of anions tested, nitrate inhibited ATPase activity but not PPase activity. PPase activity was inhibited by sulfate and sulfite. Further purification and characterization of the both enzymes are now in progress.

ATP-dependent H^+-transport activity in the tonoplast-rich vesicles (F_2/F_3 fraction) and the effects of orthovanadate, azide and nitrate are shown in Fig. 1. The effects of orthovanadate, azide, NEM and nitrate on the ΔpH formation across a membrane and the ATPase activity are summarized in Table III. The F_2/F_3 fraction was partially contaminated by plasmalemma and chloroplast/mitochondrial fractions as evidenced by the inhibition of the ATPase activity by azide and orthovanadate. The ATP-dependent H^+-transport activity was, however, little affected by azide (8% inhibition) and was somewhat inhibited by orthovanadate (22%). About 70% of the H^+-transport activity driven by ATP was, therefore, attributable to a vacuolar ATPase in the fraction.

Evidence that the ATP-dependent H^+-pump belongs to V type

Cold inactivation was performed using the F_2 and F_3 fractions as described in Ref. 19. ATPase activity in the both fractions decreased with time and reached 29% (F_2) and 27% (F_3) of the initial activities after 24 h both in the presence and absence of Mg^{2+}-ATP.

After cold inactivation of the F_2 fraction in the presence and absence of Mg^{2+}-ATP, the suspension was centrifuged at 17000 × *g* for 30 min. Both the supernatants and the pellets were subjected to SDS-PAGE and immunoblotting. The results are shown in Fig. 2. Essentially the same protein patterns were observed for the supernatants and pellets treated in the presence or absence of Mg^{2+}-ATP (Fig. 2a). Bowman et al. [20] reported for *N. crassa* vacuolar ATPase that the inhibition of the ATPase activity by cold inactivation in the presence of nitrate led to a partial release of the subunits into the supernatant. As shown in Table II, the ATPase activity in the F_2/F_3 fraction was partially inhibited by NaCl. A long exposure to NaCl might cause dissociation of subunits into the supernatant similar to that in *N. crassa* vacuolar ATPase in the presence of KNO_3. The 66 kDa and 56 kDa polypeptides were released into the supernatants and clearly cross-reacted with the antibodies against the 68 kDa (A) and 57 kDa (B) subunits of mung bean vacuolar ATPase, respectively (Fig. 2b). Thus, the 66 kDa and 56 kDa polypeptides were possible candidates for the A and B subunits of the vacuolar ATPase in *A. acetabulum*.

A 66 kDa polypeptide released into the supernatant also cross-reacted with the respective anti-α and anti-β

TABLE I

Distribution of the ATPase activity and PPase activity in the F_2, F_3 and F_4 fractions

One unit is defined as μmol P_i liberated/min at 37 °C. Specific activity represents U/mg protein.

frs.	ATPase activity		PPase activity	
	total	specific	total	specific
F_2	77.7 mU	0.56 U	218 mU	1.56 U
F_3	101	0.71	319	1.53
F_4	19.8	0.20	34.7	0.35

TABLE II

Effect of anions on the ATPase and PPase activity in the F_2/F_3 fraction

The F_2/F_3 fraction (2.5 μl) was assayed for ATPase and PPase activities.

Additions	ATPase act.	PPase act.
None	100%	100%
Na_2SO_4 (0.2 M)	85	37
Na_2SO_3 (0.2 M)	110	36
KCl (0.2 M)	76	137
KNO_3 (0.1 M)	53	125
NaCl (0.2 M)	84	82

