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Improvement of reconstitution of the Cl^--translocating ATPase isolated from *Acetabularia acetabulum* into liposomes and several anion pump characteristics

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The improved reconstitution of the Mono Q-III fraction, a Cl^--translocating ATPase, isolated from *Acetabularia acetabulum* (Ikeda et al. (1990) Biochemistry 29, 2057–2065) into liposomes rendered transport properties of this enzyme clear. The liposomes were prepared by the reversed-phase method using egg lecithin and cholesterol in a molar ratio of 2:1 and the purified ATPase was incorporated into the liposomes by a dialysis for 3 h. About 80% of the ATPase was incorporated into the liposomes. The weight ratio of the enzyme to lipid was 1:400–600. A sigmoid curve was obtained when the Cl^--transport activity of the enzyme was plotted against Cl^- concentration. Hill's plot afforded a half-substrate concentration ($[S]_{0.5}$ of 45 mM and a Hill's coefficient n of 2.33. Effects of Br^- and F^- on the Cl^--transport were also examined in the reconstituted system, both halide ions decreased the $^{36}Cl^-$ efflux significantly. These kinetic data are in good agreement with the electrophysiological data presented by Tittor et al. (1983) J. Membr. Biol. 75, 129–139).

Introduction

In our previous report [1], a novel and unique type of ATPase has been isolated and characterized from *Acetabularia acetabulum*. The ATPase consisted of two subunits, a (54 kDa) and b (50 kDa), showing catalytic properties attributable to all of the well known P, V and F types of ATPase [2]. The enzyme has been demonstrated by reconstitution studies to be an ATP-driven chloride translocator with an electrogenic nature [3]. Electrophysiological studies [4,5] have revealed that a Cl^- pump in *Acetabularia* translocates Cl^- from outer medium into cytoplasm to maintain the membrane potential of the cells (around -170 mV in the dark). When the Cl^- pump was reconstituted in liposomes, the protein arrangement turned opposite, leading to the ATP-driven Cl^- movement from inside to outside of the liposomes [3].

So far, cation-translocating ATPase has been well characterized after reconstitution of the purified enzyme into liposomes as cited in Ref. 3. As for anion-translocating proteins, band 3 protein from erythrocytes [6,7] and halorhodopsin from *Halobacterium halobium* [8] have been successfully reconstituted into liposomes, but these proteins are not ATP-driven chloride translocators. Recently, a Cl^--translocating ATPase from *Aplysia* gut was reconstituted into black lipid membrane [9]; however, the protein was not purified after solubilization of ATPase from membranes.

In our previous report [3], we have established a mini-scale measurement system of a Cl^--translocation through liposomes using $^{36}Cl^-$. In the present paper, we describe improvement of our system and several characters of the Cl^--ATPase as an anion pump in the reconstituted system.

Materials and Methods

Materials

ATP (disodium salt, orthovanadate-free), Pipes, Tris and valinomycin were purchased from Sigma Chemical Co. (St. Louis, MO). Egg lecithin was kindly supplied by Nippon Oil & Fats Co., Ltd. (Tokyo, Japan). Other reagents of analytical grade were obtained from Wako

Abbreviations: ATPase, adenosine triphosphatase; DEAE-Sephacel, *O*-(diethylaminoethyl)-Sephacel; FPLC, fast protein liquid chromatography; MEGA-9, nonanoyl-*N*-methylgluconamide; DTT, dithiothreitol.

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Pure Chemicals (Osaka, Japan). DEAE-Sephacel and the FPLC system were from Pharmacia (Uppsala, Sweden), and MEGA-9 was from Oxyl Co. (Bobingen, Germany). A microdialysis chamber was self-built as described previously [3], and an Aloka LSC-701 liquid scintillation counter (Tokyo, Japan) was used for counting the radioisotope which was from Amersham ($^{36}Cl^-$, 21.2 mCi/g of Cl) (Tokyo, Japan).

Purification of the Cl^--ATPase from A. acetabulum

The Cl^--ATPase was solubilized with MEGA-9 and purified in the presence of MEGA-9 through DEAE-Sephacel, Superose 12 and Mono Q column chromatography [1]. The Mono Q-III fraction was used for further experiments. ATPase activity was assayed at 30°C by colorimetric determination of inorganic phosphate liberated by the hydrolysis of ATP [10]. One unit of enzyme acitivity is defined as 1 μmol of phosphate liberated per min at 30°C.

