A Cl^--Translocating Adenosinetriphosphatase in *Acetabularia acetabulum*. 1. Purification and Characterization of a Novel Type of Adenosinetriphosphatase That Differs from Chloroplast F_1 Adenosinetriphosphatase

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ABSTRACT: ATPases were solubilized from membranes of *Acetabularia acetabulum* using nonanoyl-*N*-methylgluconamide and purified by ion-exchange and gel permeation chromatography. Three fractions of ATPase, Mono Q-I, -II, and -III, were separated. Activity in fraction Mono Q-I was very labile and could not be accurately determined. Fractions Mono Q-II and -III had specific activities of 0.6 and 6 units/mg of protein, respectively. By SDS–polyacrylamide gel electrophoresis, isoelectric focusing, and peptide mapping, it was shown that fractions Mono Q-II and -III consisted of the same polypeptides with molecular masses of 54K (a-subunit) and 50K (b-subunit). Fractions Mono Q-II and -III had the following catalytic properties: pH optimum at 6.0; substrate specificity, ATP = GTP = ITP ≫ UTP = CTP (K_m for ATP 0.6 mM); divalent cation requirement, $Mn^{2+} = Mg^{2+} > Co^{2+} > Zn^{2+} \gg Ca^{2+}, Ni^{2+}$. Both activities were inhibited by monovalent anions, while monovalent cations had neither inhibitory nor stimulatory effects. Orthovanadate inhibited both activities to 50% at 1 mM, and the most effective inhibitor of both was azide (95% inhibition at 100 μM). An enzyme–phosphate complex was formed after incubation of fraction Mono Q-III with [γ-^{32}P]ATP. The CF_1-ATPase subcomplexes were isolated from the same organism and compared with the fraction Mono Q-III. Data supported the difference of fraction Mono Q-III from CF_1-ATPase.

Cation-translocating adenosinetriphosphatases (ATPases)[1] (H^+-, K^+,Na^+-, and Ca^{2+}-ATPases) are thoroughly studied and well characterized (Amzel & Pedersen, 1983; Goffeau & Slayman, 1981; Pedersen & Carafoli, 1987). However, little is known about anion-translocating proteins except band 3 protein in erythrocytes (Ross & McConell, 1977; Lukakovic et al., 1981) and halorhodopsin in *Halobacterium halobium* (Blanck & Oesterhelt, 1987); neither is an ATPase. Several studies on a Cl^--translocating ATPase in bacteria and eukaryotes reported activities in membrane vesicles (Sakai et al., 1986; Inagaki & Shiroya, 1988) but the Cl^--translocating activity has not yet been demonstrated in reconstituted systems using purified proteins.

Acetabularia, a unicellular marine alga, has been extensively studied for its chloride transport properties and the presence of an electrogenic Cl^- pump demonstrated by detailed electrophysiological studies (Saddler, 1970; Gradmann, 1970, 1975; Mummert et al., 1981). Subsequent characterization (Gradmann et al., 1982; Gradmann, 1984; Tittor et al., 1983) and biochemical studies of partially purfied plasma membrane fractions suggested that the electrogenic Cl^- pump in *Acetabularia* is driven by ATP (Goldfarb & Gradmann, 1983; Goldfarb et al., 1984).

Thus, the Cl^- pump in *Acetabularia* is of considerable biochemical interest. In this paper, we describe the solubilization and purification of membrane-bound ATPases, and catalytic properties. The isolation and characterization of chloroplast F_1-ATPase (CF_1) subcomplexes are also described, and the difference of the membrane-bound ATPases from the CF_1-ATPase is presented.

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Materials and Methods

Chemicals and Instruments. Triphosphonucleotides (ATP, GTP, ITP, UTP, and CTP, Na salts and orthovanadate-free), ADP, AMP, Pipes, DCCD, oligomycin, diethylstilbestrol, FITC, valinomycin, choline chloride, and peroxidase-conjugated anti-rabbit goat IgG fraction were purchased from Sigma Chemical Co. (St. Louis, MO). NCS and CNBr were from Tokyo Kasei Kogyo, Ltd. (Tokyo, Japan), and 4-chloronaphthol and hydroxylamine hydrochloride were from Wako Pure Chemicals Ind. Ltd. (Osaka, Japan). Ampholines for IEF were from LKB (Bromma, Sweden). Other reagents of analytical grade were obtained from Merck (Darmstadt, FRG). DEAE-Sephacel and the FPLC system were from Pharmacia (Uppsala, Sweden). MEGA-9 was purchased from Oxyl Co. (Bobingen, FRG).

Preparation of Membrane Fractions. Axenic cells (about 2–5 mm in length) of *Acetabularia acetabulum* were grown according to the method described by Schmid and Giesecke (1984), and cells were collected by filtration through a nylon net. Residual medium was removed by filter papers (Whatman 3M), and the cells were then resuspended in 20 volumes

[1] Abbreviations: ATPase, adenosinetriphosphatase; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; UTP, uridine 5′-triphosphate; CTP, cytosine 5′-triphosphate; FITC, fluorescein isothiocyanate; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE-Sephacel, *O*-(diethylaminoethyl)Sephacel; MEGA-9, nonanoyl-*N*-methylgluconamide; FPLC, fast protein liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; OVA, ovalbumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IEF, isoelectric focusing; NCS, *N*-chlorosuccinimide.

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of disruption buffer as described previously (Goldfarb & Gradmann, 1983) with the exception that BSA, aprotinin, and Tris–sulfate were omitted but 0.25 mM PMSF (100 mM in ethanol) and 50 mM Pipes–Tris buffer (pH 7.6) were added. Disruption in a Parr bomb was carried out as described by Goldfarb and Gradmann (1983) but repeated 3 times. The homogenate was filtrated through a nylon net (0.5-mm pore size) for removal of cell debris, and portions (20 mL) were layered on a two-step sucrose gradient consisting of 5 and 50% (w/v) sucrose in disruption buffer, 7.5 mL of each. After centrifugation at 80000*g* for 90 min, the material at the interphase was collected by a Pasteur pipet and homogenized with a Teflon pestle homogenizer. The membrane fraction thus obtained could be stored at −70 °C without any significant loss of ATPase activity for at least 4 months.

Solubilization of ATPase Activities. The membrane fraction obtained from 10 g of cells (1 mg of protein/mL, 30–35 mL) was adjusted to 2% (w/v) MEGA-9 with a 30% (w/v) aqueous stock solution of the detergent. The suspension was stirred at room temperature for 30 min and centrifuged at 140000*g* for 1 h at 15 °C. Purification was continued at room temperature except for concentration of eluates (at 4 °C).

DEAE-Sephacel Chromatography. The supernatant was applied to a DEAE-Sephacel column (2.7 × 9 cm) previously equilibrated with a buffer consisting of 25 mM Pipes–Tris buffer (pH 7.0), 0.25 M sorbitol, 2 mM EGTA, 6 mM $MgSO_4$, 2 mM DTT, 0.1 mM PMSF, and 0.25% (w/v) MEGA-9. The column was washed with this buffer until the absorbance at 280 nm became negligible. The proteins were subsequently eluted stepwise with 0.2 M Na_2SO_4 and 0.4 M Na_2SO_4 in the buffer (about 100 mL of each). The flow rate was 40–50 mL/h. The active fractions designated as DE-I eluted with 0.2 M Na_2SO_4 and DE-II with 0.4 M Na_2SO_4 were pooled and concentrated by ultrafiltration to about 0.4 mL using Amicon's YM-10 membrane filter and Centricon YM-30, respectively.

