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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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to low permeability for chloride and the extent of incorporation of the ATPase.

Materials and Methods

Chemicals and Instruments. A negative liposome kit was purchased from Avanti Polar-Lipids, Inc. (Birmingham, AL); ATP (disodium salt, orthovanadate-free), Pipes, and valinomycin were from Sigma Chemical Co. (St. Louis, MO). Other reagents were obtained from Merck (Darmstadt, FRG), all of analytical grade. DEAE-Sephacel and the FPLC system were from Pharmacia (Uppsala, Sweden), and MEGA-9 was from Oxyl Co. (Bobingen, FRG). A microdialysis chamber was self-built as described below, and a Kontron liquid scintillation counter was used for counting the radioisotopes which were from Amersham ($^{36}Cl^-$, 12.5 mCi/g of Cl) (Braunschweig, FRG) and New England Nuclear ($^{35}SO_4^{2-}$, 30 mCi/g of sulfur) (Boston, MA).

Purification of ATPase. Membrane-bound proteins were solubilized with MEGA-9 and ATPase (Mono Q-III fraction) purified through DEAE-Sephacel, Superose 12, and Mono Q column chromatography as described previously (Ikeda et al., 1990). ATPase activity was assayed at 30 °C by colorimetric determination of inorganic phosphate liberated by hydrolysis (Lanzetta et al., 1979). One unit of enzyme activity is defined as 1 μmol of phosphate liberated/min at 30 °C. The fraction with the highest specific activity [Mono Q-III fraction; see Ikeda et al. (1990)] was frozen in liquid nitrogen and kept at −70 °C. A gradual loss of ATPase activity was observed, and about 20% of the activity remained after 3 months.

Preparation of Liposomes by the Reversed-Phase Method. As lipid sources, the negative liposome kit (egg lecithin/dicetyl phosphate/cholesterol, 63:18:9 mole ratio, negative liposomes), a mixture of egg lecithin and cholesterol (63:9 mole ratio, neutral liposomes), a mixture of egg lecithin, stearylamine, and cholesterol (63:18:9 or 83:5:12 mole ratio, positive liposomes), and asolectin were used for preparation of reversed-phase liposomes according to the method of Rigaud et al. (1983). Briefly, 15 μmol of lipids dissolved in 1.5 mL of ethyl ether saturated with water was added to 0.45 mL of the transport (dialysis) buffer consisting of 25 mM Pipes–Tris buffer (pH 6.5), 0.21 M sorbitol, 20 mM KCl, 10 mM $MgSO_4$, 1 mM EGTA, and 2 mM DTT. The mixture was sonicated in a bath-type sonicator until a cloudy emulsion formed (ca. 5 min) which did not separate into two phases upon standing. Ethyl ether of the emulsion was removed under a slight reduced pressure (ca. 0.6 atm, rotatory evaporator). More transport buffer (1.05 mL) was added to the suspension which was vortexed and kept under slight vacuum (ca. 0.9 atm, rotatory evaporator) for 20–30 min. The liposomes were pelleted by centrifugation at 60000*g* for 45 min and resuspended in 0.15 mL. For efflux experiment, radioisotopes ($^{36}Cl^-$ or $^{35}SO_4^{2-}$, 2.5 μCi of each) were first added to the buffer (0.45 mL) and then the liposomes prepared as described above.

Incorporation of the Enzyme into Liposomes and Transport Measurements. The washed and sedimented liposomes suspension (0.1 mL) was dialyzed against the same buffer for 1 h in a self-made microdialysis chamber using a 24 × 14 mm i.d. sample cup (Sarstedt, FRG). A hole (9-mm i.d.) was bored into a cap, and a glass tube (8 × 6 mm i.d.) was closed in one side with a dialysis membrane (Sartorius GmbH, Goettingen) which was fixed with a polyethylene tube and placed onto the cap. The Mono Q-III fraction (see figures legends) was added to the glass chamber containing liposomes. After 30 min, valinomycin (10–30 μM using 1 mM in ethanol) was added to the glass chamber. In another 30 min, ATP (final concentration 10 mM, sufficient for at least 15-min full enzyme activity, using 500 mM aqueous stock solution, pH 7.0) was added both to the incubation mixture and to the cup filled with the dialysis buffer. At several time intervals, ATP and/or azide was optionally added to the chamber in final concentrations of 10 and 20 mM, respectively.

