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INTERACTION OF A SOLUBILIZED MEMBRANE ATPase WITH AQUEOUS DISPERSIONS OF BILAYER LIPID MEMBRANES

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SUMMARY

1. Aqueous dispersions of phosphatidylcholine have been used to study the interaction of a soluble ATPase with phospholipid bilayers. Binding of the membrane-derived ATPase to the phospholipid was demonstrated by differential centrifugation of mixtures of the soluble enzyme and multilamellar phosphatidylcholine liposomes. Mg^{2+} was found to increase the ATPase binding to the model membranes. The upper limit of the molar ratio of the phosphatidylcholine to ATPase in the bilayer–ATPase complex was approximately 8000:1.

2. The bilayer–ATPase interaction was also investigated using homogeneous phosphatidylcholine vesicles, 270 Å in diameter. Electron microscopy was employed to determine whether morphological changes occurred as a result of the vesicle–ATPase interaction. At short incubation times, it was found that large vesicles were generated in the mixture; after an hour, multilamellar structures appeared. The lamellar spacings of these phospholipid–ATPase structures were 54 Å compared with 44 Å in the absence of the protein.

3. The addition of the ATPase was also observed to cause a line broadening and a concomitant decrease in the signal intensity in the high resolution PMR spectrum of sonicated phosphatidylcholine vesicle.

INTRODUCTION

Phospholipid lamellar systems possess many physical properties in common with biological membranes, and have thus been studied in several laboratories over the past decade in a concerted effort to elucidate the structure and function of biological membranes [1,2]. In order to understand the specific interactions occurring between proteins and lipids at the membrane level, it is desirable to first characterize the interactions between membrane-derived proteins and artificial lipid bilayer systems. These membrane models are relatively well defined, permit control of the operational parameters and thus allow analysis in greater detail than is currently possible with biological membranes [2]. The use of both the planar lipid bilayer system and aqueous dispersions of phospholipids optimizes this approach; planar lipid bilayers provide a sensitive tool to study transmembrane electrical changes, while the bulk systems allow the convenient measurement of spectroscopic

perturbation accompanying the lipid–protein interaction [3,4]. Amongst the many practical uses of the liposome system it is possible to determine interaction constants for ligand binding to the bilayer [5]. Both systems permit measurement of membrane permeability changes resulting from protein interaction, and this common property may be used to evaluate the complementarity of the bilayer membrane models. Much of the work reported on the study of lipid–protein interactions in these model systems has been carried out with non-membrane proteins or polypeptides, some of which, like lysozyme, ribonuclease and polylysine, are highly basic [6]. Recently, however, several groups are focussing their attention on membrane-derived proteins [7,8] including the physiologically interesting $(Na^+–K^+)$-ATPase of mammalian membranes [9–11].

In 1965, Abrams reported the release of a bound Mg^{2+}-ATPase from isolated *Streptococcus fecalis* membranes and described the properties of the solubilized enzyme [12]. Three years later Abrams and Baron described the reversible attachment of the ATPase to the enzyme-depleted streptococcal membranes and the effect of Mg^{2+} on the binding [13]. It was subsequently discovered that this soluble, bacterial ATPase interacts with planar lipid bilayer membranes to produce a 10^2–10^4-fold increase in the electrical conductance of the membrane [14]. The characteristics of this interaction led to the tentative conclusion that the bilayer–ATPase complex might be similar to the membrane–ATPase complex in the intact organism. However, it was not possible to directly demonstrate the existence of such a membrane–protein complex in the planar bilayer system. Additional information bearing on the interaction between the ATPase and phospholipid membranes was provided by polarographic studies [15]. Homogeneous vesicles of phosphatidylcholine, prepared by the Huang technique [16], were found to perturb the characteristic d.c. polarographic wave of the soluble ATPase. A matrix rank analysis of the phase-sensitive a.c. polarogram of the mixture of homogeneous vesicles and soluble ATPase indicated the existence of a vesicle–ATPase complex. The soluble ATPase has since been highly purified and its interaction with the planar bilayer system studied in considerable detail. At extremely low enzyme concentration, 10^{-10} M, discrete conductance channels have been discerned and are thought to be aqueous pores stabilized by the lipid–protein complex [17]. In this communication we wish to present the results of direct binding experiments using the purified ATPase and phospholipid bilayers, together with further evidence for the vesicle–ATPase interaction derived from electron microscopy and proton magnetic resonance studies.

MATERIALS AND METHODS

The buffer solutions employed were: (1) 0.02 M Tris and 0.01 M $MgCl_2$ titrated to pH 7.5 with HCl; (2) 0.02 M succinic acid and 0.01 M $MgCl_2$ titrated to pH 6.2 with NaOH. KCl was added to these solutions to vary the ionic strength. ATP was obtained as the disodium salt from Sigma, Tris and $(NH_4)_2SO_4$ were special enzyme grade from Mann Research Laboratory, and all other chemicals were analytical reagent grade. Doubly distilled water was used for all solutions except for the PMR studies where 99.8% ^2H_2O (Mallinckrodt) was employed.

The ATPase preparations used in these studies were generously provided

by Dr T. E. Thompson. The enzyme was extracted from *S. fecalis*, purified according to the procedures of Schnebli and Abrams [18] and stored in the succinic acid–$MgCl_2$ buffer (pH 6.2) from the final DEAE-cellulose column at a salt concentration of 0.2 M KCl. The ATPase for the PMR studies was obtained from the above purified preparation by precipitation with 80% saturated $(NH_4)_2SO_4$ and redisolved in ^2H_2O solution containing 0.1 M KCl and 0.02 M $MgCl_2$ at p^2H 6.4. Dialysis against the same solution was employed to remove $(NH_4)_2SO_4$ and water. ATPase activity was measured by the release of inorganic phosphate from Mg^{2+}–ATP using the method of Abrams [12]. Protein concentration was determined by the method of Lowry et al. [19].