Gel Permeation Chromatography. The concentrated fractions DE-I and DE-II were applied in two batches, 0.2 mL each, to a FPLC-Superose 12 column which was previously equilibrated with a buffer containing 25 mM Pipes–Tris buffer (pH 6.5), 0.25 M sorbitol, 2 mM EGTA, 6 mM $MgSO_4$, 2 mM DTT, 0.05 mM PMSF, 0.25% (w/v) MEGA-9, and 50 mM Na_2SO_4. The flow rate was 0.25–0.50 mL/min. An aliquot (0.5–5 μL) of each 0.5-mL fraction was assayed for the ATPase activity. Active fractions eluted from the column after application of the DE-II fraction were combined (see Figure 1c) and concentrated to about 0.2 mL, followed by 10-fold dilution with the same buffer but without Na_2SO_4 and reconcentration of the sample. This procedure was repeated twice, and the desalted solution was concentrated to a final volume of about 0.5 mL. The active fractions from the DE-I fraction were treated in the same manner.

For the determination of molecular masses, γ-globulin from human (160K), BSA (66K), Ovalbumin (45K), and cytochrome *c* (14K) were used as standard proteins dissolved in the equilibration buffer.

Mono Q Chromatography. Each desalted concentrate above was applied to a Mono Q column (1-mL bed volume) on the FPLC system which was previously equilibrated with the same buffer (pH 6.5) used for gel permeation chromatography but without Na_2SO_4. Proteins were eluted by buffer (1 mL), followed by a linear increase in Na_2SO_4 to 0.2 M (total volume of 16 mL) and 5 mL of 0.2 M Na_2SO_4-containing buffer. The flow rate was 0.5 mL/min. An aliquot (0.5 and 5 μL) of each 0.5-mL fraction was assayed for enzyme activity of the gel-filtrated DE-II and DE-I fraction, respectively. Two major protein peaks, Mono Q-I and -II (see Figure 1b), were obtained from the gel-filtrated DE-I fraction and one major, Mono Q-III (see Figure 1d), from the gel-filtrated DE-II fraction. They were concentrated and desalted as described above. The final preparations were frozen in liquid nitrogen and stored at −70 °C.

Preparation of a Fraction Containing Chloroplasts from Cells 3–5 cm in Length and Isolation of the CF_1-ATPase Components. About 20 cells were tied together with a thread around the cell tips and the rhizoids cut off with a razor blade. The threads of several cell bundles were passed through small holes punched into the cap of a centrifuge tube and tied. The centrifuge tube was filled with about 25 mL of disruption buffer. Cell contents were collected by centrifugation at 1500*g* for 5–10 min. About 1000 cells were treated this way; the green pellets obtained were resuspended in about 200 mL of disruption buffer, passed through four layers of cheesecloth, and centrifuged again. The washed pellets were then homogenized in about 15 mL of disruption buffer with a Teflon pestle. ATPase activity was detected in the supernatant after centrifugation at 7000*g* for 15 min when the washed pellets were homogenized by using a mortar in the presence of EGTA (1 mM) in the buffer.

Alternatively, the chloroplast suspension was treated with MEGA-9 and centrifuged as described above. The supernatant was applied onto a DEAE-Sephacel column (2.7 × 3 cm), and proteins were eluted with about 50 mL of buffer containing 0.2 M Na_2SO_4. Fractions with absorbance at 280 nm were combined, concentrated, and desalted as described above. The desalted concentrate was subjected to Mono Q chromatography and the elution of proteins conducted in the same manner as described above. Each 0.5-mL fraction was concentrated, desalted, and subjected to SDS–PAGE, Western blotting, and reactions with the anti-CF_1 complex serum from spinach or with the anti-α and -β sera of *Escherichia coli* F_1-ATPase as described below.

Western Blotting and Reaction with Antiserum against the CF_1 Complex from Spinach or Antisera against the Respective α- and β-Subunits of E. coli F_1-ATPase. The rabbit antiserum against the CF_1 complex lacking the ε-subunit from spinach was kindly supplied by Prof. W. Junge and the rabbit antisera against the α- and β-subunits of *E. coli* F_1-ATPase by Prof. K. Altendorf, both from the University of Osnabrueck.

After SDS–PAGE on a mini gel (see below), Western blotting was carried out according to the method of Burnette (1981) and Towin et al. (1979), except that the transfer buffer contained 0.005% SDS instead of 20% methanol (Gershoni & Palade, 1983). The antisera were diluted 1:250 (CF_1 complex) and 1:500 (α- and β-subunits) and reacted with the electroblotted proteins for 1 h at 37 °C. Peroxidase-conjugated IgG was diluted 1:250 before use; the incubation was 1 h at 37 °C.

Analytical Gel Electrophoresis. Analytical SDS–PAGE was performed on a mini gel system (12.5% or 15% gel, 7–8 cm in length, 1–1.5 mm thick) using the procedure of Laemmli (1970). For the molecular mass determination, a calibration kit from Bio-Rad (phosphorylase B, BSA, OVA, human carbonic anhydrase, soybean trypsin inhibitor, and cytochrome *c*) was used.

For PAGE of native proteins, a 5% gel containing 0.25% (w/v) MEGA-9 was used and the buffer system of Davis (1964) which also contained the same concentration of MEGA-9.

IEF under denaturing conditions using urea was carried out according to the method of O'Farrell (1975).

Protein bands were stained by silver (Wray et al., 1981) or Coomassie blue. Enzyme activity on the native gels was visualized by the method of Weinbaum and Markman (1966).

Chemical Cleavage and Peptide Mapping. Chemical cleavages on gel slices were performed according to the method of Nikodem and Fresco (1979) for CNBr cleavage, of Saris et al. (1983) for hydroxylamine cleavage, and of Lischwe and Ochs (1982) for NCS cleavage.

The second SDS–PAGE was run on a mini gel (12.5 or 15% polyacrylamide), and polypeptides were visualized by silver staining.

Assay of ATPase Activity. The assay mixture for membrane fractions contained 25 mM Pipes–Tris buffer (pH 6.5), 0.25 M sorbitol, 3 mM ATP-Na (pH 7.0), 3 mM $MgSO_4$, and enzyme (2 μL) in a total volume of 0.1 mL. Solubilized enzyme samples were supplemented with MEGA-9 [0.1–0.15% (w/v)] in the above mixture. All samples were incubated for 15 min at 30 °C. Inorganic phosphate liberated by ATPase action was colorimetrically determined as described by Lanzetta et al. (1979). In a control sample, the enzyme first was omitted and the sample incubated for the same time. The enzyme (2 μL for the membrane fraction and the supernatant after solubilization or 0.5–5 μL for the eluates from the columns) was added and inorganic phosphate content determined. The phosphate in this control sample accounts for spontaneous ATP hydrolysis and phosphate present in the enzyme preparation. Thus, the difference in phosphate content between the sample and the control mixture represents the enzymatic ATP hydrolysis. One unit of enzyme activity is defined as 1 μmol of phosphate liberated/min at 30 °C (difference between sample and control).

