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Reconstitution of the purified ($Na^+ + Mg^{2+}$)-ATPase from *Acholeplasma laidlawii* B membranes into lipid vesicles and a characterization of the resulting proteoliposomes

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The purified ($Na^+ + Mg^{2+}$)-ATPase from *Acholeplasma laidlawii* B membranes was reconstituted with dimyristoylphosphatidylcholine using a cholate solubilization and dialysis procedure. The incorporation of this enzyme into the phospholipid bilayer is accompanied by an enhancement of its specific activity and by a restoration of its lipid phase state-dependent properties which were lost during solubilization and purification from native membranes. Moreover, reconstitution of this ATPase with phospholipid also stabilizes it against cold inactivation at low temperatures ($\simeq 0\,^\circ C$), oxidative degradation at room temperature, and thermal denaturation at elevated temperatures ($\simeq 55\,^\circ C$). The elution profile from a Sepharose 4B-CL column indicates that all of the ATPase protein is associated with the phospholipid vesicles and that the Stoke's radius of the proteoliposomes formed is smaller than that of the lipid vesicles formed in the absence of any protein. The latter conclusion is supported by sedimentation velocity measurements and by an electron microscopic examination of negatively stained preparations. The electron microscopic studies demonstrate that sealed vesicles are formed only at low protein-to-lipid ratios. These observations indicate that the *Acholeplasma laidlawii* B ($Na^+ + Mg^{2+}$)-ATPase has been structurally and functionally reconstituted into lipid vesicles and that the proteoliposomes formed are amenable to studies aimed at the clarification of its proposed role as a sodium ion pump.

Introduction

The plasma membrane of the simple cell wall-less procaryote *Acholeplasma laidlawii* B has been widely used in the study of membrane lipid organization and dynamics (see Ref. 1 for a review). This plasma membrane contains a Na^+-stimulated, Mg^{2+}-dependent ATPase which is believed to be involved in the maintenance of the osmotic balance of the organism [2]. The ATPase is known to be an integral membrane protein [3] and has been purified to homogeneity from a detergent-solubilized extract of isolated plasma membranes [4]. This enzyme has since been shown to be an assembly of five subunits [4,5] of which at least one is known to penetrate the hydrophobic domain of the lipid bilayer [6] and to be involved in the binding of ATP [7,8]. The available evidence (see Refs. 2, 4, 7, 8 and references cited therein) also suggest that this ATPase is structurally and kinetically dissimilar to other bacterial or mitochondrial ATPases studied to date.

A number of studies have shown that the hydrolytic activity of this membrane enzyme is de-

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pendent upon the phase state and possibly the fluidity of the lipids in its native membrane [9–12] and this has served as a powerful tool in the study of the relationship between membrane lipid structure and membrane function [13]. In those studies alterations in the physical properties of the membrane lipids were achieved by the in vivo manipulation of the lipid fatty acid composition and cholesterol content of this organism. However, such studies are constrained by the limits to which there can be in vivo manipulation of the membrane lipid fatty acid composition and cholesterol content of the orgnaism and have been severely limited by the inability to make significant alterations in the phospholipid and gycolipid headgroup composition of its native membranes independently of fatty acid compositional variations. Furthermore, the inability to make sealed vesicles from *A. laidlawii* B plasma membranes has precluded any definitive attempts at linking ATPase activity with ion translocation. Thus, in order to understand the nature of the interaction of the ATPase protein with membrane lipids and to develop a system with which ion translocation can be studied, we have purified the enzyme and reconstituted it into a defined phospholipid model membrane system. Some properties of the resultant ATPase containing proteoliposomes are described in this paper.

Materials and Methods

The materials and methodology pertinant to the culturing of the organism *Acholeplasma laidlawii* B, the isolation of its plasma membrane, the solubilization and purification of the ATPase and the determination of its activity have been described in full detail elsewhere [2,4,6,7,8]. The ATPase was reconstituted with dimyristoylphosphatidylcholine (DMPC) by a modification of the cholate solubilization and dialysis procedure originally reported by Kagawa and Racker [16]. DMPC was spread as a thin film on the sides of a clean glass test tube by the slow removal of the solvent (chloroform) under a stream of nitrogen, and the trace amount of residual solvent were removed under vacuum overnight. The dried lipid was resuspended in reconstitution buffer (10 mM Hepes (pH 7.6), 10 mM NaCl, 1 mM EDTA, 1 mM NaN_3) using a vortex mixer while the temperature was kept above the phase transition temperature of the lipid. To this suspension purified ATPase was added to yield the desired protein-to-lipid ratio, and potassium cholate (10% w/v (pH 7.5)) was added until the suspension became optically clear. This usually required a weight ratio of cholate to phospholipid of approx. 1.5. After incubation at 37 °C for one hour, the cholate-solubilized lipid/protein mixture was transferred to dialysis bags and dialyzed extensively at room temperature (22 °C) against 4–6 litres of reconstitution buffer with three changes over a period of 48 h. To minimize oxidative degradation of the material, nitrogen was bubbled through the buffer during the dialysis and the resultant proteoliposomes were stored at room temperature under nitrogen.