Preparation of the phospholipid dispersions

Chromatographically pure phosphatidylcholine was prepared from egg yolk extract by the procedure of Rouser et al. [20], and lyophilized from recrystallized benzene. The lyophilized phosphatidylcholine was suspended in cold aqueous solution at a concentration of 2.5% (w,w) and the solution flushed with pure argon, then mixed mechanically for 10 min to generate liposomes. These multilamellar structures can be easily separated from solution by centrifugation and, therefore, provide a convenient matrix for the study of protein binding. These solutions were prepared fresh daily. Homogeneous, unilamellar vesicles were prepared by the process of 2 h ultrasonication of the liposome preparation under argon at 4 °C, followed by molecular sieve chromatography on a Sepharose 4B column (2.5 cm × 50 cm) according to the procedure of Huang [16].

Phosphatidylcholine vesicles were prepared for the PMR studies by sonicating the lyophilized phosphatidylcholine under argon in 99.8% ^2H_2O containing 0.1 M KCl and 0.02 M $MgCl_2$, p^2H 6.4, at 4 °C. After 2 h of sonication the solution was centrifuged, then passed through 0.05-μm Sartorius membrane filters.

Measurement of ATPase binding to liposomes

Aliquots of the liposome preparation were added by means of a microliter syringe to 1-ml solutions of the purified enzyme contained in 10 ml polycarbonate centrifuge tubes. The tubes were gently swirled for 10 s, stored at 4 °C for 1 h, then centrifuged at 40 000 rev./min for 30 min. The supernatants were carefully removed without disrupting the tightly packed pellets and transferred to separate tubes. The pellets were resuspended in the original buffer, and both the supernatant and pellet fractions were analyzed for ATPase activity using the appropriate controls.

Electron microscopy

Samples of the homogeneous phospholipid vesicles at approximately 0.2% (w,w) were placed on Formvar-carbon coated copper grids, allowed to adhere for 1 min, then negatively stained with an equivalent volume of a 2% solution of potassium phosphotungstate at pH 7, and the excess solution removed. A similar method was used to prepare samples of a 0.45% (w,w) solution of the ATPase for electron microscopy. In some cases, a 2% solution of ammonium molybdate in 0.1 M ammonium acetate, pH 7, was employed as the staining reagent. The ionic strengths of the ATPase and vesicle solutions were approximately equal in these studies. Mixtures of the above vesicles and ATPase solutions in volume ratios of

3:1 were allowed to incubate from 1 to 12 h in glass tubes prior to sampling for electron microscopy. In order to examine the vesicle–ATPase solution without prior incubation, enzyme was added directly to a grid containing the vesicle solution, and stained within 15 s of mixing, then the excess solution drained away. After air-drying, the grids were examined in a Philips 300 electron microscope operating at 80 kV. Latex spheres, 1090±27 Å (S.D.) in diameter, from Ernest F. Fullam (Schenectady, N.Y.) were added to some of the grids containing the negatively stained vesicles for calibration. Areas of the electron micrograph negatives showing multilamellar forms were enlarged on photographic film to provide positive transparencies, which were scanned in a Beckman microsome densitometer (Model R 110). The chart recordings of the lamellar, representing magnifications up to $6 \cdot 10^6$, were measured for lamellar spacing.

Proton magnetic resonance

High resolution PMR spectra were obtained with the freshly filtered vesicle preparations, using a Varian A60A spectrometer, operating at 60 MHz. An external reference consisting of 1% (v/v) benzene in carbon tetrachloride was used. The sample size of the vesicle preparation was 0.3 ml and the ATPase solution was added to the vesicles directly in the spectrometer tubes with the aid of a microliter syringe. The probe temperature was 40 °C. Viscosity measurements were carried out on the vesicle suspensions and mixtures of vesicles and ATPase in ^2H_2O from the PMR studies, using a Wells-Brookfield micro-viscometer operating at 40 °C.

RESULTS

Binding studies

Fig. 1 illustrates typical data obtained from the binding experiments using the purified ATPase in 0.02 M succinic acid–0.01 M $MgCl_2$ buffer (pH 6.2) containing 0.2 M KCl, and liposomes generated from chromatographically pure phosphatidylcholine. Centrifugation of the mixtures of the liposomes and soluble ATPase resulted in a significant lowering of the concentration of the ATPase in the supernatant fractions. No detectable decrease in ATPase concentration occurred following centrifugation of the enzyme controls which did not contain the phos-

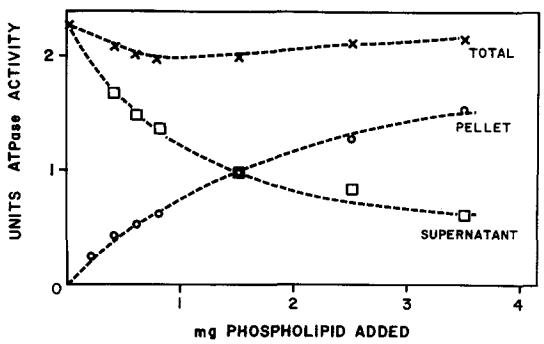


Fig. 1. Binding of the soluble ATPase to phosphatidylcholine liposomes. Each tube contained 90 μg of soluble ATPase and aqueous dispersions of phospholipid in the amount shown in a final volume of 1 ml succinic acid–$MgCl_2$ buffer (pH 6.2) with 0.2 M KCl. The mixtures were incubated for 1 h, then centrifuged at 40000 rev/min for 30 min at 4 °C.