Every 10 or 15 min, one-tenth volume of the dialysis buffer was taken, mixed with Aquasol (3 mL), and counted in a liquid scintillator for radioactivity. The dialysis buffer was maintained at constant volume of 2.5 mL with continuous stirring and kept at 30 °C in a circulated cell-type chamber. The control experiment was performed identically except that buffer used for purification replaced the Mono Q-III fraction.

Assay for Incorporation of ATPase Activity into Liposomes. ATPase activity was assayed after separation of proteoliposomes and free ATPase by centrifugation at 60000*g* for 45 min using an Airfuge (Beckman Inc.). The sediment was washed once with about 0.2 mL of the transport buffer and resuspended in the original volume of buffer. An aliquot of each the supernatant and the suspension was assayed for ATPase activity.

Measurement of Proton-Translocating Activity. Proton-translocating activity of Mono Q-III fraction was tested according to Vara and Serrano (1982). Briefly, liposomes were prepared from asolectin (20 mg/mL). An aliquot (40-fold excess over protein) was mixed with the Mono Q-III fraction 50 μg, 2 mg/mL, 1.8 units/mg of protein). Proteins were then incorporated into liposomes by freezing and thawing. Activity was measured by fluorescence quenching of an acridine dye (9-ACMA).

Results

Stability of ATPase Activity in the Presence of Liposomes. Among several methods for incorporation of membrane proteins into liposomes, detergent dilution, freeze–thaw, and detergent dialysis methods were tested for ATPase purified from *Acetabularia*. Detergent dilution caused an immediate loss of the ATPase activity, and the remainder was unstable and further reduced by about 90% after 2 h. The freeze-thaw method with the same liposomes gave more satisfactory results; 70–80% ATPase activity remained after incorporation, and only a slight decrease (20%) was observed after 2 h at 4 °C. The most satisfactory results were found for the detergent dialysis method at a lipid to protein ratio of about 80:1 (w/w). As shown in Figure 1, the lipid under these conditions preserved the ATPase activity to 70% in the Mono Q-III fraction and even activated it in the Mono Q-II fraction. Without liposomes, the activity in the Mono Q-III fraction remained stable, but Mono Q-II lost activity completely (Figure 1). This result found for negatively charged liposomes was confirmed with liposomes prepared from asolectin (dashed lines in Figure 1). All samples, however, had lost activity after 24 h.

Tightness of the Reversed-Phase Liposomes. The small diameter of liposomes prepared by sonication in general makes the vesicles less leaky for cations and anions compared with reversed-phase liposomes (Eytan, 1982). The permeability of these liposomes was therefore tested by measuring the spontaneous $^{36}Cl^-$ efflux. The results are shown in Figure 2. Liposomes prepared from asolectin alone and positive liposomes containing 20 mol % stearylamine were considerably leaky more than the negative and neutral liposomes. Reducing the addition of stearylamine to 5% also lowered the permeability of the liposomes to that of negative or neutral liposomes. After 1 h, about 6% of $^{36}Cl^-$ in the liposomes was released in the latter cases. The addition of cholesterol to asolectin, reported by Papahadjopoulos et al. (1971) to improve the tightness of the liposomes, was not tried. All types of liposomes