Preparation of liposomes by the reversed-phase method

As lipid sources, egg lecithin and cholesterol with 2:1 mole ratio were used for preparation of reversed-phase liposomes according to the method of Katsu et al. [11].

Inner and outer aqueous phases of liposomes consisted of 25 mM Pipes-Tris (pH 6.5), 0.21 M erythritol, 20 mM KCl, 10 mM $MgSO_4$, 1 mM EGTA and 2 mM DTT, when the $^{36}Cl^-$ efflux was measured. When the liposomes or proteoliposomes were monitored with K^+- and Cl^--selective electrodes, the liposomes were suspended in a Cl^--free and DTT-free buffer which contained 0.25 M erythritol instead of 0.21 M erythitol, 20 mM KCl and 2 mM DTT.

Incorporation of the enzyme in liposomes and transport activity measurements

The reversed-phase liposomes contained the inner aqueous phase as described above and 2 μCi of $^{36}Cl^-$ was added to the lipid suspension prior to sonication. The liposomes were centrifuged at 105000 × *g* for 30 min, and the resulting pellet was washed twice with the $^{36}Cl^-$-free solution. The Mono Q-III fraction was then mixed with the $^{36}Cl^-$-loaded liposomes in a protein to lipid weight ratio of 1:580 in a glass chamber, and valinomycin (1.5 μM) was further added. The valinomycin was required to cause $^{36}Cl^-$ efflux effectively [3]. The liposomes were dialyzed against the above Cl^--containing buffer for three changes every hour in a dialysis cup (total volume of 2.5 ml). The dialysis buffer was then changed and measurement of $^{36}Cl^-$ efflux was started. Every 10 min, one-tenth volume of the dialysis buffer was taken, mixed with Scintisol (5 ml), and counted in a liquid scintillator for radioactivity (^{14}C window). The dialysis buffer was maintained at constant volume (2.5 ml) with continuous stirring at 30°C. After 1 h, ATP (10 mM) was added to the glass chamber and to the dialysis cup. To evaluate the percentage of $^{36}Cl^-$ efflux, melittin (30 μM) was added to the glass chamber after 1 h of the ATP addition to disrupt the liposomal membrane structure [12], causing the complete efflux of Cl^- trapped in liposomes. ATP-dependent Cl^--transport activity was calculated from the difference between the $^{36}Cl^-$ efflux with ATP and spontaneous $^{36}Cl^-$-efflux before addition of ATP. Being normalized per 100 mU of ATPase activity in the proteoliposomes, the value was expressed as in terms of percentage-efflux of $^{36}Cl^-$ trapped in the liposomes.

Construction of ion-selective electrodes

A K^+-selective electrode was constructed by the use of poly(vinyl chloride)-based membrane as previously reported [11]. The electrochemical cell arrangement was Ag, AgCl/0.01 M KCl (internal solution)/sensor membrane/ sample solution/1 M NH_4NO_3 (salt bridge)/0.01 M KCl/Ag, AgCl [11]. A Cl^--selective electrode was based on an Ag/AgCl electrode [11], which was prepared by anodic oxidation of silver plate (30 mm long, 3 mm broad and 0.5 mm thick) in a solution containing 0.1 M NaCl and 0.1 M HCl at 1.5 mA for 5 h. The cell arrangement was Ag, AgCl/sample solution/1 M NH_4NO_3 (salt bridge)/0.01 M KCl/Ag, AgCl. The electromotive force between the Ag/AgCl electrodes was measured with an appropriate field-effect-transistor operational amplifier (input resistance > 10^{12} Ω) and recorded. Calibration graphs of the electrodes are plotted at the right axis of Fig. 1. Tightness, stability and trapped volumes of liposomes were examined by using these electrodes.

Other analytical methods

SDS-PAGE on mini-gels and protein determination were performed as described previously [1].