Demonstration of Enzyme–Phosphate Complex. An incubation mixture was prepared according to Amory et al. (1980) which contained Mono Q-III fraction (about 10 μg of protein) and 10 μCi of [γ-^{32}P]ATP in a final volume of 0.1 mL with the exception that the buffer system was the same as described under Assay of ATPase Activity. After 2-min incubation at room temperature, the reaction was stopped by addition of 50% (w/w) trichloroacetic acid (25 μL) and the sample processed as described by Amory et al. (1980). SDS–PAGE under acidic conditions was performed according to Fairbanks and Avruch (1972), and the gel was incubated for 5 min in 1% (v/v) glycerol at room temperature, dried, and exposed to Kodak XR-1 X-ray film at −70 °C for 10 days.

Protein Determination. Protein was determined by amido black staining of proteins dotted onto nitrocellulose membrane (Heil & Zillig, 1970). BSA was used as the standard protein.

Results

Solubilization of Membrane-Bound ATPases with Detergents. Several detergents were examined for their ability to solubilize ATPase activity in the membrane fraction. Satisfactory solubilization was obtained with Triton X-100, Lubrol PX, *n*-octyl glucoside, dodecyl β-*O*-maltoside, and MEGA-9 (Table I), but in all cases, the activity in the supernatant was labile at 4 °C. Among the detergents tested, MEGA-9 was selected since it had several advantages such as high cmc, removability by dialysis, relatively low costs, and relatively high stability of the enzyme activity (20% remained after 4 h at 4 °C). This was sufficient to obtain a stable enzyme fraction in the next step of purification without significant loss of activity.

Purification of Membrane-Bound ATPases. Two ATPase-containing fractions were obtained through DEAE-Sephacel chromatography. The total activities of DE-I and DE-II fractions had a ratio of about 1 to 4. The activity in the DE-I fraction was very labile and mostly lost upon concentration by ultrafiltration.

Table I: Solubilization Rate (%) of ATPase Activity with Different Detergents[a]

detergent	detergent concn (%)	ATPase act. in supernatant (%)
lysolecithin	0.10	29.7
LDAO	0.10	7.2
Triton X-100	0.25	87.1
Lubrol PX	0.25	87.6
n-octyl glucoside	0.50	100
dodecyl β-*O*-maltoside	0.25	100
MEGA-9	1.0	96.4

[a] Each detergent was added to membrane suspensions (100 μL) in the final concentration indicated. After 30 min at room temperature, the mixture was centrifuged at 100000*g* for 1 h in a Beckman Airfuge. The ATPase activity was assayed in both the supernatant and the mixture before centrifugation in the presence of sorbitol as described under Materials and Methods.

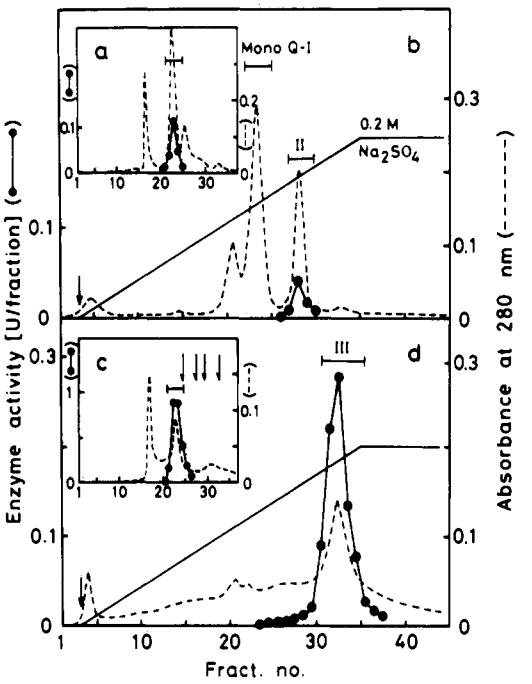

FIGURE 1: Separation of fractions DE-I and DE-II on a Superose (a and c) and on a Mono Q column (b and d). Each concentrate of fractions DE-I and DE-II was applied to a Superose column [(a) for DE-I and (c) for DE-II] and subsequently to a Mono Q column [(b) for the gel-filtrated DE-I and (d) for gel-filtrated DE-II]. The enzyme activity (●—●) of each fraction from DE-I and DE-II fractions was assayed for the ATPase activity, and protein was monitored by its absorption at 280 nm [A_{280} (- - -)]. The solid lines in (b) and (d) indicate the linear salt gradient of Na_2SO_4 used for chromatography and the arrows the start of the linear gradient from 0 to 0.2 M Na_2SO_4. The arrows in (c) from left to right indicate elutions of human γ-globulin (160K), BSA (66K), OVA (45K), and cytochrome *c* (14K) for calibration of molecular masses.

The DEAE-Sephacel column chromatography was very effective in removing green pigments from chloroplasts and throughout the purification. The next step, Superose gel permeation chromatography, removed mainly smaller molecular mass of proteins and residual green pigments and revealed an apparent molecular mass of about 230K for the ATPase (Figure 1a,c). This step was followed by a final chromatography on Mono Q (Figure 1b,d). As summarized in Table II, the Mono Q-II fraction contained an ATPase activity about 16-fold enriched in 5% yield and the Mono Q-III fraction a 190-fold-enriched ATPase in 34% yield. The specific

Table II: Purificaton of ATPases[a]

step	total volume (mL)	total protein (mg)	total act. (units)	sp act. (units/mg of protein)	yield (%)	purification (*x*-fold)
(1) membrane fraction	30	31	1.1	0.035	23	1
(2) supernatant	32	22	4.7	0.22	100	6
(3) DEAE column						
DE-I	55	1.4	0.5	0.36	11	10
DE-II	61	0.35	3.0	8.6	64	246
(4) Superose column (concentrate)						
gel-permeated DE-I	0.5	1.3	0.3	0.27	6.4	7.7
gel-permeated DE-II	0.5	0.32	2.0	6.3	43	180
(5) Mono Q column (concentrate)						
Mono Q-I	0.20	0.57	nd[b]			
Mono Q-II	0.16	0.43	0.24	0.56	5.1	16
Mono Q-III	0.20	0.24	1.6	6.7	34	191

[a] The membrane fraction obtained from ca. 10 g of cells (wet weight) was subjected to the purification. Enzyme activity at each step was assayed at pH 6.5 using ATP-Na (3 mM) and $MgSO_4$ (3 mM) as substrates and in the presence of 0.25 M sorbitol (see text). [b] nd, not detectable.

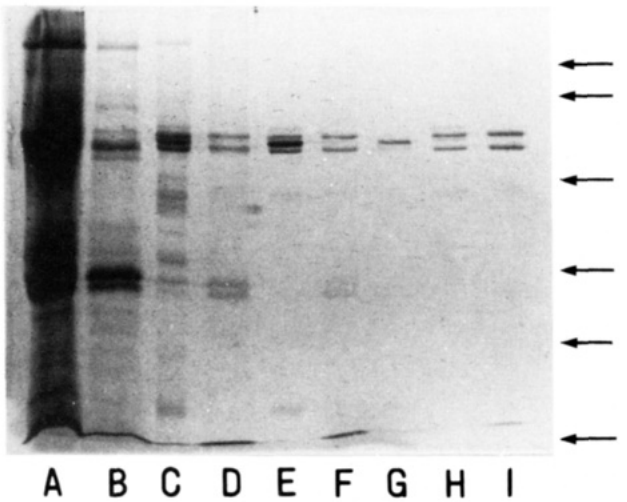

FIGURE 2: SDS–polyacrylamide gel electrophoretograms (Coomassie stain) of various enzyme samples. About 15 μg of the membrane fraction and the supernatant, and 2–4 μg of protein for all other samples, was applied to the gel and electrophoresis performed as described under Materials and Methods. Lane A, membrane fraction; lane B, the supernatant after solubilization; lane C, fraction DE-I; lane D, fraction DE-II; lane E, DE-I after a Superose column; lane F, DE-II after a Superose column; lane G, fraction Mono Q-I; lane H, fraction Mono Q-II; lane I, fraction Mono Q-III. The apparent molecular masses of the protein bands were determined with the help of marker proteins in a separate experiment. The arrows from top to bottom indicate phosphorylase B (92.5K), BSA (66K), OVA (45K), carbonic anhydrase (29K), soybean trypsin inhibitor (21K), and cytochrome *c* (14.3K).