pholipid. The total ATPase activity recovered in the pellet and supernatant fractions was approximately equal to the amount of enzyme added to the system (Fig. 1). The volume of trapped solution in the pellets was measured by equilibration with [^{14}C]sucrose and found to be less than 3% of the total volume for the range of phospholipid employed in these binding experiments. The substantial proportion of the total ATPase activity which is associated with the pellets could not be explained by solution capture and is, therefore, regarded as direct evidence for binding of ATPase to the liposomes. Ancillary measurements of the effect of phospholipid on the enzymatic activity of the ATPase have shown that phosphatidylcholine neither activates nor inhibits the enzyme (unpublished results). The amount of enzyme associated with the liposomes can thus be calculated directly from the ATPase activity of the pellet fractions. An estimate of the binding capacity of the liposomes, based on the initial slope of the pellet activity in Fig. 1, is approximately 1 unit of ATPase per mg of phospholipid. These binding experiments were repeated at higher enzyme concentrations in the interactant mixture and saturation of the liposomes was found to occur at approximately 1.6 ± 0.4 (S.D.) units/mg phospholipid. The specific activity of the soluble ATPase used in the studies was 25 units/mg protein (at 2/3 substrate saturation), where 1 unit of ATPase activity is defined as that amount of enzyme which catalyzes the release of 1 μmole of inorganic phosphate from ATP per minute at a Mg^{2+}–ATP concentration of 5 mM at 38 °C. Assuming the molecular weights of the ATPase and phosphatidylcholine to be 385000 and 770, respectively [21], the molar ratio of phospholipid to ATPase in the liposome–ATPase complex is approx. 8000 to 1. It is not known what proportion of the phosphatidylcholine in the multilamellar liposomes is exposed to the ATPase, and at present this binding ratio must be regarded as an upper limit.

Abrams and Baron have shown that the binding of the soluble ATPase to the enzyme depleted streptococcal membranes increased as a function of Mg^{2+} concentration and reached saturation at 0.01 M $MgCl_2$ [13]. In order to check the effect of Mg^{2+} on the interaction between the ATPase and liposomes, the binding studies were carried out in Mg^{2+}-free solutions containing Tris–KCl adjusted to the same pH and ionic strength as the 0.02 M Tris–0.01 $MgCl_2$ buffer (pH 7.5). Sufficient EDTA was added to chelate the Mg^{2+} present in the ATPase stock solution and controls were run with the EDTA treated ATPase preparation in the Tris–$MgCl_2$ buffer (pH 7.5) in the presence and absence of phospholipid. Representative results of these experiments are listed in Table I. The binding of the ATPase to the liposomes is significantly increased by Mg^{2+}, albeit, the magnitude of this enhancement is somewhat lower than that reported by Abrams and Baron in their reconstitution experiments [13]. Native *S. fecalis* membranes are thought to contain a limited number of specific binding sites for the soluble ATPase and reconstitution experiments probably involve the reinsertion of the soluble ATPase into these sites [13]. Clearly the interaction of the soluble ATPase with phosphatidylcholine liposomes cannot involve specific membrane receptor sites. Nevertheless, it appears that Mg^{2+} plays a role in stabilizing the membrane–ATPase complexes in both systems.

Electron microscopic evidence for vesicle–ATPase interaction

The elution profile of the sonicated dispersion of phosphatidylcholine from

TABLE I

EFFECT OF Mg^{2+} ON THE LIPOSOME–ATPase INTERACTION

The soluble ATPase was added in the amounts shown to liposomes in either Tris–$MgCl_2$ or Tris–KCl buffer, pH 7.5 at 4 °C. In each case the amount of phospholipid used was 0.5 μmole, and the total volume of interactant mixture 0.365 ml. The specific activity of the enzyme was 33 units/mg protein (at 2/3 substrate saturation). The liposome–ATPase mixtures were incubated for 1 h, then centrifuged for 30 min at 40000 rev./min and 4 °C. Amounts of ATPase bound were determined from enzymatic assay of the supernatant fractions. The data presented are mean values of 3 runs, with an experimental variation of less than 5%.

Units ATPase added	Units ATPase bound	
	0.02 M Tris–0.01 M $MgCl_2$	0.02 M Tris–0.03 M KCl
0.56	0.54	0.46
0.74	0.71	0.63
0.92	0.80	0.66
1.11	0.87	0.69