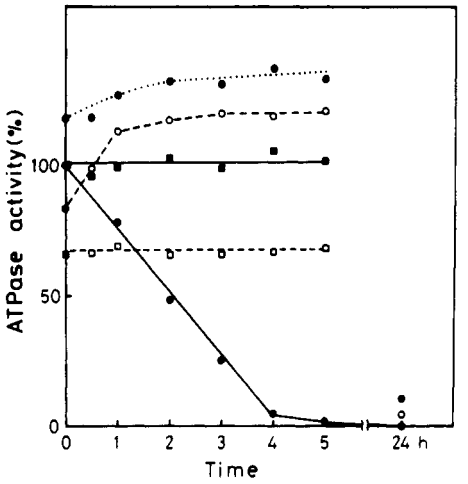

FIGURE 1: Stability of ATPase activity in the presence of liposomes. The negatively charged reversed-phase liposomes were prepared as described in the legend of Figure 2, except that no radioactive $^{36}Cl^-$ was added. The enzyme (48 μg in 12.5 μL, 1.8 units/mg of protein) was added to liposomes (7.5 μmol of lipid) in a total volume of 100 μL in a microdialysis chamber. At timed intervals, an aliquot (2 μL) of the mixture was taken and assayed for ATPase activity. (■—■) ATPase activity in fraction Mono Q-III [see Ikeda et al. (1990)] in the absence of liposomes; (□---□) ATPase activity of fraction Mono Q-III in the presence of negative liposomes; (●—●) ATPase activity in fraction Mono Q-II [see Ikeda et al. (1990)] in the absence of liposomes; (○---○) ATPase activity in fraction Mono Q-II in the presence of negative liposomes; (○···○) ATPase activity in fraction Mono Q-II in the presence of liposomes prepared from asolectin and cholesterol (9:1 mole ratio).

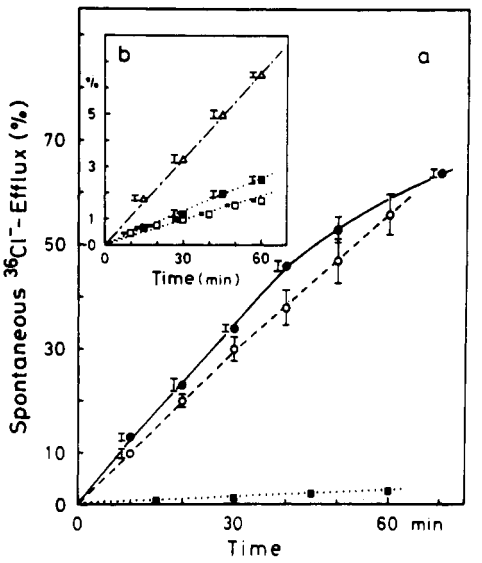

FIGURE 2: Chloride permeability of reversed-phase liposomes prepared from various lipid mixtures. The liposomes prepared as described under Materials and Methods (100 μmol/mL, 150 μL) contained about 300000 cpm of $^{36}Cl^-$. The suspension was transferred to a microdialysis chamber, and one-tenth of the dialysis buffer (2.5 mL) was taken at various times and counted for $^{36}Cl^-$. After each sampling, the volume was readjusted to 2.5 mL by addition of buffer, and the cpm values are corrected for these additions. Vertical bars in the figure indicate the standard deviation of triplicate separate experiments. (●—●) Liposomes prepared from asolectin; (○---○) positive liposomes containing 20 mol % stearylamine; (△-·-△) positive liposomes containing 5 mol % stearylamine; (□···□) neutral liposomes; (■···■) negative liposomes.

with low permeability consist of pure lipids with known composition, and no significant difference between their abilities to stabilize the ATPase activity exists (see Figure 1). The ability to incorporate the ATPase in an active form was tested, and the results are summarized in Table I. Incorporation in this context is defined as sedimentable ATPase activity after dialysis. This includes insertion into the lipid bilayer of the liposomes as well as adsorption to the liposomal surface. The ATPase was incorporated into the negative liposomes at about 15–20% after 1-h dialysis, into neutral liposomes at about 15–20% after 1-h dialysis, and 30–40% after 5-h dialysis. The positive liposomes showed an apparent incorporation greater than 95%. All three preparations were used for the transport experiments described below.