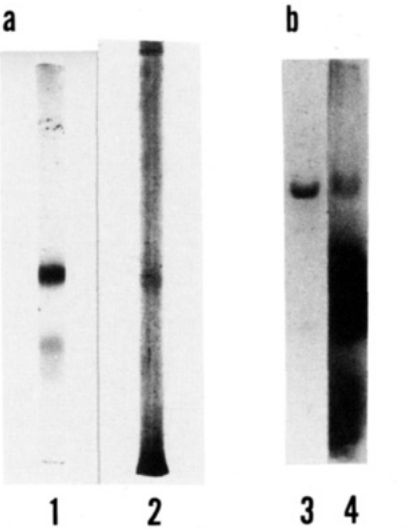

FIGURE 3: Polyacrylamide gel electrophoresis of fraction Mono Q-III (a) and formation of the enzyme–phosphate complex after incubation of fraction Mono Q-III with [γ-^{32}P]ATP. (a) Approximately 10 μg of the protein was applied per gel. Lane 1, Coomassie-stained gel; lane 2, activity-stained gel using the method of Weinbaum and Markman (1966). (b) Lane 3, fraction Mono Q-III stained by Coomassie after SDS–PAGE under acidic conditions; lane 4, fraction Mono Q-III (about 10 μg of protein) labeled by ^{32}P as described under Materials and Methods.

activities of the final preparation ranged from 0.5 to 0.7 unit/mg for the Mono Q-II fraction and from 6 to 6.7 units/mg of protein for the Mono Q-III fraction. The Mono Q-I fraction had little enzyme activity and was very unstable. Its properties were not further investigated.

Figure 2 shows SDS–polyacrylamide gel electrophoretograms of the membrane fraction, the supernatant after solubilization, and the concentrates after each purification step. Fraction Mono Q-I contains essentially a single polypeptide species with a molecular mass of 52K, while fractions Mono Q-II and -III mainly show two polypeptide species with molecular masses of 54K and 50K, and a fainter band at 39K. Throughout the purification, the addition of PMSF was necessary to avoid the proteolytic degradation of the 54K polypeptide (data not shown).

Figure 3a shows the activity and protein staining of fraction Mono Q-III. Only the main band showed ATPase activity. ^{32}P activity was detected when the enzyme was incubated with [γ-^{32}P]ATP prior to SDS–PAGE (see Figure 3b), and the phosphate turnover was observed by a cold chase (data not shown). However, due to the acidic conditions of electrophoresis for prevention of ^{32}P release, the resolution did not allow the separation of 54K and 50K bands.

IEF of Fractions Mono Q-I, -II, and -III under Denaturing Conditions, Successive Chemical Cleavage, and Peptide Mapping. As seen in Figure 2, fractions Mono Q-II and -III yielded the same pattern on the SDS gel (lanes H and I). By IEF, we attempted to obtain more information on these polypeptides. The results are shown in Figure 4a. Fractions Mono Q-II and -III gave essentially the same patterns with at least seven bands between p*I* 6.3 and 5.4 (group I) and about five bands between p*I* 5.2 and 5.0 (group II) (lanes B and C in Figure 4a). Fraction Mono Q-I is shown to give different patterns from those of fractions Mono Q-II/III (lane A). The pattern of fractions Mono Q-II and -III upon SDS–PAGE in the second dimension showed that group I bands migrated with molecular masses around 54K and that of group II around 50K (data not shown).

From the results of IEF, fractions Mono Q-II/III appeared to be heterogeneous. When IEF was performed at a narrow p*I* range (6.5–4) using fraction Mono Q-III, about six bands

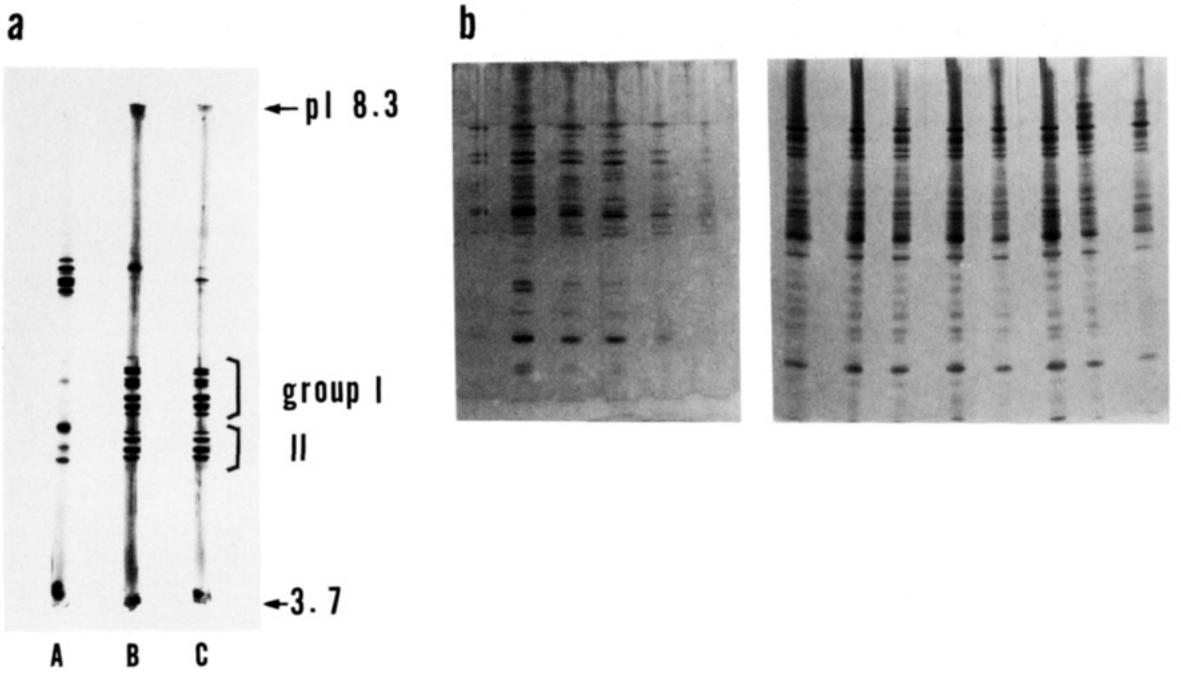

FIGURE 4: Isoelectric focusing of fractions Mono Q-I, -II, and -III (a) and chemical cleavage and peptide mapping of fraction Mono Q-III after isoelectric focusing (b). (a) About 10 μg of the three fractions was subjected to IEF under denaturing conditions according to the method of O'Farrell (1975). Lane A, fraction Mono Q-I; lanes B and C, fraction Mono Q-II and -III in the p*I* range from 8.3 to 3.7. (b) Each band of the gel separated in the p*I* range from 6.5 to 4 (confer the text) was carefully excised and subjected to CNBr cleavage for groups I and II. After SDS–PAGE, polypeptides were stained by silver. The left side of electrophoretograms shows peptide maps of group I and the right side those of group II.