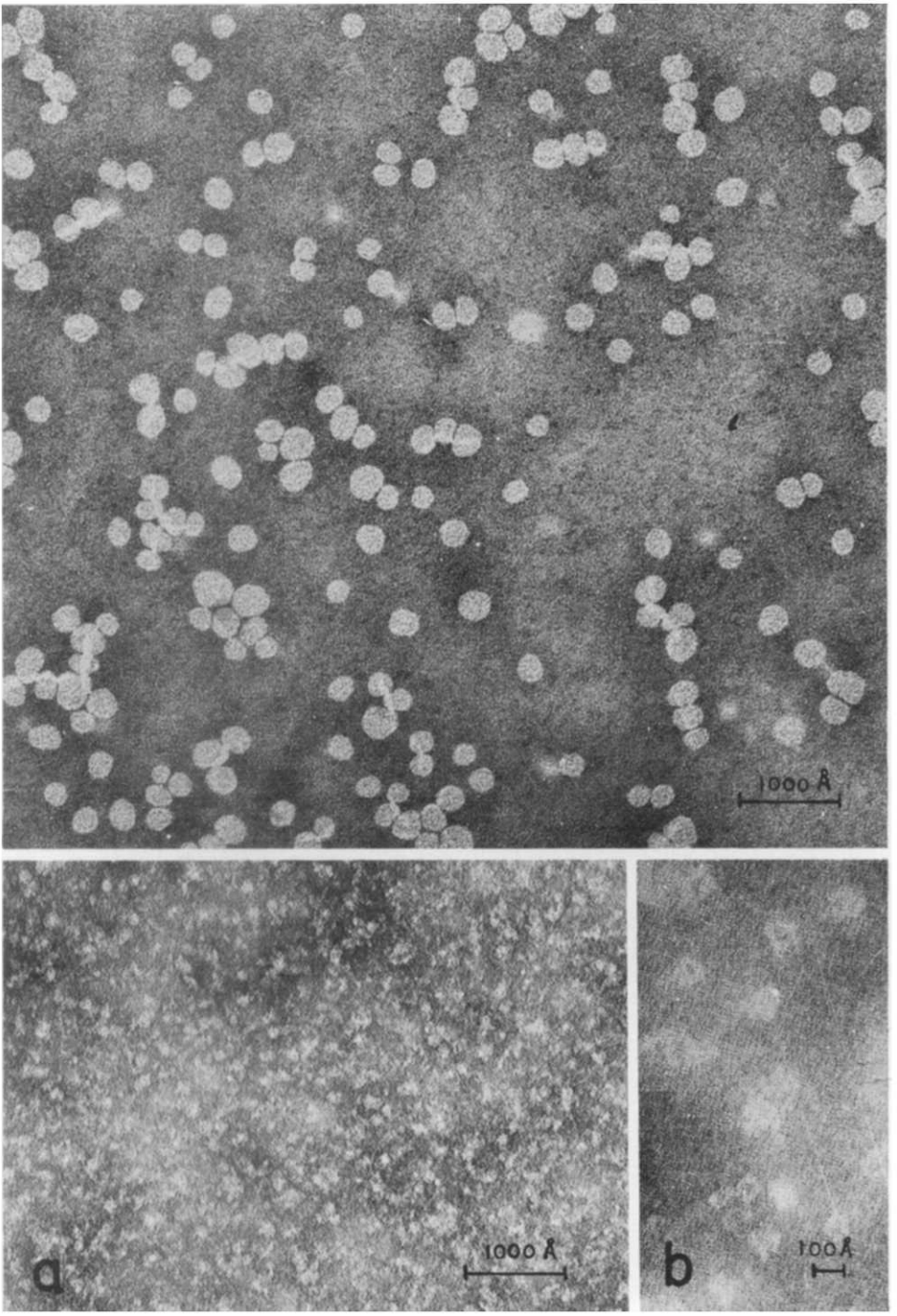
the Sepharose 4B column, measured in a spectrophotometer flow cell at 300 nm, consisted of two well resolved fractions. The size homogeneity of the vesicles taken from the internal volume fraction is demonstrated in Fig. 2. Large numbers of these negatively stained phospholipid vesicles were measured in order to determine the size distribution. The mean value obtained for the vesicle diameter was 268 ± 8 Å (S.E.), in satisfactory agreement with the early estimate of 300 ± 30 Å reported by Huang for this system [16].

Fig. 3a illustrates a representative field of the purified ATPase stained with ammonium molybdate. At higher magnifications (Fig. 3b) the subunit structure of the enzyme is discernible. The staining of the enzyme in these preparations was too indistinct for precise measurement of the molecular dimensions, but the indications were that the longest dimension of the macromolecule was 120 Å, in agreement with the value found by Schnebli et al. [21].

Addition of the soluble ATPase to the homogeneous vesicle preparation resulted in the formation of large vesicles, ranging in diameter up to approximately 900 Å. Fig. 4 is a view of the vesicle–ATPase mixture, stained and examined as close to zero incubation time as was practical. Some of the larger vesicles appear to be negatively stained on the inside, in contrast to the homogeneous small vesicles which exclude the stain in these preparations. The unusual structure shown in the insert may be a large vesicle in the process of fusing with one of the smaller

Fig. 2. Electron micrograph of phosphatidylcholine vesicles, obtained by Sepharose 4B chromatography of sonicated liposomes, negatively stained with 1% potassium phosphotungstate (× 141000).

Fig. 3. a. Electron micrograph of the *S. fecalis* membrane ATPase solution, negatively stained with 1% ammonium molybdate (× 141000). b. The enzyme preparation stained with phosphotungstate at a higher magnification (× 455000) to demonstrate the individual molecules of ATPase (cf. ref. 21).