Table I: Extent of Incorporation for ATPase into Liposomes of Different Net Charges[a]

liposomes	ATPase activity (milliunits) supernatant	precipitate	incorporation ratio (%)
negative	23 (32)[b]	5.4 (7.1)[b]	19 (18)[b]
neutral	23 (21)[c]	5.7 (9.3)[c]	20 (31)[c]
positive	0.4	46.5	99

[a] Mono Q-III fraction (12.5 μL, 2 units/mg of protein, 4 mg of protein/mL) was added to the reversed-phase liposomes (75 μL, 100 μmol/mL) in a microdialysis chamber and dialyzed against the transport buffer (2.5 mL) for 1 h. An aliquot (50 μL) of the mixture was centrifuged at 60000*g* for 45 min. The pellet was washed twice with the transport buffer (0.2 mL of each) and centrifuged again. The pellet was suspended in the transport buffer to the original volume (50 μL). The supernatant and the suspension were assayed for ATPase activity (1 μL of each). [b] Results of the freeze–thaw method for incorporation. [c] The dialysis was performed for 5 h.

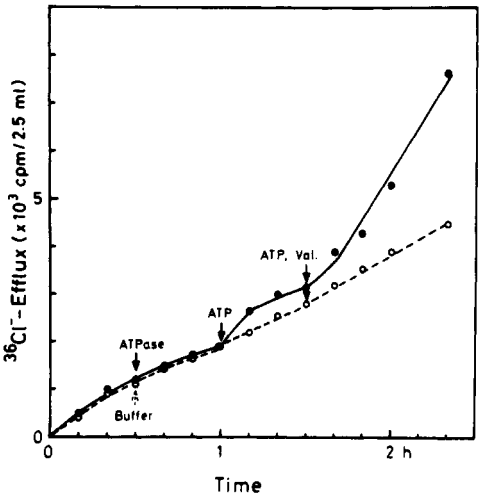

FIGURE 3: ATP-dependent $^{36}Cl^-$ efflux from proteoliposomes containing protein of the Mono Q-III fraction. Mono Q-III fraction (120 μg, 3 mg/mL, 0.8 unit/mg of protein) was reconstituted into the reversed-phase negative liposomes containing $^{36}Cl^-$ by detergent dialysis, and the $^{36}Cl^-$ efflux was measured as function of time. The dashed line shows a control experiment without addition of the Mono Q-III fraction and the solid line the transport activity (extrusion of $^{36}Cl^-$) by addition of ATP in the absence and presence of valinomycin (Val.). The total radioactivity of the liposomes was 1.4×10^5 cpm, and the rapid efflux of $^{36}Cl^-$ was observed after addition of Triton X-100 which destroyed the liposomes (data not shown).

Transport Activity of the ATPase. Transport experiments were in most cases carried out according to the following protocol. The $^{36}Cl^-$-loaded reversed-phase liposomes were kept 30 min in the microdialysis chamber before the enzyme was added. After further dialysis for 30 min, valinomycin was mixed in and the transport started after 90 min by addition of ATP. Optionally, azide was added after some more time for demonstration of transport inhibition. Radioactivity leaking from the vesicles and passing through the dialysis membrane was analyzed by measuring aliquots from the dialysis chamber. For increasing radioactivity, the term efflux is used independent of whether it is due to passive permeation or ATP-driven transport.

