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BINDING OF A SOLUBILIZED MEMBRANE ATPase TO PHOSPHOLIPID BILAYERS

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SUMMARY

Interaction of purified *Streptococcus fecalis* membrane ATPase with lipid bilayer membranes was investigated using aqueous dispersions of phospholipids. A liposome–ATPase complex was demonstrated by gel filtration analysis. Binding of the solubilized ATPase to liposomes was studied by differential ultracentrifugation and the determination of binding parameters was based on the measurement of ATPase activity in the liposome–ATPase complex and the concentration of free ATPase in equilibrium with the complex. ATPase combined in phosphatidylcholine liposomes was essentially as active as the solubilized enzyme, and the binding of the ATPase to these liposomes was not reduced by increasing the ionic strength. The ratio of phosphatidylcholine to ATPase in the liposome complex was approximately 11/1 (w/w). The intrinsic association constant for the interaction, determined from Scatchard plots was $7.4 \cdot 10^7 \pm 1.4 \cdot 10^7\ M^{-1}$.

Phosphatidylcholine liposomes containing stearylamine or cardiolipin were prepared to investigate the effects of surface charge on binding. Stearylamine increased the ATPase binding at low ionic strength. In contrast, cardiolipin did not appear to change the binding characteristics. Phosphatidylethanolamine also had little effect on the ATPase binding. Cholesterol incorporated into phosphatidylcholine liposomes lowered ATPase binding. ATPase binding to synthetic dimyristoyl phosphatidylcholine liposomes was lower than with natural phosphatidylcholine liposomes at 4 °C but not significantly different at 30 °C, above the gel-liquid crystalline phase-transition temperature.

INTRODUCTION

Characterization of lipid–protein interactions at the membrane level is essential to the understanding of the structure and function of biological membranes. Several recent reviews on biomembranes have dealt with this problem, and considerable progress has been made in investigating the role played by lipid–protein interactions in membrane functions [1–5]. The complex composition and heterogeneity of membrane preparations, however, has hindered the elucidation of the nature of

these forces in biological membranes. An alternative approach has involved the study of lipid–protein interactions in model membrane systems. Each of the lipid membrane systems, the monolayers, bilayers, and liposomes has been used in this type of investigation [6–8]. The underlying structural component common to many biological membranes may be a phospholipid bilayer, albeit discontinuous, containing globular proteins [9]. Phospholipid lamellar systems may, therefore, provide the more appropriate model systems with which to study lipid–protein interactions relevant to biological membranes. Many of the earlier studies with these systems have involved nonmembrane proteins or polypeptides, some of which, like lysozyme, ribonuclease and polylysine are highly basic [10–12]. The data from these studies are often explainable in terms of initial electrostatic interactions between protein and phospholipid, followed by other types of interaction leading to changes in the system properties, such as membrane permeability. Recently, attention has focussed on membrane derived proteins [13–16], including the physiologically interesting ATPase of mammalian membranes which have been obtained associated with lipid by detergent extraction [17–20]. The membrane-derived protein we have chosen to study is the Mg^{2+}-activated membrane ATPase of *Streptococcus fecalis*. This solubilized, lipid-free enzyme has been highly purified and shown to interact with planar lipid bilayers and sonicated phospholipid vesicles [21–24]. In this paper, we present a detailed description of the interaction of the purified ATPase with phospholipid liposomes including the influence of surface change, cholesterol and acyl chain fluidity on the ATPase binding.

MATERIALS AND METHODS

The buffer solution employed for the binding studies was 0.02 M Tris and 0.01 M $MgCl_2$ titrated to pH 7.5 with HCl. ATP was obtained from Sigma, Tris and $(NH_4)_2SO_4$ were special enzyme grade from Mann Research Laboratories, [^{3}H]acetic anhydride (50 Ci/mole) was purchased from New England Nuclear, and all other chemicals were analytical reagent grade.