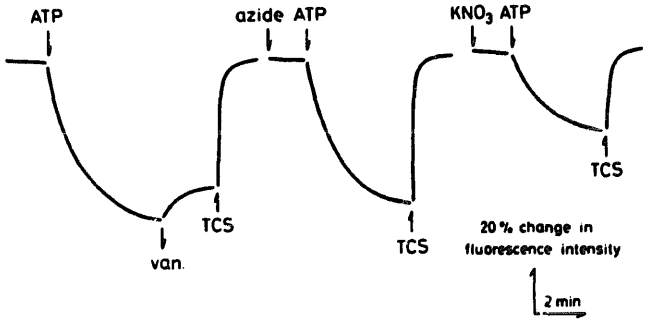

Fig. 1. ATP-dependent H^+-transport activities in the tonoplast-rich vesicles. Tonoplast-rich vesicles (15 μg protein in 25 μl) were subjected to an acridine orange fluorescence quenching assay as described under Materials and Methods. Inhibitors (azide and KNO_3) were mixed with the enzyme source, then valinomycin and acridine orange were added to the mixture. Concentrations of additions: ATP, 2.5 mM; orthovanadate (van.), 5 mM; azide, 1 mM; KNO_3, 50 mM; TCS, 1 μM.

TABLE III

Effect of azide, orthovanadate, NEM and KNO_3 on the H^+ transport activity and on the ATPase activity in the F_2/F_3 fraction

Twenty-five μl of the F_2/F_3 fraction (15 μg protein) were assayed for ATP-dependent H^+ transport activity (azide, 1 mM; vanadate, 5 mM; NEM, 1 mM; KNO_3, 50 mM). Five μl of the F_2/F_3 fraction were assayed for ATPase activity in the presence and absence of the same amounts of inhibitors used for H^+-transport activity measurement.

Addition	% fluorescence quenching	ATPase activity
None	100	100
Azide	92	69
Vanadate	78	67
NEM	68	75
KNO_3	52	62

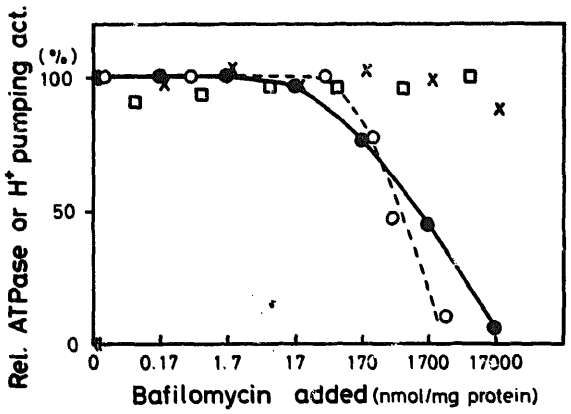

Fig. 3. Effect of bafilomycin on ATPase activity in the F_3 fraction, the Cl^--ATPase and the chloroplast ATPase, and on the H^+-transport activity in the F_2/F_3 fraction. The F_3 fraction (0.6 μg protein), the Cl^--ATPase (Mono Q-III fraction in Ref. 10, 0.5 μg protein) and the chloroplast ATPase (1.4 μg protein of chloroplast-rich fraction prepared by the method of Bidwell et al. [22]) were preincubated with various concentrations of bafilomycin at room temperature for 10 min in the assay mixture (97 μl) without ATP, the reaction was started by addition of ATP (100 mM, 3 μl) and incubated at 37 °C for 15 min. pH Values of the buffers were 8.0, 6.5 and 8.9 (25 mM Pipes-Tris), respectively. ●———●, ATPase activity in the F_3 fraction; ×, ATPase activity in the Mono Q-III fraction; □, ATPase activity in the chloroplast-rich fraction; ○------○, H^+-transport activity in the F_2/F_3 fraction.

IgG fraction of *Halobacterium halobium* ATPase and the mixture of both (Fig. 2c). Several archaebacterial ATPases have been isolated and the primary structures elucidated from the cloned genes [3,4]. They showed significant sequence similarities to the V type ATPase in eukaryotes. The possible candidate of the B subunit (56 kDa) of the vacuolar ATPase in *A. acetabulum*, however, did not cross-react with the anti-β IgG fraction of *H. halobium* ATPase.