Sedimentation velocity experiments were carried out at 20 °C in a Beckman Spinco model K analytical ultracentrifuge using the Schlieren optical system. Samples for electron microscopy were stained on a formvar coated grid with a 2% solution of phosphotungstic acid (pH 6.0) and were examined with a Philips EM 400T transmission electron microscope. Phosphatidylcholine was quantitated by the procedure described by Raheja et al. [17]. Protein was estimated by the method of Hartree [14] after precipitation according to Bensadoun and Weinstein [15] with bovine serum albumin as standard. The purified, detergent-solubilized ATPase contains approx. 88 nmol glycerolipid per mg protein [4] and 35% of the lipid is phospholipid. At the protein-to-lipid ratios generally used in these studies, this comprises less than 1% of the added phospholipid and was ignored in phospholipid determinations.

Results

An important consideration in this type of study is the establishment of viable criteria for a successful reconstitution of this ATPase. To this end we have examined the physical properties of the lipid/ATPase complex formed by gel filtration chromatography, analytical ultracentrifugation, and electron microscopy. Fig. 1 shows the elution profiles of DMPC vesicles, and of the proteoliposomes composed of DMPC and the purified

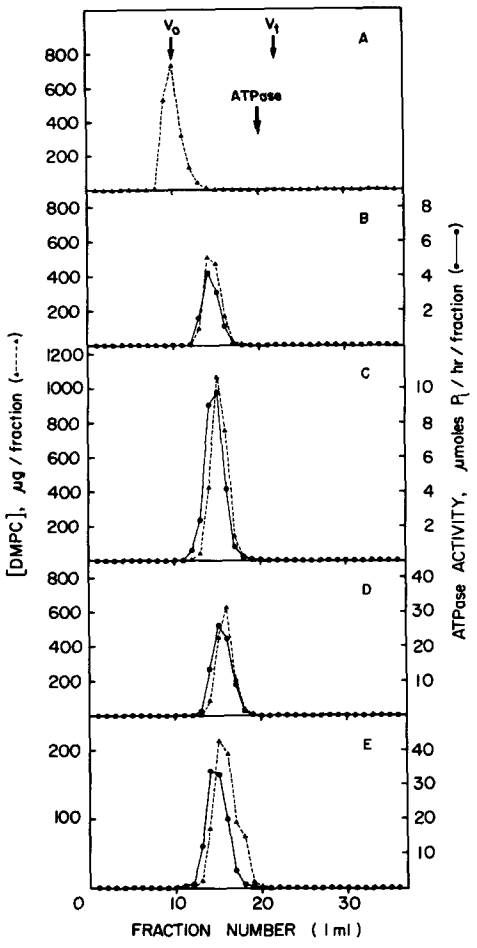

Fig. 1. Elution profile of DMPC/ATPase vesicles from Sepharose 4B-CL. The elution volume of the purified detergent-solubilized ATPase as well as the void (V_0) and total (V_t) volumes of the column are indicated by the arrows in panel A. The weight ratios of ATPase:DMPC were: (A) 0; (B) 0.084; (C) 0.145; (D) 0.51; (E) 0.76.

ATPase, from a Sepharose 4B-CL gel filtration column. In the absence of any protein the DMPC vesicles elute in the void volume of the column and are well resolved from the detergent-solubilized ATPase. However, once reconstituted with the phospholipid, the elution volume of the ATPase activity and the added phospholipid coincide and differ from that of either the pure lipid or that of the purified unreconstituted ATPase. The elution profiles presented in Fig. 1 show that the lipid and protein are associated over the entire range of protein-to-lipid ratios studied and that the average Stoke's radius of the resultant proteoliposomes is smaller than that of the protein-free DMPC vesicles and is significantly larger than that of the purified detergent-solubilized ATPase.