Preparation of the solubilized ATPase

The soluble ATPase was extracted from *S. fecalis* membrane ghosts and partially purified by a heat treatment step, followed by column chromatography on DEAE-cellulose (Whatman 32) according to the procedure of Schnebli and Abrams [25]. Further purification was achieved by molecular sieve chromatography on Agarose A 1.5 m (BioRad) in 0.02 M Tris and 0.01 M $MgCl_2$ at pH 7.5, as reported earlier [22]. The specific activity of the peak fractions was approximately 30 units/mg. ATPase activity was measured by the release of inorganic phosphate from Mg^{2+}-ATP at 2/3 substrate saturation using the method of Abrams [26]. One unit of ATPase activity is defined as that amount of enzyme which catalyzes the release of 1 μmole of inorganic phosphate from ATP per min, at a Mg^{2+}-ATP concentration of 5 mM at 38 °C. Inorganic phosphate was measured by the method of Bartlett [27], and protein concentration determined by the method of Lowry et al. [28]. Radio-labeled ATPase was prepared with [^{3}H]acetic anhydride using a modification of the acetylation method described by Riordan and Vallee [29]. The radioactivity (cpm) of the ATPase was measured in a liquid scintillation counter and found to be $3 \cdot 10^3$ cpm/unit of ATPase after extensive dialysis to completely remove the free [^{3}H]acetic anhydride.

Preparation of lipids

Phosphatidylcholine and phosphatidylethanolamine were isolated from fresh egg yolks. The crude mixture of phospholipids obtained by solvent extraction with chloroform–methanol (2 : 1, v/v) [30] was partially purified by three successive acetone precipitations from petroleum ether [31]. Column chromatography on silicic acid (Unisil, 200–235 mesh, Clarkson Chemical Co.) was used to separate the phospholipids, which were eluted by increasing the proportion of methanol in the chloroform–methanol solvent. The purified phospholipids were stored under acetone at −65 °C. Synthetic dimyristoyl phosphatidylcholine was purchased along with purified cholesterol from Sigma. Stearylamine was obtained as the hydrochloride from Eastman Organic Chemicals, and cardiolipin was purchased from Applied Science Laboratories. Thin layer chromatography on silica gel G was employed to check purity. Chloroform–methanol–water (65 : 25 : 4, v/v/v) was used to develop the plates, and the lipids were stained with I_2 vapors. The phospholipids, cholesterol, and stearylamine appeared to be pure, but the cardiolipin contained a trace of phosphatidylcholine.

Formation of liposomes

Aliquots of the purified phosphatidylcholine were lyophilized from recrystallized benzene and suspended in cold Tris–$MgCl_2$ buffer at a concentration of 2.5 % by weight. The solution was flushed with pure argon, then mixed mechanically for 10 min to generate liposomes. Incorporation of phosphatidylethanolamine, cholesterol, stearylamine or cardiolipin into phosphatidylcholine liposomes was accomplished by lyophilizing homogeneous solutions of the mixed lipids, followed by mechanical dispersion in the Tris–$MgCl_2$ buffer as above. The liposomes generated by mechanical agitation could be completely separated from solution by centrifugation at 100 000 × *g* for 30 min as determined by phospholipid analysis on the supernatants [31, 32] and, therefore, provided a convenient lipid matrix for the study of protein binding. Liposomes intended for use in the gel filtration studies were subjected to ultrasonic irradiation for 10 min to reduce the particle size heterogeneity. Ultrasonication was carried out under argon in a jacketed vessel maintained at 4 °C by a circulating water bath.

Formation and analysis of liposome–ATPase complexes

Mixtures of the ATPase and liposomes used in the column chromatography studies were prepared by adding the soluble ATPase to the ultrasonicated liposomes and the interactant mixtures were incubated at 4 °C for 1 h prior to the chromatography.

A standard procedure was adopted for the binding studies, involving the differential ultracentrifugation of preincubated mixtures of the liposomes and soluble ATPase. Aliquots of the freshly prepared phospholipid dispersion were added to the Tris–$MgCl_2$, pH 7.5, buffer solution contained in 10 ml polycarbonate centrifuge tubes. The soluble ATPase was added by means of a microliter syringe and the volume of buffer in each tube varied in order to maintain a constant total volume in the interactant lipid–proteins mixtures and controls. The mixtures were gently swirled for 30 s, incubated at 4 °C for 1 h, then centrifuged at 100 000 × *g* for 30 min. After centrifugation, 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 duplicate samples from both the supernatant and pellet fractions analyzed for ATPase activity using appropriate blanks for each set. It was not found to be necessary to wash the pellets free of supernatant, since they were very small, containing usually 0.3 mg lipid. The volume of the trapped solution in the pellets was measured by equilibration with [^{3}H]sucrose and found to be only 1 % of the initial volume of the interactant mixtures. The incubation step was included because it was found that up to 50 % more ATPase became bound to the liposomes following 1 h incubation compared with the liposome–ATPase samples which were centrifuged immediately after mixing. However, no significant increase in binding occurred when incubation periods longer than 1 h were used. There was no observable decrease in the soluble ATPase concentration upon centrifugation of the enzyme solutions that did not contain the phospholipid liposomes. All liposome solutions were freshly prepared for each binding experiment, and to reduce lipid oxidation, argon was used to displace oxygen from the solutions wherever possible.