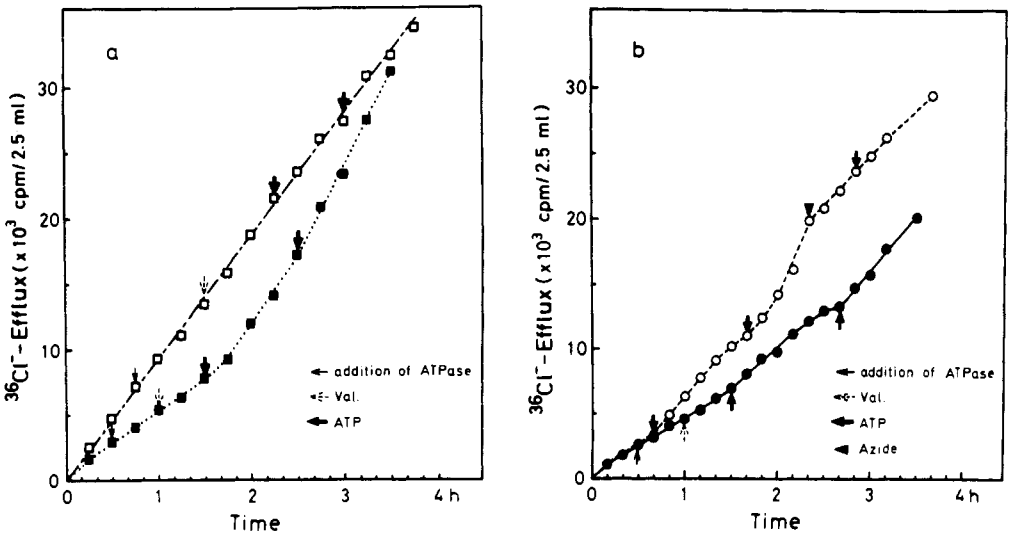

FIGURE 4: Chloride transport experiments with liposomes after incorporation of the purified ATPase. Negative and positive (a) and neutral liposomes (b). The reversed-phase liposomes were prepared as described under Materials and Methods and transferred to a microdialysis chamber. An amount of 48 μg in 12.5 μL of the purified ATPase (1.8 units/mg of protein) was added to neutral and positive liposomes (75 μL corresponds to 7.5 μmol and 1.35×10^5 cpm of $^{36}Cl^-$). One hundred micrograms in 40 μL of ATPase (2 units/mg of protein) was added to negative liposomes (100 μL corresponds to 10 μmol and 1.35×10^5 cpm of $^{36}Cl^-$). Then to all preparations in a total volume of 140 μL were added valinomycin (10 μM), ATP (10 mM), and azide (20 mM) at times indicated by the arrows. One-tenth of the dialysis buffer was taken at constant time intervals, counted for $^{36}Cl^-$, and corrected as described in legend of Figure 2. (a) (■···■) Negative liposomes; (□-·-□) positive liposomes. (b) (●—●) Neutral liposomes; (○- - -○) neutral liposomes in the chamber for 5 h before the start of the experiment [the same mixture as in (●—●) was used for experiment].

(*a*) *Requirement of Ionophore for Continuous Transport Activity.* Initial evidence for the Cl^- transport activity was obtained in the negative proteoliposomes in the presence of valinomycin (Figure 3). Without the addition of valinomycin, ATP-specific $^{36}Cl^-$ ceased within 10 min. The result supports that the ATPase is a Cl^- translocator of an electrogenic nature.

(*b*) *Effect of Net Charges of Liposomes on Reconstitution.* Figure 4 shows that no $^{36}Cl^-$ efflux was driven by ATP with positive liposomes as the lipid source although these liposomes showed the highest extent of ATPase incorporation. Neutral as well as negative proteoliposomes gave transport activity, although their ATPase content was low. In both preparations, a second addition of ATP enhanced $^{36}Cl^-$ efflux. This effect is presumably due to the action of free ATPase hydrolyzing ATP without transport since no separation of the incorporated and the free ATPase was attempted during proteoliposome preparation. The time of dialysis also affected the ATP-specific $^{36}Cl^-$ efflux in the case of neutral liposomes. After 5-h dialysis, the ATP-specific increase of the efflux was significantly greater than after only 1-h dialysis. Freezing and thawing after 1-h dialysis did not improve the incorporation ratio.

The results presented so far suggest neutral and negative liposomes as the best experimental material for studying ATP-driven chloride translocation.

(*c*) *Effect of Azide and Phenylglyoxal on the Cl^--Translocating Activity.* Azide was the most effective inhibitor for the ATPase, and its effect on the transport activity was therefore tested. Complete inhibition of ATPase by 20 mM azide occurred within 10 min (Figure 5b). The ATP-specific $^{36}Cl^-$ efflux was also inhibited by this concentration (Figures 4 and 5a).