1000 Å
a
1000 Å
b
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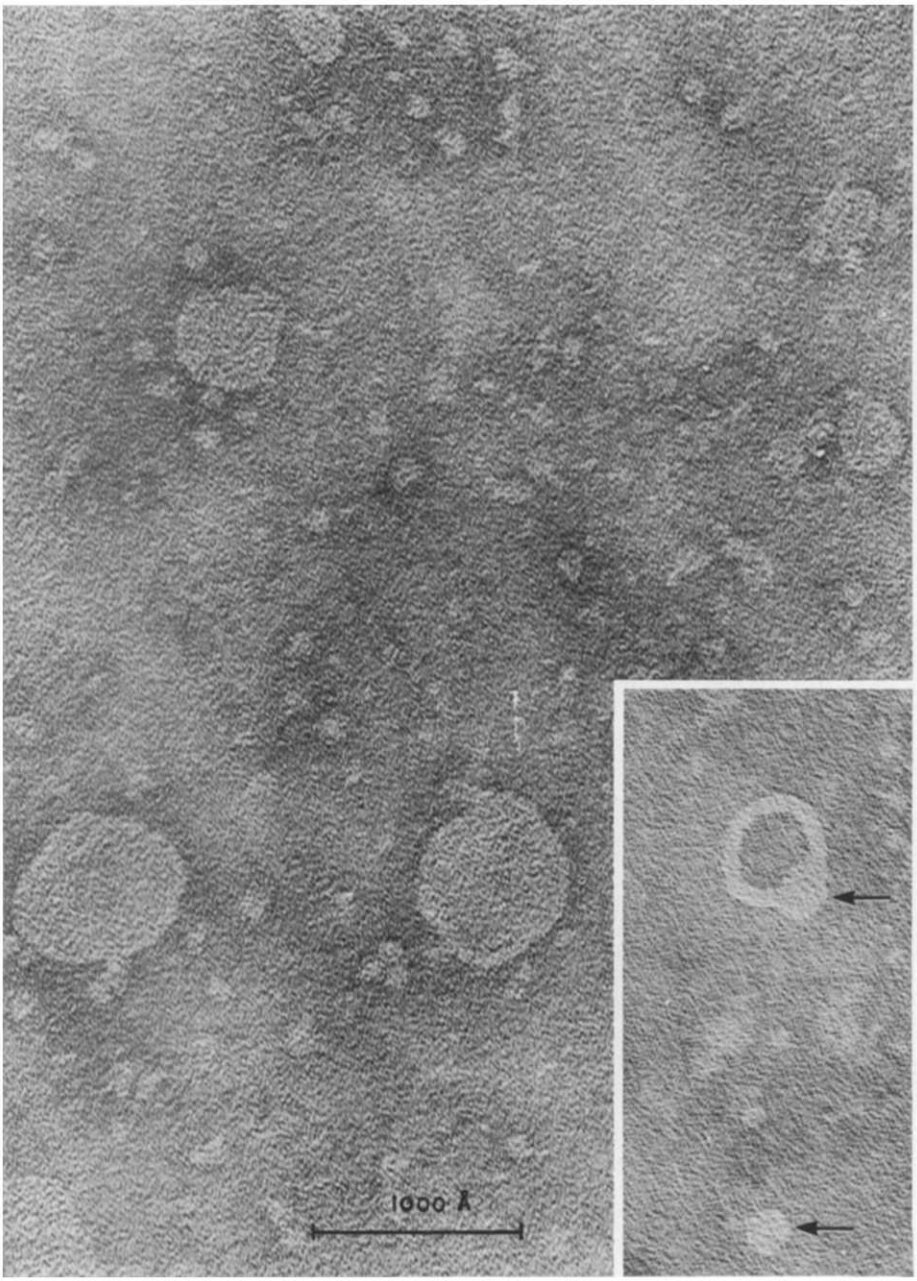

Fig. 4. A mixture of enzyme and vesicles was fixed with ammonium molybdate within a few seconds of mixing. Against a background of enzyme molecules several of the original vesicles can be seen at upper right and several larger ones at lower left. (X 273 000). Insert (at same magnification): the arrows indicate two vesicles, one of which may be fusing with a larger vesicle that the negative stain has penetrated.