In order to determine whether the ATPase activity was influenced by the phospholipid liposomes, the following experiment was conducted. A series of samples containing a constant amount (1 μg) of soluble ATPase and increasing quantities of phospholipid (0–1 mg) were assayed directly for enzymatic activity. Mg^{2+}-ATP (5 mM) in 0.1 M Tris–HCl buffer, pH 7.5, was added to the mixtures in a final volume of 2 ml, the solutions incubated at 38 °C for 20 min, then the reaction stopped by the addition of 2 ml of 5 % $HClO_4$. The precipitate was removed by centrifugation and the ATPase activity determined by the measurement of the inorganic phosphate in the supernatants [26]. All ATPase determinations were carried out in the linear range of the assay, where product inhibition was negligible [25, 26].

RESULTS

Gel filtration analysis

Fig. 1 illustrates the elution profiles from an Agarose column of the ^{3}H-labeled soluble ATPase, and a mixture of ^{3}H-labeled ATPase and ultrasonicated liposomes which had been preincubated for 1 h at 4 °C. Also shown in Fig. 1 is the elution profile of the ultrasonicated liposomes alone, as measured by their absorbance at 240 nm. The exclusion limit of the gel used in these studies was approximately 1.5 $\times 10^6$ for globular proteins (Agarose A 1.5 m, BioRad Laboratories). As expected, the ultrasonicated liposomes eluted from the column in the void volume fraction, while the peak of the radioactivity for the soluble ATPase eluted within the internal volume of the gel. An additional peak of radioactivity appeared in the void volume fraction when the liposome–ATPase mixtures were chromatographed (see Fig. 1). The elution profile of this leading fraction of radioactivity closely resembled the absorbance profile of the liposomes. This observation clearly demonstrated the existence of a liposome–ATPase complex. Similar studies were carried out with unlabeled ATPase for gel filtration analysis, by monitoring ATPase enzymatic activity in the column fractions. It was found that the ATPase eluted in the internal volume of the column, with no enzymatic activity detectable in the void volume. In the liposome–ATPase mixtures a significant proportion of the ATPase was found to be associated with the liposome fractions. The ATPase profile in the void volume was slightly retarded with

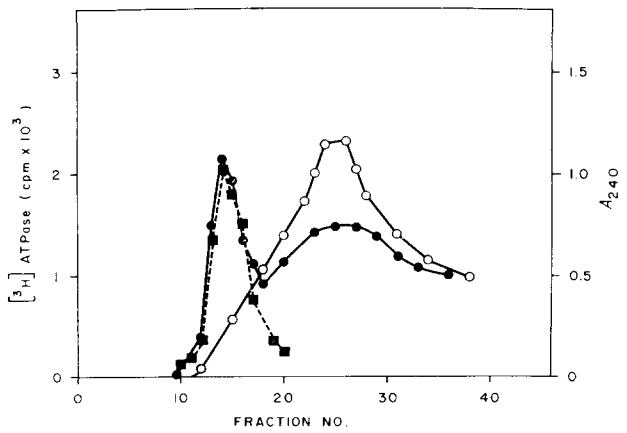


Fig. 1. Gel filtration analysis of a liposome–ATPase mixture. 10 units of [^{3}H]ATPase were incubated with 2.8 mg of phosphatidylcholine liposomes for 1 h at 4 °C, then chromatographed on Agarose A 1.5 m at 4 °C in Tris–$MgCl_2$, pH 7.5. ● (closed circles) radioactivity profile of the liposome–ATPase mixture; ○ (open circles) radioactivity profile of the [^{3}H]ATPase alone; ■ (closed squares) for the elution profile of the liposomes alone measured by their absorption at 240 nm.

respect to the absorbance profile of the liposome–ATPase mixture, which suggested that the binding of the ATPase was a reversible process.