At the higher protein-to-lipid ratios studied, the elution profiles of the proteoliposomes from the gel-filtration column suggest some heterogeneity in the proteoliposome population (see Fig. 1). This was further investigated by sedimentation velocity studies using an analytical ultracentrifuge, and the resultant Schlieren patterns are shown in Fig. 2. These results show that in the absence of the ATPase protein, there is essentially a single population of lipid vesicles, at least with respect to their sedimentation characteristics. However, at all the protein-to-lipid ratios examined there is clear evidence of heterogenity in the population of the proteoliposomes present. At low protein-to-lipid ratios (< 100 μg ATPase per mg DMPC) there are two populations of vesicles which sedimented more quickly than the pure DMPC vesicles or the lipid-protein complexes formed at the higher protein-to-lipid ratios. Two populations of complexes are also evident in the Schlieren patterns of the proteoliposome preparations with 'high' protein-to-lipid ratios, and one of these was found to sediment more quickly than the pure lipid vesicles. The rapidly sedimentating population comprised some 20–25% of the total particulate matter in the preparation, and the ratio of the slow-sedimenting to the fast-sedimenting population is apparently unaffected by the protein-to-lipid ratio, especially at the higher lipid-to-protein ratios tested.

The morphology of the proteoliposome preparations formed was also examined by electron microscopy in order to determine whether or not sealed vesicles amenable to ion translocation studies are formed. In the absence of added ATPase the cholate solubilization and dialysis procedure results in the formation of large unilamellar vesicles with diameters in the range 300–700 nm (see Fig. 3). However, the incorporation of the ATPase into the phospholipid vesicles radically alters their structure throughout the entire range of protein-to-lipid ratios studied. At low protein-to-lipid ratios (< 100 μg ATPase protein per mg DMPC), sealed unilamellar vesicles are formed (see Fig. 3). However, the presence of the protein results in a significant reduction in the size of