Bafilomycin was known to be a specific inhibitor of the V type ATPase [20]. ATPase activity in the F_3 fraction was inhibited by bafilomycin, whereas the Cl^--ATPase and chloroplast ATPase were not (Fig. 3). The effect of bafilomycin on the H^+-transport activity in the F_2/F fraction is also presented in Fig. 3, and the inhibitory curves were well correlated. The effect

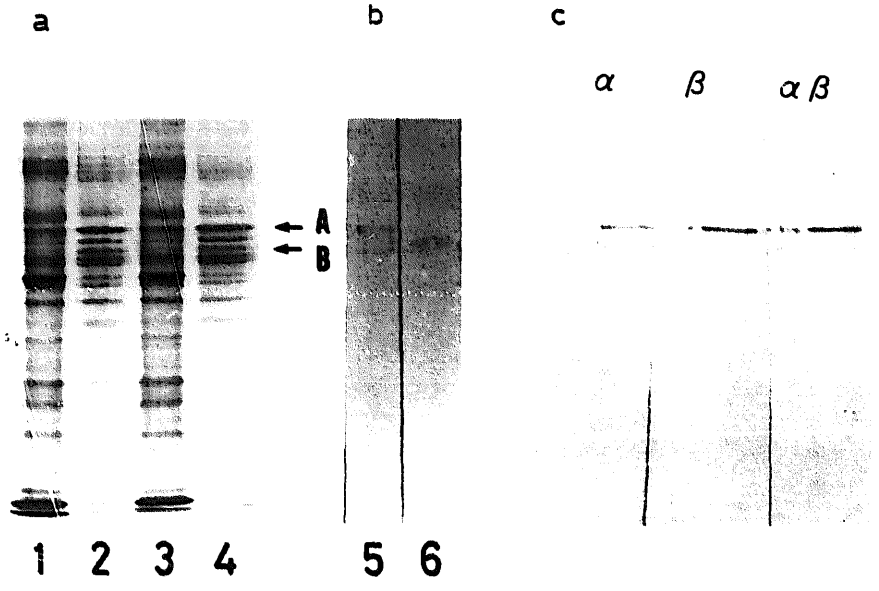

Fig. 2. Cold inactivation and immunoblotting of the F_2 fraction in the presence and absence of Mg^{2+}-ATP. The F_2 fraction (7 μg protein in 50 μl) was kept at 4 °C overnight in the presence and absence of Mg^{2+}-ATP (5 mM each). After centrifugation, one-fourth of each supernatant and pellet was subjected to SDS-PAGE and to immunoblotting. (a) Silver stain; lanes 1 and 2, precipitate and supernatant obtained after cold inactivation in the absence of Mg^{2+}-ATP; lanes 3 and 4, precipitate and supernatant obtained after cold inactivation in the presence of Mg^{2+}-ATP. (b) Reaction of the F_2 fraction supernatant after cold inactivation with antibodies against the A (lane 5) and B (lane 6) subunits of mung bean vacuolar ATPase. (c) Immunoreaction with anti-α subunit (left), anti-β subunit (middle) of *H. halobium* ATPase and a mixture of both antibodies (each 3 μg/ml) (right). The samples on each membrane strip correspond to that of lanes 3 and 4, respectively. The immunoblots of lanes 1 and 2 were essentially the same as those obtained for lanes 3 and 4, respectively.

of bafilomycin was not as drastic as reported for known V type ATPases by Bowman et al. [21]. Partial contamination of the F_2/F_3 fraction by plasmalemma and chloroplast/mitochondrial ATPases or membrane fractions probably decreased the overall effect of bafilomycin. Data in Fig. 3 also shows that the isolated Cl^--ATPase [10] and ATPase activity in chloroplast-rich fraction isolated by the method of Bidwell et al. [22] from *A. acetabulum* were not significantly inhibited by bafilomycin. The A type of ATPase was generally known for its insusceptibility to bafilomycin, even though this class of ATPase was closely related to the V type. It was also possible that a vacuolar ATPase in *Acetabularia* has properties intermediate that of the V and A type ATPases.