Results

Tightness, stability and trapped volumes of the reversed-phase liposomes

Fig. 1 shows a trapping efficiency of KCl in the reversed-phase liposomes monitored by the K^+- and Cl^--selective electrodes. At the first arrow, liposomal suspension was added in a KCl-free buffer, which increased both K^+ and Cl^- concentrations due to the untrapped KCl in the suspension. At the second arrow, melittin was added to disrupt the membrane structure of liposomes [12]. The efflux of K^+ and Cl^- occurred rapidly, and both the amounts effused in an outer medium were the same, indicating that Cl^- held in the present liposomes was stable as K^+. These liposomes were very stable, and spontaneous efflux of Cl^- after keeping at 4°C overnight was less than 15%. ATPase-

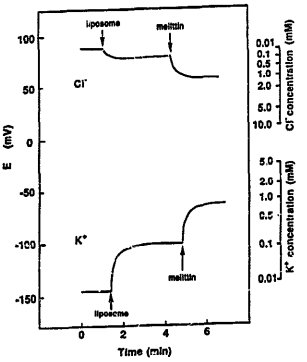


Fig. 1. Tightness and stability of the reversed-phase liposomes. The reversed-phase liposomes were prepared as described previously [11] using egg lecithin (10 μmol) and cholesterol (5 μmol). The inner aqueous phase of the liposomes contained 25 mM Pipes-Tris (pH 6.5), 0.21 M erythritol, 20 mM KCl, 10 mM $MgSO_4$ and 1 mM EGTA. The liposomal suspension was diluted with 6 volumes of the Cl^--free buffer (approx. 6 ml) containing 0.25 M erythritol instead of 0.21 M erythritol and 20 mM KCl and centrifuged at 105000 × g for 30 min. The pellet was resuspended in 1 ml of the Cl^--free buffer, the suspension was again diluted and centrifuged as described above. The resulting pellet was resuspended in 1 ml of the Cl^--free buffer. At the first arrow, 0.5 ml of liposomal suspension was added to 0.5 ml of the Cl^--free buffer. At the second arrow, melittin (30 μM) was added to a chamber. The left axis represents potential changes of electrodes and the right axis showed concentration changes calculated from the calibration curves of K^+- and Cl^--selective electrodes. The other half of the liposomal suspension was kept at 4°C overnight and the same experiment was performed to test the stability.

incorporated reversed-phase liposomes (proteoliposomes) were also found to be tight enough for both of K^+ and Cl^- ions as observed for bare liposomes (data not shown).

Incorporation of the ATPase into liposomes

As described in our previous report [3], detergent dialysis method was most appropiate for the purified Cl^--ATPase. However, in our previous system, incorporation ratio was unsatisfactory. Protein to lipid ratio was thus examined under prolonged dialysis time, 3 h. The results are summarized in Table I. Protein to lipid ratio with more than 1 to 380 (weight ratio) gave almost complete incorporation of the ATPase into liposomes.

Stability of the ATPase incorporated into the liposomes was also tested. The results are shown in Fig. 2. The incorporated ATPase was stable over more than 3 h at room temperature, but lost the activity with increasing time when kept at 4°C. Release of both subunits, a and b, from the proteoliposomes was observed during cold storage (see Fig. 3).

TABLE I

Incorporation of the ATPase into liposomes

The Mono Q-III fraction (5 μg in 3 μl, 8.3 U/mg of protein) was added to the reversed-phase liposomes (100 μl, 0.5 μmol to 4.5 μmol of lipid) in a micro-dialysis chamber and dialyzed for 3 h as described in Materials and Methods. After 3 h dialysis the suspension was collected, centrifuged and the pellet was washed again as described in the legend of Fig. 1. The washed pellet was resuspended in an original volume of the Cl^--free buffer (0.1 ml). The supernatant (10 μl) and the suspension (4 μl) were assayed for ATPase activity.

Enz. to Lipid ratio (w/w)	ATPase activity (mU)		Incorporation ratio (%)
	supernatant	precipitate	
1:64	11.5	13.5	54
1:129	9.6	17.7	64
1:193	13.1	23.0	64
1:386	6.2	21.3	77
1:580	4.7	24.0	83

Transport activity of the ATPase

(A) Effect of Cl^- concentration. Transport activities were measured with changing Cl^- concentrations inside and outside of the liposomes. The result is shown in Fig. 4, giving a sigmoid curve for Cl^- concentration. The curve was treated by Hill's plot, and a half-substrate concentration, $[S]_{0.5}$ and a Hill's coefficient, n value were calculated as 45 mM and 2.33, respectively.