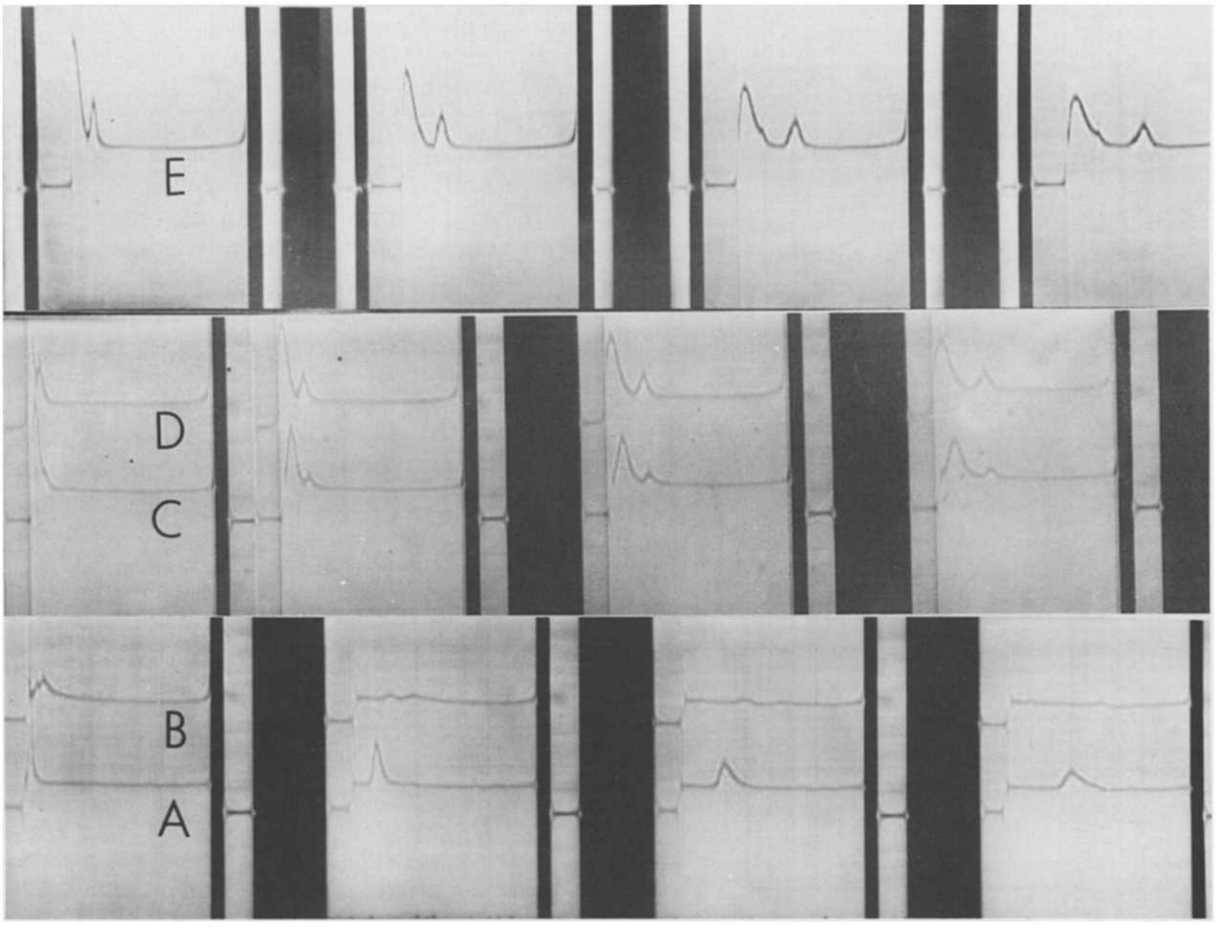

Fig. 2. Sedimentation patterns of DMPC/ATPase vesicles. The photographs were taken at 8 minute intervals and the sedimentation direction was from left to right. The weight ratios of ATPase: DMPC were: (A) 0; (B) 0.002; (C) 0.145; (D) 0.51; (E) 0.76.

these vesicles. In general, the diameters of the sealed unilamellar vesicles formed in the presence of the ATPase range from 100–300 nm and this range of vesicular sizes is not significantly affected by protein-to-lipid ratios of less than 100 μg ATPase protein per mg DMPC. These electron microscopic studies also show that there are few if any sealed vesicles in preparations with protein-to-lipid ratios greater than 100 μg protein per mg DMPC. Such preparations react very poorly with the stain, could not be easily visualized, and showed no evidence of bilayer-like or vesicular structures of the type observed at low protein-to-lipid ratios.

Experiments designed to assess the affect of phosholipid reconstitution on the functon of the $(Na^+ + Mg^{2+})$-ATPase were also performed. Specifically, measurements of the specific activity of this enzyme and of its temperature dependence were made since those kinetic properties are kown to be lipid dependent in vivo (see Refs. 4, 9). The effect of added lipid on the specific activity of the purified ATPase is illustrated in Fig. 4. The results indicate that the reconstitution of the ATPase with the phosholipid results in an increase in its specific activity, apparently in a saturable, concentration-dependent manner. The increase in the specific activity of the purified enzyme is only observed after the cholate used in the dialysis procedure is dialyzed away (see Table I). This indicates that the observed increase in specific activity is dependent upon the formation of some