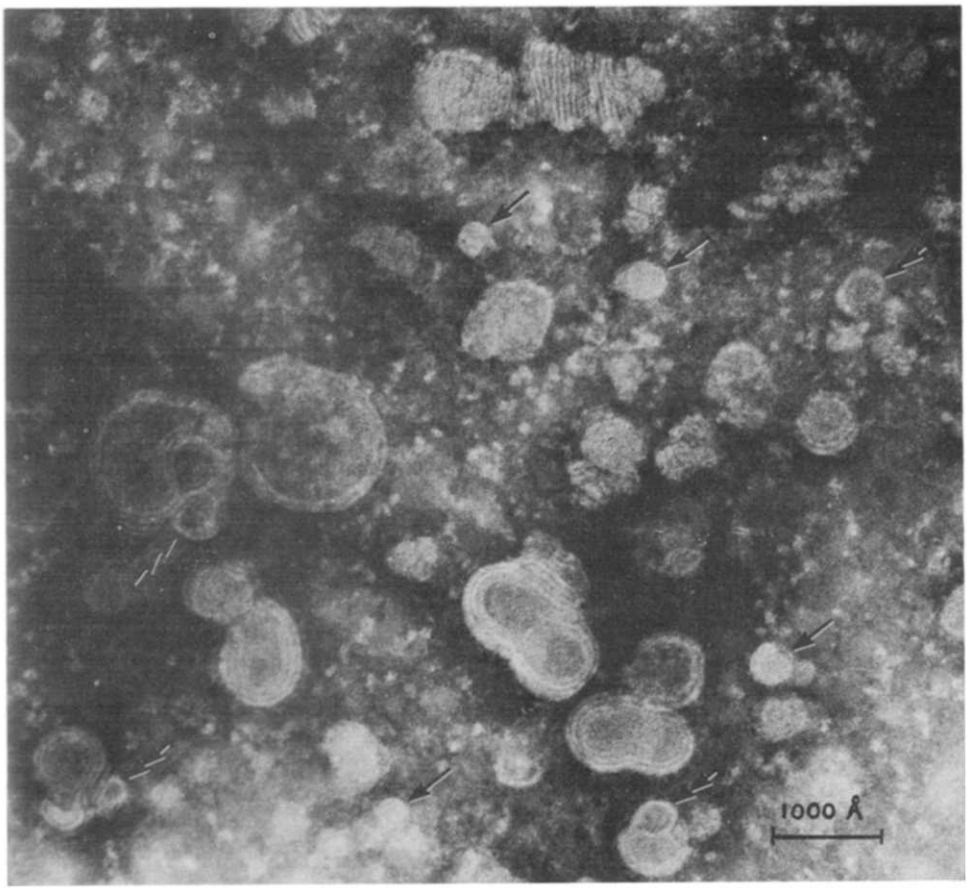

Fig. 5. A mixture of vesicle and enzyme incubated for 100 min at 4 °C shows some original vesicles (at arrows) and some vesicles penetrated by the phosphotungstate negative stain (at double-headed arrows). Several of these latter structures are seen to be associated with multilamellar figures. (× 141 000).

vesicles. Only a few seconds were allowed for the vesicle and ATPase solutions to mix in this preparation, nevertheless, these large vesicles were observed in many areas of the e.m. field. At substantially longer incubation times of the vesicle–ATPase mixtures, the electron micrographs showed greater numbers of these larger vesicles and progressively fewer smaller vesicles. Fig. 5 is a representative view of the mixture after 100 min incubation at 4 °C. The presence of the negative stain on the inside of these large vesicles reveals that in some cases the boundary membrane consists of several bilayers. In fact, there was some evidence of multilamellar structure in the vesicle–ATPase mixtures, more so in the preparations allowed to incubate for several hours. This was in contrast to the homogeneous vesicle preparation, stored under the same conditions, in which no multilamellar material was found. In order to compare the thickness of these multilamellar regions with myelin forms which did not contain the ATPase, samples of the phosphatidyl–

choline taken from the void volume fraction of the Sepharose 4B column were prepared for electron microscopic examination.

Typical high magnification pictures of the multilamellar regions of the phospholipid dispersions in the presence and absence of ATPase are shown in Figs 6a and 6b, respectively. The mottled appearance of the multilamellar structure seen in Fig. 6a may represent an array of ATPase molecules bound to the surface membranes. Several of the dark circles are approximately 40 Å in diameter, which is the estimated size of the hole in the center of the enzyme macromolecule [21].

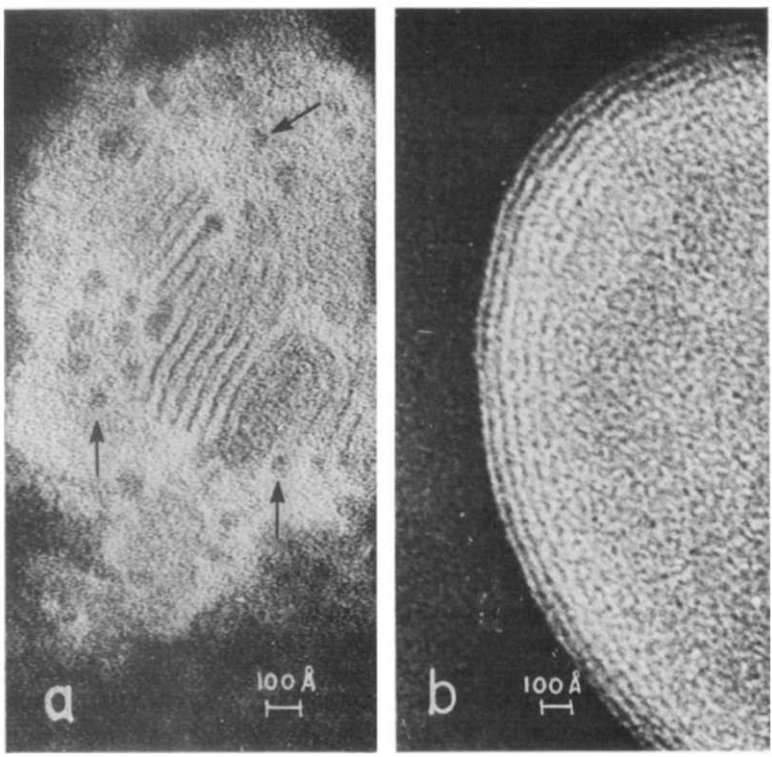

Fig. 6. Higher magnification micrographs of phosphotungstate-stained preparations. ($\times$ 405000). a. A vesicle–ATPase mixture, as in Fig. 5. Arrows indicate dark structures on the surfaces of the multilamellar figure which are suggestive of the 40 Å core of the enzyme macromolecule. b. A multilamellar figure from the excluded volume of the Sepharose 4B column preparation of phosphatidylcholine vesicles. No ATPase is present.

The periodicity of the densitometric tracings of these multilamellar phospholipid–ATPase structures based on 50 observations, was 54.4 ± 0.48 A^0 (S.E.) compared with 44.4 ± 0.44 A^0 (S.E.) for the void volume material (no ATPase present). Although it did appear that the increased spacing in the presence of the enzyme represented increases in the thickness of both the positively and negatively stained areas, the densitometric tracings were not sharp enough to clearly define the demarcations of these regions.