H^+ pumping PPase is associated with vacuolar membrane

Tonoplast-rich vesicles prepared from the F_2/F_3 fraction were tested for PP_i-dependent H^+-transport activity. The H^+-transport activity driven by PP_i was observed in the presence of valinomycin, but not in the absence of valinomycin (Fig. 4). The data support the electrogenicity of the PP_i-dependent H^+-transport activity.

A vacuolar membrane-bound inorganic PPase has been isolated and purified from mung bean by one of the authors [13]. The antibodies produced against this PPase were reacted with the supernatant and pellets of the F_2 fraction after cold inactivation. As shown in Fig. 5b, a polypeptide in the pellets with a size similar to that of the mung bean PPase clearly cross-reacted with the antibody.

Data presented in this report suggested that the vacuolar membrane of *A. acetabulum* was equipped with a similar energy transducing systems found in other higher plant vacuoles, a vacuolar ATPase and inorganic PPase. Both H^+-translocating systems are generally known to energize vacuole and function in ion metabolism and concentrating metabolites within the vacuole. H^+-ATPase and PPase in vacuole of *A. acetabulum* are expected for the presence of such a

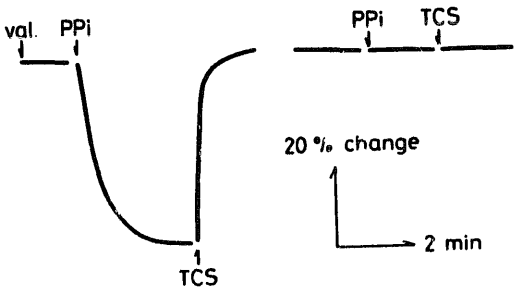

Fig. 4. PP_i-dependent H^+-transport activity in the tonoplast-rich vesicles. The experiments were performed as described in the legend in Fig. 1, except that PP_i (1 mM) was added instead of ATP and tonoplast-rich vesicles (7.5 μg protein in 12.5 μl) were assayed for H^+-transport activity.

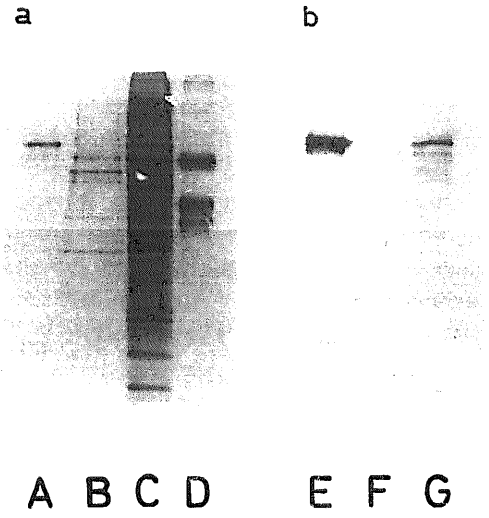

Fig. 5. Immunoblots of the pellets after cold inactivation of F_2 fraction with the antibody against mung bean vacuolar PPase. SDS-PAGE and Western blotting were carried out as described in Materials and Methods. The F_2 fraction (10 μg protein) was subjected to cold inactivation in the presence of Mg^{2+}-ATP, and the supernatant and pellet were successively analyzed by SDS-PAGE and immunoblotting. (a) Silver stain; lane A, mung bean vacuolar PPase (0.5 μg protein); lane B, supernatant after cold inactivation of the F_2 fraction, lane C, pellets after cold inactivation; lane D, standard proteins (BSA, OVA and aldolase). (b) Immunoblot and reactions with the anti-IgG fraction against mung bean vacuolar PPase; Lanes E, F and G correspond to lanes A, B and C, respectively.

secondary system utilizing energy as described above. As mentioned in Introduction, *A. acetabulum* does not belong to higher plants but to alga. This is also the first evidence for the presence of the both H^+-translocating systems in a vacuolar membrane of alga. Cloning of the genes encoding the A and B subunits of the vacuolar ATPase, and PPase is now in progress. Studies along these lines could give more informations on their primary structures, and on evolutionary aspects of the ATPase family and PPase.

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