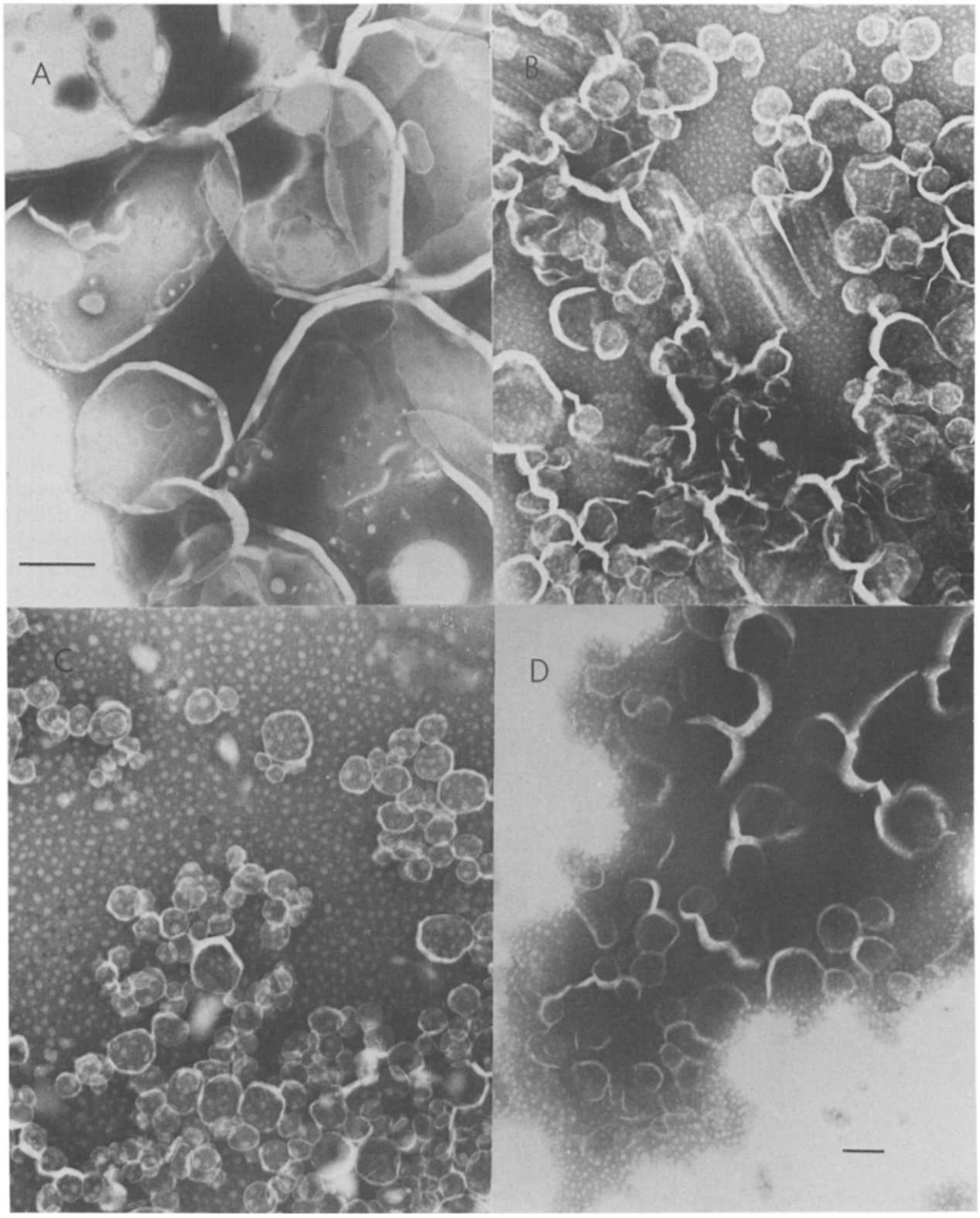

Fig. 3. Electron micrographs of negatively stained DMPC/ATPase vesicles. The DMPC concentration in the samples shown were approx. 2 mg·cm^{-3} and the weight ratios of ATPase:DMPC were: (A) 0; (B) 0.0098; (C) 0.02; (D) 0.049. The samples containing high ATPase:DMPC ratios stained poorly and are not shown here.

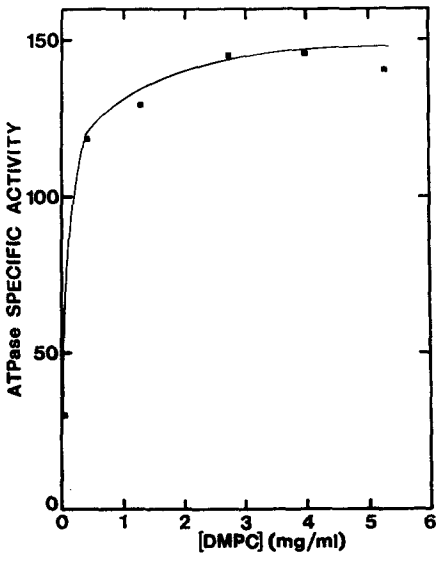

Fig. 4. Effect of lipid reconstitution on the specific activity of the purified ATPase from *Acholeplasma laidlawii* B membranes. In the case of the example shown, the protein concentration was held at 52 $\mu g \cdot cm^{-3}$ and the lipid concentration varied as indicated.

form of bilayer structure and not simply on the presence of excess phospholipid.