Proton magnetic resonance spectra of vesicle–ATPase mixtures

The high resolution PMR spectrum obtained with the homogeneous phosphatidylcholine vesicle preparation was essentially identical to that previously reported by Penkett et al. [22]. On addition of low concentrations of the soluble ATPase, however, the high resolution signals became broadened. Fig. 7 illustrates the increase in the line width ($\Delta\nu_{1/2}$) of the $N^+(CH_3)_3$ choline signals in the presence

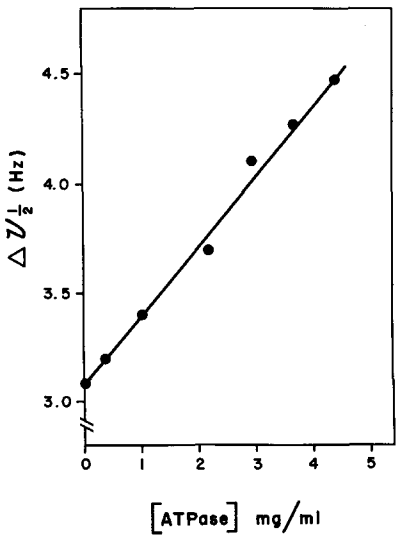

Fig. 7. The dependence of the line width of the high resolution PMR signal of the $N^+(CH_3)_3$ protons from sonicated phosphatidylcholine vesicles in the presence of the soluble ATPase.

of the enzyme. A similar line broadening was observed for the hydrocarbon chain protons, but this effect was more difficult to quantitate since the signal integral is broad and complex. Only a small rise in the bulk viscosity occurred when the ATPase was added to the vesicle suspension in ^2H_2O. The magnitude of the viscosity increase, which was linear with the protein concentration was 14% per mg/ml of ATPase. Recently, Horowitz et al. [23] have shown that the line widths of sonicated phosphatidylcholine vesicles are independent of viscosity over a 5-fold range of the bulk viscosity. The line broadening observed in the vesicle–ATPase mixture is, therefore, not attributable to viscosity effects. In addition to the line broadening, there was a concomitant decrease in the PMR signal intensities in the presence of ATPase. A comparison of the decrease in peak heights of the alkyl chain proton signals with the $N^+(CH_3)_3$ signals is presented in Table II. The peak height measurement of the (CH_2) signal integral is subject to an uncertainty in the baseline determination due to the broad-line contribution of those protons not giving a high resolution spectrum. Nevertheless, the values obtained for the percentage decrease in the peak height are not significantly different from the $N^+(CH_3)_3$ values (Table II). The addition of 2 mM $MnCl_2$ to the sonicated vesicle preparation caused the high resolution signal of the $N^+(CH_3)_3$ protons to decrease to 30% of its original peak height. This result is in agreement with the finding of

TABLE II

EFFECT OF THE SOLUBLE ATPase ON THE 60-MHz PMR SPECTRA OF SONICATED PHOSPHATIDYLCHOLINE VESICLES

The soluble ATPase was added in the concentrations shown to 0.3 ml 5% sonicated phosphatidylcholine vesicles in ^2H_2O solution containing 0.1 M KCl and 0.02 M $MgCl_2$ at p^{2}H 6.4. The continuous wave PMR spectra were recorded at 40 °C and the peak heights corrected for dilution.

ATPase concentration (mg/ml)	Molar ratio of phosphatidylcholine/ATPase	Decrease in peak height (%)		
		$(CH_2)_n$	$N^+(CH_3)_3$	$N^+(CH_3)_3$*
0.37	66 000	2.7	2.5	10.7
2.16	9 400	11.2	9.6	–
3.06	6 000	23.4	25.2	36.9
4.56	3 300	29.0	34.9	61.5

* 2 mM $MnCl_2$ present.

Finer et al. [24]. The subsequent addition of the soluble ATPase to this system led to a further decrease in the peak height of the remaining $N^+(CH_3)_3$ signal. Furthermore, the decrease in this signal, which is thought to arise from the choline moieties on the inner surfaces of the vesicles [24], was significantly larger for a given ATPase concentration than the relative decrease of the choline signal in the vesicle–ATPase solutions without $MnCl_2$ (Table II). The addition of the ATPase to the vesicle solutions in the PMR tubes caused a slight increase in the light scattering of the dispersions, but no particulate material separated when the tubes were allowed to stand for several hours.

DISCUSSION

The formation of larger vesicles by the interaction of the soluble ATPase with an initially homogeneous population of small vesicles may qualitatively explain the observed line broadening and decrease in signal intensity of the high resolution PMR spectrum of the phosphatidylcholine vesicles. Sheetz and Chan [25] have employed PMR spectroscopy to study the effect of sonication on phosphatidylcholine bilayers. These authors separated small vesicles (approx. 300 Å diameter) from large vesicles (approx. 900 Å diameter) by Sepharose 4B chromatography and examined the PMR spectra of both systems. On the basis of their findings, they argued that the observed differences in the PMR spectra of vesicles with different curvatures were due to variations in the molecular packing of the phospholipid molecules in the bilayer phase [25]. The line broadening observed in the vesicle–ATPase mixture might, therefore, be due to a tighter packing arrangement of the constituent phospholipid molecules in the larger vesicles. The observed conversion of some of the phospholipid vesicles into multilamellar forms would certainly be expected to cause line broadening and a concomitant decrease in the peak heights

of the high resolution PMR signals [22]. Although the ATPase caused the changes in the vesicle size, the extent to which lipid–protein interactions in the mixture affected the PMR spectra of the phospholipid is unclear. Due to the high molar ratios of phospholipid to ATPase in the interactant mixtures (Table II), only a small proportion of the phospholipid molecules could interact directly with the enzyme. Thus, it is not known whether lipid–protein interactions contributed directly to the immobilization of the phospholipid molecules in the larger vesicles. It should be noted that the spectral changes reported here are qualitatively similar to the PMR data for the interaction of phosphatidylcholine vesicles with alamethicin. where it was also found that an increase in particle size occurred during the vesicle–polypeptide interaction [26].