Table III: Catalytic Properties of the Protein in the Mono Q-III Fraction (% Activity)[a]

property	Mono Q-III	property	Mono Q-III
pH optimum	6.0	divalent cation requirement	
		Ca^{2+} (3 mM)	38
substrate specificity (%)		Zn^{2+} (3 mM)	50
ATP (3 mM)	100	Ni^{2+} (3 mM)	11
GTP (3 mM)	88	Co^{2+} (3 mM)	58
ITP (3 mM)	106		
UTP (3 mM)	18	monovalent cation effects	
CTP (3 mM)	6	Li^{+} (10 mM)	139
ADP (3 mM)	1	K^{+} (10 mM)	129
AMP (3 mM)	1	Na^{+} (10 mM)	110
p-nitrophenyl phosphate (3 mM)	1	NH_4^{+} (10 mM)	109
		choline (10 mM)	100
inhibitor effects			
none	100	monovalent anion effects	
FITC (100 μM)	66	none	100
vanadate (100 μM)	87	Cl^{-} (10 mM)	56
DCCD (100 μM)	104	Br^{-} (10 mM)	89
diethylsilbestrol (100 μM)	112	I^{-} (10 mM)	78
azide (100 μM)	6	HCO_3^{-} (10 mM)	101
		F^{-} (10 mM)	34
divalent cation requirement		NO_3^{-} (10 mM)	22
none	nd[b]		
Mg^{2+} (3 mM)	100	divalent anion effect	
Mn^{2+} (3 mM)	121	SO_4^{2-} (10 mM)	100

[a] pH dependence, substrate, substrate specificity, inhibitor effects, divalent cation requirements, and monovalent cation and anion effects were tested for the Mono Q-III fraction in an assay mixture containing 25 mM Pipes–Tris buffer, 0.25 M sorbitol, 3 mM each of $MgSO_4$ and ATP-Na, and 0.125% (w/v) MEGA-9. Except for the pH dependence, the buffer was pH 6.5. For the inhibitor studies, the preincubation of each inhibitor with the enzyme was carried out at 30 °C for 5 min, and the reaction was started by addition of ATP. Results are the mean of duplicate measurements. [b] nd, not detectable.

were observed for group I and about eight bands for group II (data not shown). These bands were carefully excised and subjected to CNBr cleavage. Peptide maps are shown in Figure 4b. The polypeptides of group I separated on IEF gave essentially the same patterns on SDS–PAGE in the second dimension after chemical cleavage, and also the same patterns were observed for the polypeptides of group II.

The experimentation presented here supported that fractions Mono Q-II/III both consisted of the same 54K and 50K polypeptides and were almost homogeneous preparations.

Characterization of Fraction Mono Q-III as ATPase. Fraction Mono Q-III was characterized as ATPase, and the results are summarized in Table III. All the data are presented as relative values from duplicate tests.

(*A*) *pH Optima.* The pH profile of enzyme activity in the membrane fraction was without distinct maxima and decreased from 100% at pH 5.5/6.0 to 75% at pH 9.0. The activity in fraction Mono Q-III had a pH optimum around 6.0.

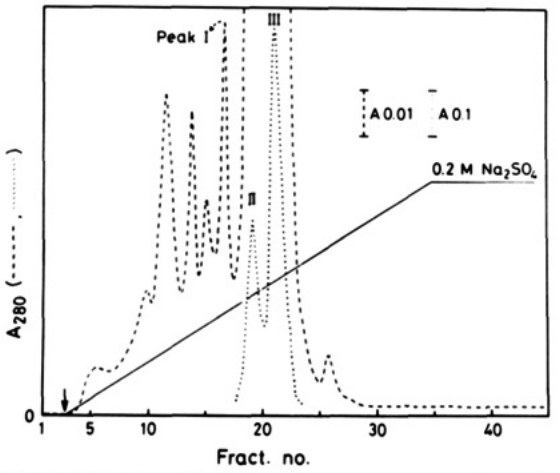

FIGURE 5: Separation of the CF_1-ATPase components by ion-exchange chromatography (Mono Q). Chloroplast-containing fraction prepared from cells 3–5 cm in length was treated with MEGA-9 and subjected to DEAE-Sephacel chromatography as described under Materials and Methods. A concentrate of the desalted 0.2 M Na_2SO_4 eluate was applied to a Mono Q column in the FPLC system. The solid line indicates the linear salt gradient of Na_2SO_4 used for chromatography and the arrow the start of the gradient from 0 to 0.2 M Na_2SO_4.

The activity in Mono Q-I fraction was very labile; its pH optimum could not be determined.

(*B*) *Substrate Specificity and Inhibitors.* Several triphosphonucleotides, ADP, AMP, and *p*-nitrophenyl phosphate were tested as substrates. As summarized in Table III, triphosphonucleotides with a purine base were judged to be good substrates for the ATPase, but triphosphonucleotides with a pyrimidine base were poor substrates as were ADP, AMP, and *p*-nitrophenyl phosphate.

Table III also lists a number of compounds tested as inhibitors for the ATPase activity. These tests were carried out with Mg-ATP as substrate. DCCD and diethylstilbestrol, typical H^+-ATPase inhibitors, did not inhibit the activity. Orthovanadate, a specific inhibitor of plasmalemma ATPases, inhibited the activity by 14% at 100 μM, by 35% at 0.5 mM, and by 50% at 1 mM.

Azide, a specific inhibitor of F_1-ATPases in mitochondria, in chloroplasts, and in bacteria, had a strong inhibitory effect on the ATPase. FITC, which is considered to interact with ATPases as an ATP analogue, inhibited the activity by about 40% at 100 μM concentration.

(*C*) *Cation and Anion Requirement.* Several divalent cations were tested as replacement of Mg^{2+}, and the results are also listed in Table III. In the absence of Mg^{2+}, the ATPases had almost no activity. Mn^{2+} and CO^{2+} could substitute for Mg^{2+}, while Zn^{2+}, Ni^{2+}, and Ca^{2+} in decreasing order diminished activity. A number of monovalent cations were tested and shown to have no significant stimulatory effect on the activity. In contrast, some monovalent anions had a significant inhibitory effect on the ATPase. F^- and NO_3^- strongly inhibited the activity, but also Cl^-, the presumed substrate, had an inhibitory effect. This was not observed in the membrane fractions where at 10 mM Cl^- almost full activity and at 500 mM 70% activity were observed.