The present studies of the effect of phospholipid reconstitution on the stability of this enzyme

TABLE I

EFFECT OF LIPID RECONSTITUTION ON THE SPECIFIC ACTIVITY OF THE PURIFIED ATPase FROM *Acholeplasma laidlawii* B PLASMA MEMBRANES

Preparation	Specific activity (μmol mg protein per h)
(i) Purified ATPase	177
(ii) ATPase with DMPC in cholate buffer	197
(iii) DMPC/ATPase proteoliposomes [a]	328

[a] The DMPC:ATPase protein ratio was 1.3 mg DMPC per 100 μg ATPase protein.

were prompted by an earlier study which reported that relipidation of the purified *Acholeplasma laidlawii* B ATPase with phospholipids resulted in a stabilization of the purified enzyme against cold inactivation [4]. Illustrated in Fig. 5 is a comparison of the stability of the purified, detergent solubilized ATPase and the purified, lipid-reconstituted ATPase. When stored at 0 °C, the activity of the detergent-solubilized preparation decays with an apparent half life of 10 h, while at room temperature (22–24 °C) ATPase activity decays with an apparent half life of 8–10 days. The reassociation of the ATPase with lipid reduces the

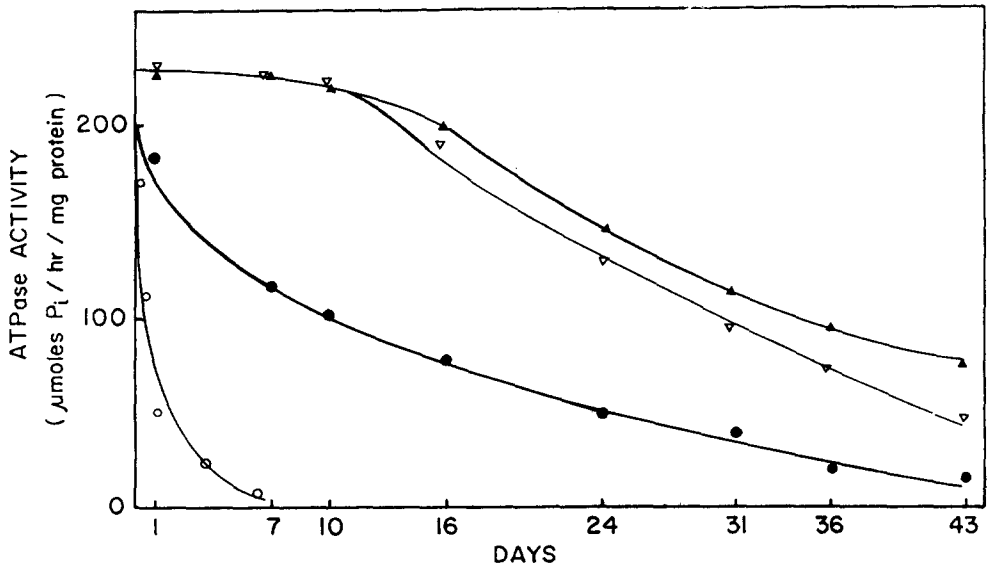

Fig. 5. Effect of lipid reconstitution on the stability of the purified ATPase from *Acholeplasma laidlawii* B membranes. ○——○, detergent-solubilized ATPase at 0 °C; △——△, lipid-reconstituted ATPase at 0 °C; ●——●, detergent-solubilized ATPase at room temperature; ▲——▲, lipid-reconstituted ATPase at room temperature.

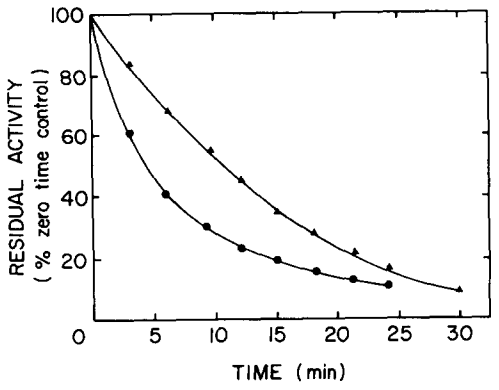

Fig. 6. Effect of lipid reconstitution on the thermal stability of the purified ATPase from *Acholeplasma laidlawii* B membrane. ●———●, detergent-solubilized ATPase; ▲———▲, lipid-reconstituted ATPase.

rate of inactivation of its enzymic activity at either temperature. When stored at either 0 °C or at room temperature, there appears to be a significant lag phase before inactivation of the lipid-reconstituted preparations begins. However, once started, the rate of inactivation is some 10–20-times slower than that of the detergent-solubilized preparation. We also investigated the effect of lipid reconstitution on the stability of the ATPase activity to thermal inactivation at 55 °C and the results are shown in Fig. 6. The rate of thermal inactivation of the lipid-reconstituted preparation is significantly slower than that of the detergent-solubilized preparations, as is evident from the apparent half lives obtained by interpolation of the data shown (about 4 min for the detergent-solubilized preparation versus about 12 min for the lipid-reconstituted preparation).