An arginine-modifying reagent, phenylglyoxal, was also tested for its effect on transport and ATPase activity. The results are shown in Figure 5a,b. The ATPase activity was not significantly affected by phenylglyoxal within 3 h, but no transport activity was observed in the presence of phenylglyoxal.

(*d*) *Ion Specificity.* It was checked by replacement of chloride versus sulfate. No appreciable transport activity was found in this case (Figure 5a). No proton-translocating activity

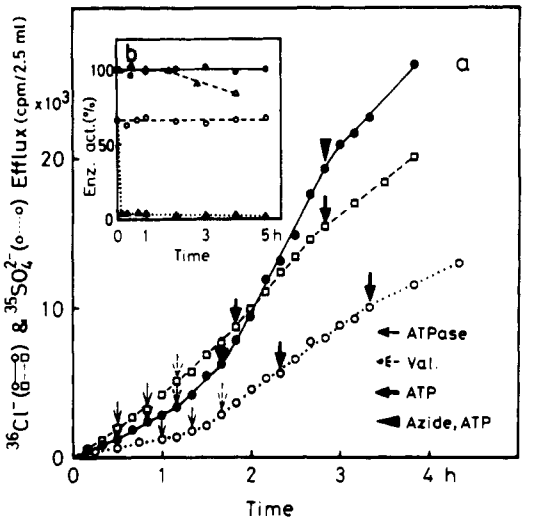

FIGURE 5: Effect of the ATPase inhibitor azide and of phenylglyoxal on $^{36}Cl^-$ efflux and $^{35}SO_4^{2-}$ efflux from proteoliposomes. Negative liposomes were prepared as described under Materials and Methods. Phenylglyoxal was added to the purified ATPase (96 μg in 25 μL, 1.8 units/mg of protein) in a final concentration of 20 mM, and the pH was adjusted to pH 8 with Tris base before the reaction was allowed to proceed for 1 h at room temperature. The transport experiments were started by addition of ATPases followed by addition of valinomycin (30 μM), ATP (10 mM), and azide (20 mM). The sample with the phenylglyoxal-treated enzyme only contained 10 μM valinomycin. The buffer for the $^{35}SO_4^{2-}$ efflux experiment consisted of 25 mM Pipes–Tris buffer (pH 6.5), 0.19 M sorbitol, 20 mM K_2SO_4, 10 mM $MgSO_4$, 1 mM EGTA, and 2 mM DTT. The assay for the ATPase activity was performed as described in the legend of Figure 1. In all transport measurements, 10 μmol of lipids was used which contained around 1.4×10^5 cpm of radioisotopes. (a) Transport measurements: (●—●) effect of azide on the $^{36}Cl^-$ efflux; (□- - -□) effect of phenylglyoxal on the $^{36}Cl^-$ efflux; (○···○) $^{35}SO_4^{2-}$ efflux. (b) Enzyme activities: (●—●) ATPase activity in the absence of liposomes; (○- - -○) ATPase activity in the presence of liposomes; (▲···▲) ATPase activity in the presence of liposomes and azide; (△- - -△) ATPase activity in the presence of phenylglyoxal.

could be detected by fluorescence quenching assay.

Discussion

Almost all cation-translocating ATPases were successfully reconstituted into a small diameter (20–30 nm) of liposomes prepared by sonication since the direction of the transport is the uptake of cations by additon of ATP into the liposomes. However, according to the electrophysiological studies (Gradmann et al., 1982; Tittor et al., 1983; Gradmann, 1984), a Cl^- pump in *Acetabularia* should translocate Cl^- ions from the inside to the outside of the liposomes using ATP. At first, we tried a Cl^- transport activity measurement using a small diameter of liposomes from asolectin prepared by sonication, a freeze–thaw procedure for incorporation, and a tetraphenyl borate sensitive electrode. A slight and slow decrease in tetraphenyl borate concentration corresponding to the extrusion of Cl^- was observed by addition of ATP, but the signal was too weak. In these systems, the volumes in the inside of the liposomes were calculated to be about 1–2 μL, when 20–40 μg of proteins was incorporated into 1–2 mg of lipids. A minimum volume for the electrode was 0.5–1.0 mL. A 10 mM change of $[Cl^-]$ in the inside, for example, corresponds to a 10–20 μM change of [tetraphenylborate] in the outside, which was near the limit of the sensitivity of the electtrode. Therefore, the size of liposomes was most critical for the reconstitution studies. A complication in this experimental approach is that we are limited in the amount of material since there is no large-scale culture system available to obtain kilogram amount of *Acetabularia*.