Isolation and Identification of α-, β-, *and* γ-*Subunits of* CF_1-*ATPase.* As described above, fractions Mono Q-II/III consisted of two polypeptides, the 54K (a-subunit) and the 50K (b-subunit). The molecular masses of both subunits are very similar to those of the α- and β-subunits of the CF_1-ATPase, and it was not clear under which conditions the components of CF_1-ATPase were eluted on anion-exchange chromatography (DEAE-Sephacel and Mono Q). Since contamination

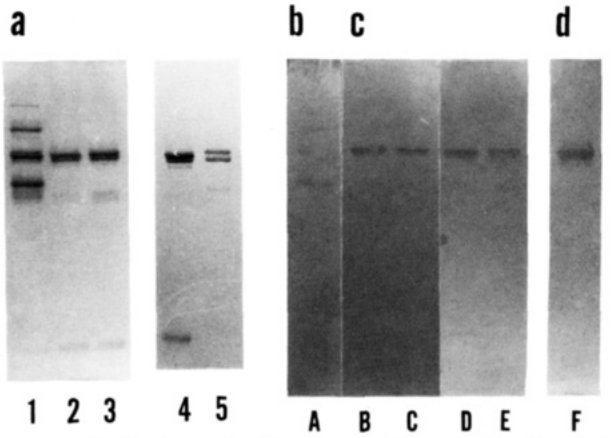

FIGURE 6: SDS–polyacrylamide gel (a) and immunoblot (b and c) of CF_1 components after separation on an anion-exchange column (Mono Q). Proteins in peaks I, II, and III were subjected to SDS–PAGE and immunoblotting. In (d), the starting material before column chromatographies was electroblotted and reacted with the antisera against the α- and β-subunits from *E. coli* F_1-ATPase. (a) Coomassie stain after SDS–PAGE: lane 1, peak I; lane 2, peak II; lane 3, peak III; lanes 4 and 5, comparison of peak III and fraction Mono Q-III. (b) Reactions with anti-CF_1 complex serum after SDS–PAGE. Lane A corresponds to peak I. (c) Reactions with anti-α and -β sera after SDS–PAGE. Lanes B and C correspond to peaks II and III, respectively. These parts of the gel were reacted with anti-α serum after electroblotting and bands visualized by the peroxidase reaction. Lanes D and E correspond to peaks II and III, respectively. These were reacted with the anti-β serum. (d) Reaction of the starting material with the mixture of the anti-α and -β sera (lane F).

Table IV: Partial Amino Acid Sequences Obtained from the CNBr-Cleaved Fragments of the a-, α-, b-, and β-Subunits[a]

species	partial amino acid sequence
a-subunit	M S L K L - A P P F
α-subunit	M S A L I D R Q P N E
tobacco CF_1, α	^{274}M S L L L R R P G R E
b-subunit	M A N A Y N Y Q V P
β-subunit	M I T A V T P Y N D E
spinach CF_1, β	^{292}M P S A V G Y Q P T L

[a] The method will be described in subsequent papers (Ikeda et al. and Moritani et al., in preparation).

of the starting material, the membrane fraction, by chloroplast fragments could not be ruled out because of its greenish color, a fraction containing chloroplasts was prepared as described under Materials and Methods and further subjected to the purification procedure established for membrane-bound ATPases. Upon homogenization of the chloroplasts in the presence of EGTA, ATPase activity becomes soluble and was found in the supernatant. Treatments with detergent, however, to solubilize the membrane-bound ATPase activity deactivated chloroplast-bound ATPase activity. Under the conditions described for purification of the membrane-bound ATPases, most proteins and traces of ATPase activity were eluted with 0.2 M Na_2SO_4-containing buffer from the DEAE-Sephacel column and further purified on Mono Q chromatography (see Figure 5). Each fraction with absorbance at 280 nm was then subjected to SDS–PAGE and Western blotting, and the results for the relevant fractions are shown in Figure 6. Peak I mainly contained a 41K polypeptide which cross-reacted with the anti-CF_1 complex serum from spinach. Peaks II and III mainly consisted of two polypeptides around 50K which cross-reacted with the anti-α and -β sera of *E. coli* F_1-ATPase. Partial peptide sequences obtained from the a-, α-, b-, and β-subunits are summarized in Table IV. Data support that

Table V: Properties of Various Mg^{2+}-ATPases

	Mono Q-III	F type	P type	V type
molecular mass ($\times 10^{-3}$)	230	450	95–120	400
subunit	54 (a) 50 (b)	55 (α), 30 (a) 50 (β), 17 (b) 31 (γ), 8 (c) 20 (δ) 14 (ϵ)	1 or 2 subunit(s)	2 or 3 subunits α, β (and γ)
pH optimum	6.0	9.5	5.5–6.5	7.0
inhibitor	azide orthovanadate FITC	azide DCCD (F_o) oligomycin	orthovanadate DCCD DES	DCCD DES KNO_3, KSCN
covalent intermediate	formed	none	aspartyl phosphate	none
ionic substrate	Cl^-	H^+	H^+, Na^+, K^+, Ca^{2+}	H^+

the α- and β-subunits clearly differ from the a- and b-subunits, respectively, but all four subunits show significant similarities to the α- and β-subunits of CF_1-ATPase of higher plants. More detailed information on partial amino acid sequences of the four subunits will be described in subsequent papers (Ikeda et al. and Moritani et al., in preparation).

It was judged from the results that the α-, β-, and γ-subunits of CF_1-ATPase had partially dissociated in the presence of MEGA-9 and eluted from the DEAE-Sephacel column as subcomplexes of varying stoichiometry. This also explains the fact that the 0.2 M Na_2SO_4 eluates from the DEAE-Sephacel column had almost no ATPase activity.

Chemical Cleavage and Peptide Mapping of the α- and β-Subunits of CF_1-ATPase: Comparison with the a- and b-Subunits in the Mono Q-II/III Fractions. Peptide maps of the α- and β-subunits of CF_1-ATPase compared with those of the a- and b-subunits of fractions Mono Q-II/III are shown in Figure 7. CNBr and hydroxylamine cleaving patterns of the a- and α-subunits were clearly different from each other. In the case of the b- and β-subunits, CNBr cleaving patterns were different but NCS cleaving patterns showed similarity.

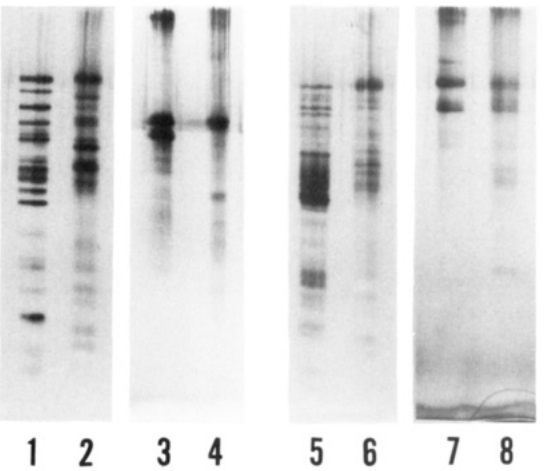

FIGURE 7: Peptide maps of the α-, β-, a-, and b-subunits after chemical cleavages. Peak III of the chromatographic separation shown in Figure 5 was subjected to SDS–PAGE, and two bands with molecular masses of 52K (α-subunit) and 51K (β-subunit) were excised. The a- and b-subunits of fraction Mono Q-III were treated the same way. The α- and a-subunits were subjected to CNBr and hydroxylamine cleavages and the β- and b-subunits to CNBr and NCS cleavages, followed by SDS–PAGE. Protein bands were visualized by silver. Lanes 1 and 2, a- and α-subunits treated with CNBr; lanes 3 and 4, a- and α-subunits treated with hydroxylamine; lanes 5 and 6, b- and β-subunits treated with CNBr; lanes 7 and 8, b- and β-subunits treated with NCS.