The temperature dependencies of the ATPase activity in the detergent-solubilized and lipid-reconstituted preparations are presented as Arrhenius plots in Fig. 7. In the absence of lipid, the Arrhenius plot of the purified, detergent-solubilized ATPase preparation consists of a smooth curve similar to those characteristic of native membranes with lipid phase transitions below 0 °C (see Refs. 2, 9). A similar result is found when this enzyme is reconstituted with dioleoylphosphatidylcholine, which exists exclusively in the liquid-crystalline state above 0 °C. When reconstituted with DMPC, the Arrhenius plot of the ATPase activity is characterized by a marked increase in

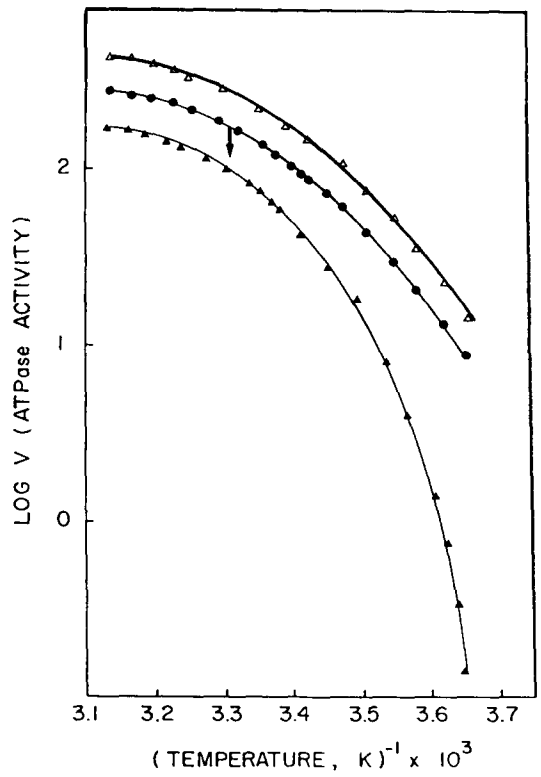

Fig. 7. Effect of lipid reconstitution on the temperature dependence of the purified ATPase from *Acholeplasma laidlawii* B membranes. The temperature dependence is shown here as Arrhenius plots for: ●———●, detergent-solubilized ATPase; △———△, DOPC-reconstituted ATPase, and ▲———▲, DMPC-reconstituted ATPase. The arrow indicates the calorimetrically determined maximum of the phase transition of the proteoliposomes. The gel/liquid-crystalline phase transition of those proteoliposomes ranges from 18 °C to 33 °C. To facilitate better comparison of the datasets the Arrhenius plot of the detergent-solubilized and DOPC-reconstituted preparations are each offset on the *y* axis by +0.4 log units.

slope at temperatures below 25 °C. This change in slope coincides with the calorimetrically determined phase transition temperature of the proteoliposomes (data not presented), just as previously observed in native membranes whose lipids undergo phase transitions above 0 °C [9]. These results indicate that the lipid phase state-dependent properties of the enzyme are restored upon reconstitution with lipid. In these lipid vesicles, the sharp decline in ATPase activity with decreasing temperature is consistent with an inactivation of the enzyme by gel-state lipid, as demonstrated previously with native membranes.

Discussion

The purification of an integral membrane protein and its successful reconstitution with defined lipid systems are generally considered to be necessary prerequisites for definitive physical studies on the nature of its interaction with lipids and on the ways in which such interaction affects its function. Of the various methods used for such reconstitutions (see Ref. 18 for a review), the procedure we have used was not deleterious to the activity of this ATPase, while being simple in its implementation and inexpensive in its use of reagents. This procedure results in the formation of large lipid-protein aggregates and, as evidenced the gel filtration chromatographic results, all of the catalytically active protein is associated with lipids throughout the entire range of lipid/protein ratios studied. While it is clear that the physical properties of the proteoliposomes formed are different from those of the pure lipid vesicles and the detergent/ATPase micellar particles, the data presented indicate that these proteoliposomes are heterogeneous, at least with respect to their sedimentation properties, and the reasons for this heterogeneity are currently unknown. However, it should be noted that the purified detergent-solubilized ATPase is itself heterogeneous in sedimentation equilibrium expeirments (see Ref. 4), and this heterogeneity may thus be an inherent property of the ATPase that has been purified by the methods used.