Sonicated phosphatidylcholine vesicles do not spontaneously fuse to form larger vesicles [16] yet they appear to be inherently unstable in the presence of the ATPase. Generation of the larger vesicles could conceivably be preceded by complete disruption of the smaller vesicles by the ATPase, followed by reaggregation. This process would account for the apparent penetration of Mn^{2+} into the phospholipid vesicles as indicated by the PMR data, and also explain the negative staining of the interiors of the large vesicles. However, preliminary permeability experiments suggest that this is unlikely to occur. The homogeneous vesicles were formed in the presence of [^{3}H]sucrose in 0.02 M Tris–0.01 M $MgCl_2$ buffer (pH 7.5), and completely separated from external tracer by gel filtration on Sephadex G 25. No significant leakage of the trapped [^{3}H]sucrose could be detected when the vesicles were dialyzed against the Tris–$MgCl_2$ buffer (pH 7.5) for several days, but the addition of the soluble ATPase to these vesicles did cause a slow release of the tracer which was linear with time. This leakage rate was less than 0.1% of the trapped [^{3}H]sucrose per hour, but was independent of the amount of ATPase added. In contrast, the addition of 1% Triton X100 to the vesicles caused an immediate release of the radioactive sucrose. The effect of ATP on the vesicle–ATPase system was of particular interest in the light of the bilayer electrical data [14]. It was found that the addition of ATP to the Tris–$MgCl_2$ buffer (pH 7.5) reversibly stimulated the release of the [^{3}H]sucrose from the vesicles in the presence of the ATPase, but had little effect in the absence of the enzyme. The enhanced leakage of [^{3}H]sucrose was linear with time, and roughly proportional to both the ATPase and ATP concentrations. At a phospholipid to ATPase molar ratio of approximately 6000:1, with 5 mM ATP in Tris–$MgCl_2$ (pH 7.5) at 4 °C the [^{3}H]sucrose leakage rate was 3% per h, approximately 30-fold larger than the control without the enzyme. The fact that the low level diffusion of the trapped sucrose was linear within the time studied and that there was a stimulation of efflux rate by ATP indicates that the interaction of the ATPase with the phospholipid vesicles is quite specific and probably does not simply involve the breakdown of the lipid vesicles. Fusion of the phospholipid vesicles to form larger membranous structures, without the significant release of trapped sucrose is an interesting phenomenon requiring further study. The data available indicate that the ATPase combines with the vesicles to form larger membrane vesicles which are more permeable to solute molecules. It is tempting to speculate that the ATPase interacts with the vesicles to form large conducting channels in the membranes similar to those found in the planar bilayer ATPase interactant system. In this context, it is significant that Mg^{2+} stabilizes

the liposome–ATPase complex and is also required to elicit the increase in membrane conductance in the planar bilayer–ATPase system [14].

Schnebli et al. [21] have proposed a model for the ATPase, which consists of a hexagonal array of globular subunits, each 40 Å in diameter, with a length of 120 Å for the long axis of the hexagon. Clearly, geometric factors could hinder the interaction of the ATPase with the phospholipid molecules in the relatively small homogeneous vesicles unless these membrane structures were capable of major distortion and fusion to form larger structures. Hydrophobic interactions between the ATPase molecules and the phosphatidylcholine molecules could well be the driving force behind the formation of larger vesicles and the ultimate formation of the lowest energy configurations, namely, the multilamellar structures. This action of the membrane ATPase is contrary to the effect of the lytic polypeptide, mellitin which appears to disrupt phospholipid multilamellae and generate fragmented structures [27]. Although micrographs of the negatively stained preparations are difficult to interpret in molecular terms, the increase in the lamellar spacings seen in the phospholipid–ATPase mixtures does suggest that the enzyme is in some way incorporated into the membrane structures.

In conclusion, it may be stated that we have demonstrated that an interaction occurs between phosphatidylcholine bilayers in aqueous dispersions and the soluble ATPase derived from *S. fecalis* membranes. On the basis of the evidence, it would appear probable that a bilayer–ATPase complex should exist in the planar bilayer system previously studied [14]. Accordingly, the electrical changes observed in the membrane upon the addition of the ATPase, reflected the properties of this complex. That the membrane ATPase is strongly implicated in the active transport of cations and other solutes across the plasma membrane of the intact organism is well documented [28, 29]. The *S. fecalis* membrane ATPase [21] has properties that are remarkably similar to the properties of the membrane ATPase of beef heart mitochondria [30]. Since the ultimate significance of our work is related to the synthesis of membrane pumps, it is necessary to recognize that Racker has shown that a large number of protein F fractions are needed in addition to the ATPase to reconstitute oxidative phosphorylation and ion pumping [31, 32]. Baron and Abrams [33] have in fact isolated a small molecular weight protein, termed nectin, which is required for the attachment of the membrane ATPase, to enzyme-depleted membranes. Future work with this membrane-derived ATPase will be directed towards reconstitution of the biological activity in model membrane systems, and will employ mixtures of the bacterial lipids and appropriate coupling factors, including nectin. The present work represents an initial step in this direction.

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