Discussion

In the membrane fraction of *Acetabularia* prepared by the N_2 cavitation method, at least two forms of ATPases (Mono Q-I and Mono Q-II/III fractions) existed, which were separable by anion-exchange column chromatography (DEAE-Sephacel and Mono Q). The molecular masses of the native enzymes were indistinguishable (230K), while those of their subunits were determined to be 52K for fraction Mono Q-I and 54K and 50K for fractions Mono Q-II/III on the SDS gels. The low and unstable ATPase activity in fraction Mono Q-I was apparently a contamination because N-terminal sequencing of a fragment after NCS cleavage of the 52K band provided evidence that Mono Q-I fraction contained the large subunit of ribulose-1,5-bisphosphate carboxylase.

By IEF, fractions Mono Q-II and -III appeared to be identical, but the difference between their specific activities was significant (see Table II). The catalytic properties of the Mono Q-II fraction were essentially the same as the Mono Q-III fraction (data not shown).

Cation-translocating ATPases are classified into three categories (Pedersen & Carafoli, 1987): (1) P type in *Neurospora crassa* (Bowman et al., 1981), in *Saccharomyces cerevisiae* (Malpartida & Serrano, 1980; Foury et al., 1981), and in *Saccharomyces pombe* (Dufour & Goffeau, 1978, 1980); (2) V type in *N. crassa* (Bowman & Bowman, 1982), in *S. cerevisiae* (Kakinuma et al., 1981; Uchida et al., 1985), in corn roots (Dupont et al., 1982), in oat roots (Churchill & Sze, 1983), in *Hevea brasiliensis* (D'Auzac, 1977), and in *Daucus carota* (Zimniak et al., 1988); (3) F-type ATPases. General properties of the three type of ATPases are summarized in Table V and also compared with the properties of the fraction Mono Q-III. Judged from the molecular masses of subunits, the fractions Mono Q-II/III showed similarity to the α- and β-subunits of F-type ATPases. Azide, a specific inhibitor for F-type ATPases, was also the most effective inhibitor for Mono Q-III. However, phosphorylation of the enzyme specific for P-type ATPases (Amory et al., 1980; Amory & Goffeau, 1982; Scalla et al., 1983) could be demonstrated for the fraction Mono Q-III. Orthovanadate, a specific inhibitor for P-type ATPases, also had an inhibitory effect on Mono Q-III, and the extent of inhibition was similar to that of myosin ATPase (Goodno, 1979). V-type ATPases are generally composed of two or three subunits: 89K and 64K and a DCCD binding polypeptide of 19.5K in *S. cerevisiae* (Uchida et al., 1985), 66K and 57K in *N. crassa* (Bowman & Bowman, 1986). DCCD is a strong and specific inhibitor to those ATPases, and monovalent and/or divalent anions inhibit the activity. The fractions Mono Q-II/III were not inhibited by DCCD, but monovalent anions, especially fluoride and nitrate, had inhibitory effects.

Recently several H^+-ATPases in archaebacteria were purified and characterized: in *Halobacterium halobium* (Nanba & Mukohata, 1987), in *Sulfolobus acidocardarius* (Konishi et al., 1987), and in *Methanosarcina barkei* (Inatomi, 1986). Their molecular masses are estimated to be 320K in *Halo-*

bacterium halobium, 360K in *Sulfolobus acidocardarius*, and 420K in *Methanosarcina barkei*. These, in general, consist of two or three subunits [α, β (and γ)], have acidic pH optima, and are stimulated by bisulfite, sulfate, and bicarbonate, but are inhibited by nitrate. Neither azide nor orthovanadate is an inhibitor of these ATPases.

In comparison with the above observations, the ATPase reported here has novel and unique properties which do not belong to any category so far described. It showed mixed properties of all the ATPases mentioned above.

During our studies, however, the most intriguing observation was that the Mono Q-II/III fractions showed strong similarities to CF_1-ATPases of higher plants, in molecular sizes of subunits and inhibition by azide as described above. Therefore, we isolated and characterized the CF_1-ATPase from *Acetabularia acetabulum*. A fraction containing chloroplasts prepared by low-speed centrifugation of whole cells contained considerable amounts of ATPase activity when measured at pH 8.5 after freezing and thawing. The activity was 5-fold higher than at pH 6.0 and was lost after treatment with MEGA-9. The expected presence of the α-, β-, and γ-subunits of CF_1-ATPase could be confirmed by cross-reactions with the anti-CF_1 complex serum from spinach and the respective antiserum against the α- and β-subunits of *E. coli* F_1-ATPase. The amino acid sequences of these two subunits of F_1-ATPase are known to be well conserved in several species. A 55% homology between tobacco α-subunit and *E. coli* α-subunit has been reported by Deno et al. (1983), and the β-subunit of several higher plants also shows over 80% homology (Runswick & Walker, 1983). In this approach, the three subunits of CF_1-ATPase were shown to elute with different salt concentrations from the Mono Q-III fraction in DEAE-Sephacel and Mono Q chromatography (see Figures 1 and 5). The peptide maps and partial amino acid sequences of the four polypeptides around 50K after chemical cleavages showed that the α- and β-subunits were clearly different from the a- and b-subunits, respectively (Figure 7 and Table IV). The results obtained from IEF of fractions Mono Q-II/III under denaturing conditions and subsequent chemical cleavage also supported that no CF_1-ATPase components contaminated these fractions.

The proposal that the active fraction (Mono Q-III) contains the Cl^- pump was substantiated by the reconstitution studies as described in the following paper (Ikeda & Oesterhelt, 1990).

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REFERENCES

Amory, A., & Goffeau, A. (1982) *J. Biol. Chem. 257*, 4723–4730.

Amory, A., Foury, F., & Goffeau, A. (1980) *J. Biol. Chem. 255*, 9353–9357.

Amzel, L. M., & Pedersen, P. L. (1983) *Annu. Rev. Biochem. 52*, 801–824.

Blanck, A., & Oesterhelt, D. (1987) *EMBO J. 6*, 265–273.

Bowman, E. J., & Bowman, B. J. (1982) *J. Bacteriol. 151*, 1326–1337.

Bowman, B. J., & Bowman, E. J. (1986) *J. Membr. Biol. 94*, 83–97.

Bowman, B. J., Blasco, F., & Slayman, C. W. (1981) *J. Biol. Chem. 256*, 12343–12349.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Churchill, K. A., & Sze, H. (1983) *Plant Physiol. 71*, 610–617.

D'Auzac, J. (1977) *Phytochemistry 16*, 1881–1885.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Deno, H., Shinozaki, K., & Sugiura, M. (1983) *Nucleic Acids Res. 11*, 2185–2191.

Dufour, J.-P., & Goffeau, A. (1978) *J. Biol. Chem. 253*, 7026–7032.

Dufour, J.-P., & Goffeau, A. (1980) *Eur. J. Biochem. 105*, 145–154.

Dupont, F. M., Giorgi, D. L., & Spanswick, R. M. (1982) *Plant Physiol. 70*, 1694–1699.

Fairbanks, G., & Avruch, J. (1972) *J. Supramol. Struct. 1*, 66–75.

Foury, F., Amory, A., & Goffeau, A. (1981) *Eur. J. Biochem. 119*, *395*–400.

Gershoni, J. M., & Palade, G. E. (1983) *Anal. Biochem. 131*, 1–15.

Goffeau, A., & Slayman, C. W. (1981) *Biochim. Biophys. Acta 639*, 197–223.