We have thus tried to prepare liposomes of relatively large diameter and to establish the minimum system for the transport activity measurement using radioisotope. There are several methods for preparing liposomes of a large diameter: the reversed-phase method (100 nm), a spontaneous formation by mixing long- and short-chain fatty acids containing lipids (300 nm) (Gabriel & Roberts, 1984; Dencher, 1986), and the pH adjustment method (300–600 nm) (Aurora et al., 1985). The tightness of the larger diameter of liposomes is also critical for the reconstitution studies (Eytan, 1982). Taking all of the above parameters into consideration, we chose the reversed-phase method for the preparation of liposomes of larger diameter.

As shown in Figure 2, relatively tight reversed-phase liposomes were obtained with the negatively charged and neutral lipid mixtures. The positive liposomes containing 5 mol % of stearylamine were not as tight as negative and neutral liposomes but nevertheless could be used. In these systems, the inside volumes were calculated to be 5–10 μL (corresponding to 5–10 μmol of lipids). The ratio of the inside to the outside is 1 to 15–30. These systems are presumably much more favorable for measurement of the Cl^- efflux when compared with the liposomes of small diameter.

There are several methods for incorporation of membrane proteins into liposomes: (i) detergent dilution (Racker et al., 1975); (ii) freeze–thaw–(sonication) (Kasahara & Hinkle, 1976; Pick & Racker, 1979; Malpartida & Serrano, 1981; Vara & Serrano, 1982; Serrano, 1984); (iii) cholate (detergent) dialysis (Kagawa & Racker, 1971; Sone et al., 1977; Villalobo et al., 1981; Graber et al., 1982); and (iv) other methods (Racker, 1973; Racker et al., 1979; Eytan, 1982). The three methods, (i), (ii), and (iii), were tested for the ATPase. The detergent dialysis method gave satisfactory results and judged to be best for our reconstituted system using radioisotopes.

The transport activity measurements could not be repeated enough for statistical analysis because of the limitation of the material. The same enzyme preparation was reconstituted into positive and neutral liposomes in Figure 4 and into negative liposomes in Figure 5. ATP-driven $^{36}Cl^-$ efflux was roughly evaluated as not detectable for positive liposomes, 2300 cpm/h for neutral liposomes (half of the enzyme was used in comparison with the experiment using negative liposomes), and 6400 cpm/h for negative liposomes. These relative values are significant in evaluating the effect of net charges of vesicles on the reconstitution of the Cl^--translocating ATPase. No transport activity with positive liposomes could be explained as follows: The purified and native ATPase has an acidic p*I* (around 4.5) (data not shown), and the strong electrostatic binding of the ATPase to positive liposomes could therfore occur. The reconstitution studies into negative liposomes were repeated 4 times with the ATPase of different activity. The relation between ATP-driven $^{36}Cl^-$ efflux and the ATPase activity is shown in Figure 6, indicating a good correlation between them.