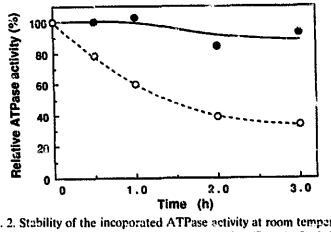


Fig. 2. Stability of the incoporated ATPase activity at room temperature (●) and at 4°C (○). The Mono Q-III fraction (7 μg in 5 μl, 3.7 units/mg of protein) was added to liposomes (6.3 μmol of lipid) in a total volume of 140 μl in a microdialysis chamber and dialyzed against the Cl^--free buffer for 3 h with changes in every 1 h. The proteoliposome suspension was collected and centrifuged at 105000 × g for 30 min. The pellet was resuspended in the Cl^--free buffer to an original volume of 140 μl. A half of the suspension was kept at room temperature and the other half at 4°C. At several time intervals, a 5 μl aliquot was assayed for ATPase activity. The enzyme activity in the suspension after centrifugation was expressed as 100% activity (17 mU in total).

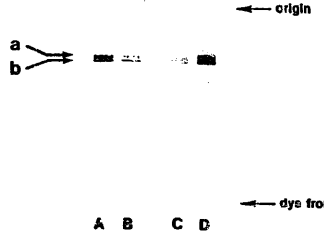


Fig. 3. Release of the subunit a and b of the Mono Q-III fraction from the proteoliposomes by cold treatment. The Mono Q-III fraction was incorporated into the liposomes in the same manner as described in the legend of Fig. 2, except that the buffer contained 20 mM KCl. After being kept at room temperature or at 4°C for 3 h, the suspensions were centrifuged at 105000× *g* for 30 min. Aliquots of the supernatant (10 μl) and the pellet after resuspension in an original volume of the buffer (15 μl) were subjected to SDS-PAGE on a mini-gel (12.5%) and polypeptides were stained by silver. Lane A: the pellet kept at room temperature; lane B: the pellet kept at 4°C; lane C: the supernatant kept at room temperature; lane D: the supernatant kept at 4°C.

(B) Effect of Br^- and F^- on Cl^- transport. The Cl^- concentration inside and outside of the liposomes was fixed to 20 mM, and effects of 10 mM of Br^- and F^- on the Cl^- transport were examined in the reconstituted system. The results are summarized in Table II. The presence of Br^- and F^- decreased Cl^--transport activity without affecting the ATPase activity.

TABLE II

Effect of Br^- and F^- on the Cl^- transport

Effect of Br^- and F^- on the Cl^--transport was measured in the same manner as described in the legend of Fig. 4, except that the buffer contained 20 mM KCl and 10 mM KBr or KF.

	Control	Br^-	F^-
$^{36}Cl^-$ efflux (cpm/h)	1000	665	563
$^{36}Cl^-$ trapped in liposomes (cpm)	55000	61800	48500
ATPase activity (mU)	70	75	71
$^{36}Cl^-$ efflux (%)	1.8	1.1	1.2

Discussion

As discussed in our previous report [3], measurements of anion-efflux from liposomes caused some difficulties with respect to spontaneous permeability of anions through liposomes, trapped volumes of liposomes and sensitivity. Among the liposomes tested (negatively charged, neutral and positively charged) negative and neutral liposomes were successfully applicable to measurement of $^{36}Cl^-$ efflux caused by an ATP-driven Cl^- pump isolated from *A. acetabulum* [3]. In our previous system, however, the incorporation ratio of Cl^--ATPase into liposomes was unsatisfactory, and the incorporated and free ATPase were not separated in the reconstitution studies. In the present study, these points were improved as summarized in Table I. Preparation of the reversed-phase liposomes was conducted by the method of Katsu et al. [11], which shortened the time required for preparation. The liposomes were proved to be tight and stable enough as shown in Fig. 1 and from measurement of spontaneous $^{36}Cl^-$-efflux (Table II).