Goldfarb, V., & Gradmann, D. (1983) *Plant Cell Rep. 2*, 152–155.

Goldfarb, V., Sanders, D., & Gradmann, D. (1984) *J. Exp. Bot. 35*, 626–644.

Goodno, C. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2620–2624.

Gradmann, D. (1970) *Planta 93*, 323–353.

Gradmann, D. (1975) *J. Membr. Biol. 25*, 183–208.

Gradmann, D. (1984) in *Chloride transport coupling in biological membranes and Epithelia* (Gerencser, G. A., Ed.) pp 13–61, Elsevier Science Publishers B.V., Amsterdam, The Netherlands.

Gradmann, D., Hansen, U.-P., & Slayman, C. L. (1982) *Curr. Top. Membr. Transp. 16*, 257–276.

Heil, A., & Zillig, W. (1970) *FEBS Lett. 11*, 165–168.

Ikeda, M., & Osterhelt, D. (1990) *Biochemistry* (following paper in this issue).

Inagaki, C., & Shiroya, T. (1988) *Biochem. Biophys. Res. Commun. 154*, 108–112.

Inatomi, K. (1986) *J. Bacteriol. 167*, 837–841.

Kakinuma, Y., Ohsumi, Y., & Anraku, Y. (1981) *J. Biol. Chem. 256*, 10859–10863.

Konishi, J., Wakagi, T., Oshima, T., & Yoshida, M. (1987) *J. Biochem. 102*, 1379–1387.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 95–97.

Lischwe, M. A., & Ochs, D. (1982) *Anal. Biochem. 127*, 453–457.

Lukacovic, M. F., Feinstein, M. B., Sha'afi, R. I., & Perrie, S. (1981) *Biochemistry 20*, 3145–3151.

Malpartida, F., & Serrano, R. (1980) *FEBS Lett. 111*, 69–72.

Mummert, H., Hansen, U.-P., & Gradmann, D. (1981) *J. Membr. Biol. 62*, 139–148.

Nanba, T., & Mukohata, Y. (1987) *J. Biochem. 102*, 591–598.

Nikodem, V., & Fresco, J. R. (1979) *Anal. Biochem. 97*, 382–386.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Pedersen, P. L., & Carafoli, E. (1987) *Trends Biochem. Sci. 12*, 146–150.
Ross, A. H., & McConnell, H. M. (1977) *Biochem. Biophys. Res. Commun. 74*, 1318–1325.
Runswick, M. J., & Walker, J. E. (1983) *J. Biol. Chem. 258*, 3081–3089.
Saddler, H. D. W. (1970) *J. Gen. Physiol. 55*, 802–821.
Sakai, Y., Sumida, Y., Tsuda, M., Shinoda, S., & Tsuchiya, T. (1986) *Jpn. J. Pharmacol. 40(8)*, 377.
Saris, C. J. M., van Eenbergen, J., Jenks, B. G., & Bloemers, H. P. J. (1983) *Anal. Biochem. 132*, 54–67.
Scalla, R., Amory, A., Rigaud, J., & Goffeau, A. (1983) *Eur. J. Biochem. 132*, 525–530.
Schmid, R., & Giesecke, K. (1984) in *Acetabularia 1984* (Bonotto, S., Cinelli, F., & Billau, R., Eds.) pp 319–326, CEN-SCK, Brussels, Belgium.
Tittor, J., Hansen, U.-P., & Gradmann, D. (1983) *J. Membr. Biol. 75*, 129–139.
Towbin, H. H., Staeheling, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Uchida, E., Ohsumi, Y., & Anraku, Y. (1985) *J. Biol. Chem. 260*, 1090–1095.
Weinbaum, G., & Markman, R. (1966) *Biochim. Biophys. Acta 124*, 207–209.
Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.
Zimniak, L., Dittrich, P., Gogarten, J. P., Kibak, H., & Taiz, L. (1988) *J. Biol. Chem. 263*, 9102–9112.

A Cl^--Translocating Adenosinetriphosphatase in *Acetabularia acetabulum*. 2. Reconstitution of the Enzyme into Liposomes and Effect of Net Charges of Liposomes on Chloride Permeability and Reconstitution

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ABSTRACT: The Mono Q-III fraction, a Mg^{2+}-ATPase, isolated from *Acetabularia acetabulum* was reconstituted into liposomes of various net charges prepared by the reversed-phase method and tested for a Cl^--translocating activity. The liposomes from a mixture of egg lecithin, dicetyl phosphate, and cholesterol (63:18:9 mole ratio, negative liposomes) and from a mixture of egg lecithin and cholesterol (63:9 mole ratio, neutral liposomes) were less leaky than positive liposomes from asolectin, and from a mixture of egg lecithin, stearylamine, and cholesterol (63:18:9 mole ratio). A significant increase in $^{36}Cl^-$ efflux from the negative and neutral liposomes was observed by addition of ATP in the presence of valinomycin after incorporation of the enzyme by short-term dialysis. The ATP-driven $^{36}Cl^-$ efflux was inhibited by addition of azide, an inhibitor of the ATPase. The preincubation of the enzyme with phenylglyoxal, an arginine-modifying reagent, inactivated ATP-mediated $^{36}Cl^-$ efflux, but the ATPase activity of the preparation was not affected. When chloride was replaced by $^{35}SO_4^{2-}$, no ATP-dependent $^{35}SO_4^{2-}$ efflux was detectable from the proteoliposomes. Proton-translocating activity of the enzyme was also tested, and no fluorescent quenching of 9-ACMA was observed.

Cation-translocating adenosinetriphosphatase (ATPases)[1] have been well characterized after reconstitution of the purified enzymes into liposomes for F_0F_1-ATPases (Sone et al., 1977; Pick & Racker, 1979; Graeber et al., 1982; Nyren & Baltscheffsky, 1983), for H^+-ATPases from plasma membrane (Malpartida & Serrano, 1981; Villalobo et al., 1981; Vara & Serrano, 1982; Benett & Spanswick, 1983; Serrano, 1984), and for Ca^{2+}- and Na^+,K^+-ATPases (Zimniak & Racker, 1978; Racker et al., 1975, 1979). Reconstitution studies on several anion-translocating proteins, band 3 protein from erythrocytes (Ross & McConnell, 1977; Lukacovic et al., 1981), and halorhodopsin from *Halobacterium halobium* (Bamberg et al., 1984) have been reported, but these proteins are not ATP-driven chloride translocators.

Acetabularia acetabulum has been electrophysiologically characterized as having an electrogenic Cl^- pump (ATPase) in its plasmalemma that maintains the membrane potential (around −170 mV in the dark) of the cell (Gradmann et al., 1982; Tittor et al., 1983; Gradmann, 1984). In the preceding paper (Ikeda et al., 1990), we have reported on the purification and characterization of a novel-type ATPase from this organism.

In the present paper, we describe the substantiation of a Cl^--translocating activity of the purified enzyme, the Mono Q-III fraction in reconstituted systems, and the effect of lipid sources of various net charges on reconstitution with respect

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[1] Abbreviations: ATPase, adenosinetriphosphatase; ATP, adenosine 5′-triphosphate; DEAE-Sephacel, *O*-(diethylaminoethyl)Sephacel; FPLC, fast protein liquid chromatography; MEGA-9, nonanoyl-*N*-methylgluconamide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; 9-ACMA, 9-amino-6-chloro-2-methoxyacridine.

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