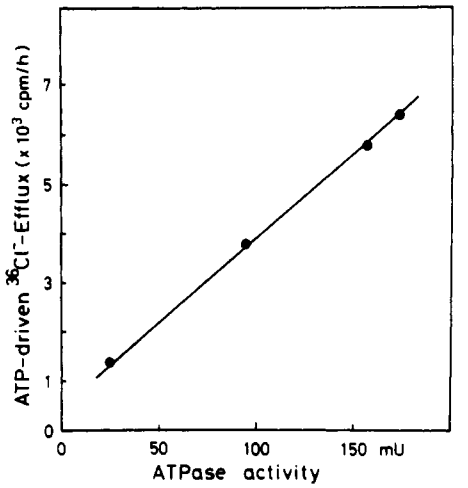

FIGURE 6: Relation between ATP-driven $^{36}Cl^-$ efflux and the ATPase activity reconstituted into negative liposomes. From the results shown in Figures 3, 4a, and 5a, ATP-driven $^{36}Cl^-$ efflux was calculated as the difference between the efflux before and after the first addition of ATP. The ATP-driven $^{36}Cl^-$ efflux with 25 milliunits of ATPase was also plotted in the figure (1400 cpm/h).

The addition of valinomycin is expected to be necessary only in electrogenic transport processes through membranes which are impermeable to charge-compensating ions. Such a situation was reported for H^+-ATPase (valinomycin) (Vara & Serrano, 1982), for halorhodopsin (CCCP) (Bamberg et al., 1984), and for Ca^{2+}-ATPase (stimulation by valinomycin) (Zimniak & Racker, 1978). Indeed, in the absence of valinomycin, only a small $^{36}Cl^-$ efflux was observed upon addition of ATP but completely ceased within 10 min (see Figure 3). The addition of valinomycin (10 μM) enhanced the chloride efflux continuously. CCCP was also tested as ionophore for compensating ions, but the $^{36}Cl^-$ efflux of proteoliposomes prior to ATP addition was stronger than valinomycin at 10 μM. The addition of 30–40 μM valinomycin had a comparable effect.

From the results presented here, the characteristics of the purified ATPase from *Acetabularia* are summarized as follows: (1) An electrogenic Cl^- pump has been demonstrated. (2) No transport activities for SO_4^{2-} and H^+ were found. (3) Arginine residues play an essential role for the Cl^- transport.

The reconstituted system presented here is not the best one and should be further improved. The results in the present paper, however, could give bases in reconstituting an anion pump into liposomes which should be taken into account, anion permeability and net charges of liposomes. We also believe that the present paper gives the first successful reconstitution

of a Cl^--translocating ATPase.

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Evidence for Reactivity of Serine-74 with *trans*-4-(*N*,*N*-Dimethylamino)cinnamaldehyde during Oxidation by the Cytoplasmic Aldehyde Dehydrogenase from Sheep Liver

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ABSTRACT: A nucleophilic group in the active site of aldehyde dehydrogenase, which covalently binds the aldehyde moiety during the enzyme-catalyzed oxidation of aldehydes to acids, was acylated with the chromophoric aldehyde *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde (DACA). Acyl-enzyme trapped by precipitation with perchloric acid was digested with trypsin, and the peptide associated with the chromophoric group was isolated and shown to be Gln-Ala-Phe-Gln-Ile-Gly-Ser-Pro-Trp-Arg. After redigestion with thermolysin, the chromophore was associated with the C-terminal hexaresidue part. If the chromophore is attached to this peptide, serine would be expected to bind the aldehyde and lead to the required acylated derivative. Differential labeling experiments were performed in which all free thiol groups on the acylated enzyme were blocked by carboxymethylation. The acyl chromophore was then removed by controlled hydrolysis and the protein reacted with [^{14}C]iodoacetamide. No ^{14}C-labeled tryptic peptides were isolated, suggesting that the sulfur of a cysteine cannot be the acylated residue in the precipitated acyl-enzyme.

Aldehyde dehydrogenase catalyzes the irreversible oxidation of a wide variety of aldehyde substrates (MacGibbon et al., 1977a,b) including the chromophoric aldehyde *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde (DACA)[1] (Buckley & Dunn, 1982) by the ordered pathway shown in Scheme I. For DACA at pH 6, since the rate of hydrolysis of the acyl-enzyme

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[1] Abbreviation: DACA, *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde.

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