The data presented also indicate that the physical properties of the proteoliposomes formed are dependent upon the protein/lipid ratio. Of particular interest in this regard are the observations that sealed vesicles are not formed nor is there full enhancement of ATPase activity when the protein to lipid ratio exceeded 100 μg protein per mg of DMPC. We suspect that this may be largely due to the presence of increasing amounts of Brij-58 (the non-ionic detergent used for solubilization and purification of the ATPase, see Ref. 4) in the proteoliposomes with increasing protein/lipid ratios. Brij-58 is a so-called non-dialysable detergent, and will thus be concentrated along with the protein if, for example, ultrafiltration techniques are used to concentrate the enzyme. To date, our attempts at the complete removal of this detergent using standard chromatographic and detergent-exchange procedures while retaining the catalytic activity of the ATPase have been unsuccessful. However, we find that the cholate solubilization and dialysis procedure used for the lipid reconstitution results in a complete ($> 99.7\%$) removal of the added cholate and a 50% reduction of the Brij-58 levels associated with the ATPase. Thus, at the maximum protein/lipid ratios at which sealed vesicular structures are formed (100 μg ATPase protein per mg DMPC), the residual Brij-58 comprises no more than 5 mol% of the lipid molecules present. In spite of the above, we are of the opinion that such vesicular preparations are amenable to studies of the nature of the interaction between this enzyme and the lipid bilayer. Indeed, a similar preparation has been used to demonstrate that the α subunit of this enzyme complex does penetrate into the hydrophobic domain of the lipid bilayer [6].

The effects of the lipid reconstitution on the kinetic properties of the ATPase and the morphology of the proteoliposomes are themselves very interesting. Firstly, maximal reactivation of ATPase activity upon reconstitution was only observed at the low protein/lipid ratios at which bilayer-like structures are formed. Since there is likely to be significant levels of residual Brij-58 present in preparations having high protein/lipid ratios, our failure to detect vesicular structures in their negatively stained electron micrographs and the poor reactivation of ATPase activity may have been the result of the insertion of the protein into mixed micellar particles composed of lipid and detergent. Such structures would probably react very poorly with the 'stain' and may not be able to support the full activity of the ATPase. Secondly, the reconstitution of this ATPase with lipid confers a greater stability of its catalytic activity to cold inactivation and to heat denaturation, as well as to the random oxidative degradation which occurs at room temperature. In native membranes the ATPase activity is usually very resistant to cold inactivation and is considerably more stable to oxidative degradation and thermal inactivation than that in detergent-solubilized preparations. Thus the reconstitution of the ATPase into lipid bilayers has clearly resulted in the restoration of those in vivo properties. The above also indicates

that thermal unfolding of the ATPase protein is affected by its association with membrane lipids. Thus preparations in which this ATPase has been reconstituted into structurally and compositionally different lipid bilayers may be very useful systems with which to probe the relationship between membrane lipid 'fluidity' and the thermal stability of membrane proteins.

Finally, the data reported here also shows that the reassociation of the ATPase with membrane lipids restores its lipid phase state-dependent properties and provides further evidence for a full functional reconstitution of this enzyme. The availability of purified, fully functional lipid-reconstituted ATPase preparations thus make possible a wider range of investigations than is feasible in native membranes, since we have a considerably greater control of the lipid environment in which the protein is embedded. Of particular interest is the demonstration that the ATPase could be reconstituted into sealed vesicular structures, albeit at very low protein-to-lipid ratios. Thus, for the first time, it is possible to address the question of whether or not this ATPase is the ion pump responsible for the extrusion of sodium ions from the intact microorganism. This is a significant advance since, in the past, such studies have been impeded by the inability to form sealed vesicular structures from the membrane fragments derived from cell lysates of this organism. Indeed, our preliminary studies using ^{23}Na-NMR spectroscopy and ^{22}Na tracer techniques have shown that the vesicular preparations described here are sealed vesicles which maintain a permeability barrier to sodium ions at temperatures where the lipids are all in the liquid-crystalline state, and that this ATPase is capable of catalysing the transport of Na^+ ions (data not presented). However, it is clear that the low density of ATPase sites in the sealed vesicular preparations which can now be formed does set limits to the usefulness of such preparations in ion translocation studies, and that some refinements are needed to enable the effective exploitation of this system. Such studies are currently in progress.

Acknowledgements

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