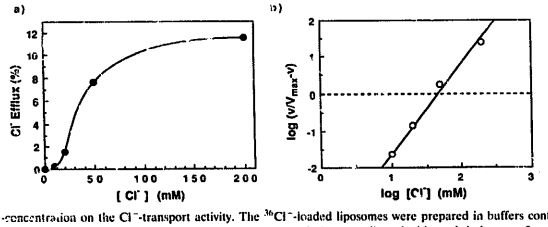


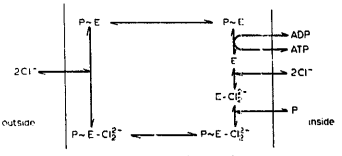
Fig. 4. Effect of Cl^--concentration on the Cl^--transport activity. The $^{36}Cl^-$-loaded liposomes were prepared in buffers containing various Cl^- concentrations (10, 20, 50 and 200 mM) using 2 μCi of the isotope. The osmolarity was adjusted with erythritol except for the case of 200 mM KCl where erythritol was not added at all. The other components added in the same amounts. The Mono Q-III fraction (25 μg in 15 μl, 8.4 units/mg of protein) was incorporated into the liposomes (13.5 μmol of lipid) in a total volume of 315 μl and dialyzed against the respective buffer adjusting for Cl^- concentrations (10 to 200 mM). The ATP-dependent $^{36}Cl^-$ efflux (cpm/h) was corrected for 100 mU of ATPase activity and for Cl^- concentrations, and represented as % values of the trapped $^{36}Cl^-$ inside the liposomes at the start of the experiments. Total ATPase activities before correction were 125, 100, 85 and 82 mU for 10, 20, 50 and 200 mM Cl^- concentrations. (a) [Cl^-] vs. transport activity curve; (b) Hill's plot.

Here we also tested the stability of enzyme activity in the reconstituted system. A new finding was that the incorporated Cl^--ATPase was released from the liposomes when kept at 4°C (see Fig. 2) and lost the activity. In this case, both the subunits, a and b of the Cl^--ATPase were released from the proteoliposomes, suggesting that the both subunits were in part incorporated into the liposomes.

Measurements of Cl^- transport was also attempted using a fluorescence probe and a Cl^--selective electrode. Several anion-sensitive fluorescence probes such as 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) [13,14] and acridine derivatives [15,16] have recently been developed and applied to measure anion transport [17,18]. A fluorescence probe, SPQ, was tested for measurement of Cl^- translocation through liposomes, but the method was not applicable because of its lower sensitivity to Cl^- and drifts of base-line in the reconstituted system. A 0.5 mM change in Cl^- concentration was at most measurable, and this was not the case for our experimental system (confer Discussion in the Ref. 3). In the system using either a fluorescence probe or an electrode, a Cl^--free buffer outside of the liposomes was required for measurement. The presence of Cl^- outside of the liposomes was, however, found to be necessary for the Cl^- transport: no significant increase in the $^{36}Cl^-$ efflux was observed by addition of ATP, when the Cl^--free buffer was used as the outer medium (data not shown). As discussed below, a Hill's coefficient of 2.33 supported the binding of $2Cl^-$ to the enzyme as another driving force in addition to ATP for Cl^- translocation through the proteoliposomes.

From intensive electrophysiological studies by Gradmann and his co-workers [4,5], anion specificity of the electrogenic Cl^- pump in *Acetabularia* has been reported to be $Cl^- > NO_3^- > Br^- > SO_4^{2-} > I^- > HCO_3^-$ > benzensulfonate > F^-, and a stoichiometry of two Cl^-/one ATP has been supported. In the present report, kinetic experiments were performed with the purified Cl^--ATPase in the reconstituted system. The results are shown in Fig. 4 and Table II. Other anions tested here, Br^- and F^- showed inhibitory effects on the $^{36}Cl^-$ efflux but not on enzyme activity. Sulfate has not been transported by the Cl^--ATPase (see our previous report [3]). Anion specificity of the Cl^--ATPase was similar to the case of whole cells except for SO_4^{2-}. *Acetabularia* is equipped with sulfate permease as an anion transport system independent of the Cl^--ATPase [19]. The uptake of SO_4^{2-} observed in whole cells may occur through this enzyme. Effect of I^- could not be tested because of facile oxidation of I^- in the reconstituted system.

Analysis of effects of Cl^- concentrations on the Cl^- transport by Hill's plot also showed tendency that binding of $2Cl^-$ to the enzyme was required for the transport. Gradmann and his co-workers presented a kinetic model of the Cl^- pump in *Acetabularia* as described in Scheme I (cited from Ref. 18).



Scheme I. E, enzyme; P, phosphate.

The present results giving a Hill's coefficient of 2.33, supported this model for Cl^- translocation utilizing ATP. We also found that the Cl^--ATPase required Cl^- outside the liposomes, i.e. inside the cells, for Cl^- transport, suggesting that the binding of Cl^- to both sides is required for Cl^- translocation.

Because of limitation of materials, statistical analysis of the data was not available, but the $^{36}Cl^-$ effluxes from the proteoliposomes in the presence of 20 mM KCl inside and outside the liposomes were 1.8 and 1.5% from duplicate separate experiments, respectively. In conclusion, the data presented here reflected the characteristics of the Cl^- pump obtained by in vivo electrophysiological studies.

Acknowledgments

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