Effect of liposomes on the ATPase activity

Addition of pure phosphatidylcholine in the form of multilamellar liposomes to the soluble ATPase neither activated nor significantly inhibited the enzyme activity, see Fig. 2. It is known from the present studies that the ATPase binds to the liposomes; therefore, the activity of the membrane-bound ATPase must be similar to that of the soluble ATPase. In contrast, a marked inhibition of the ATPase activity was observed in the presence of phosphatidylcholine liposomes containing stearylamine (Fig. 2). The pH dependence of the soluble ATPase is illustrated in Fig. 3, and is similar to that reported by Abrams [26]. In the presence of pure phosphatidylcholine liposomes, there was no significant change in the enzyme activity over the pH range studied. However, the incorporation of 10 % by weight stearylamine into the phosphatidylcholine liposomes depressed the ATPase activity over the same pH range. Furthermore, the maximum in the enzyme activity was less well defined and appeared to be shifted to lower pH. The pH at the surface of the positively charged liposomes should be greater than in the bulk solution [33, 34], which could qualitatively account for the observed shift of the pH maximum for the ATPase activity in the stearylamine–phosphatidylcholine liposomes–ATPase mixture.

Binding of the ATPase to pure phosphatidylcholine liposomes

Figs 4a and 4b illustrate the correlation between the concentration of the soluble ATPase in the interactant mixture and the amount of enzyme which sedimented as the liposome–ATPase complex upon centrifugation. It is evident that the phosphatidylcholine liposomes bind the ATPase and become saturated with the ATPase at relatively low concentrations of the soluble ATPase, namely, at less than 5 units/ml.

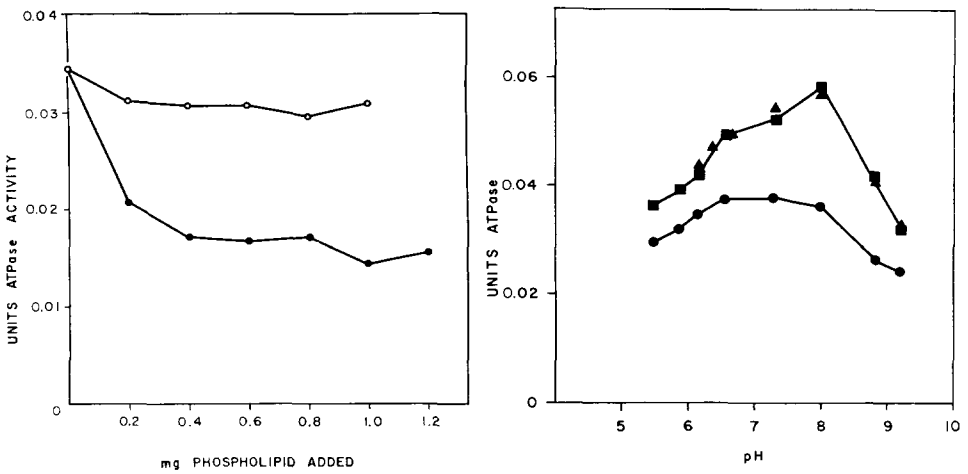
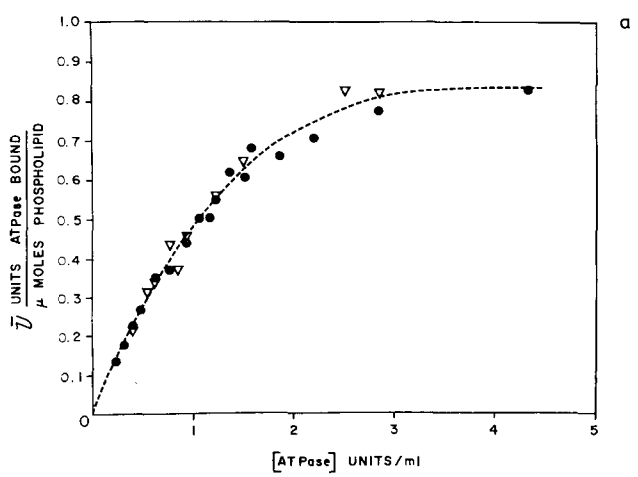


Fig. 2. Influence of neutral and positively charged liposomes on ATPase activity. ○ (open circles) phosphatidylcholine liposomes; ● (closed circles) phosphatidylcholine liposomes containing 10 % (w/w) stearylamine. Each assay tube contained 1 μg ATPase in Tris-$MgCl_2$ buffer at pH 7.5.

Fig. 3. pH dependence of the solubilized ATPase alone, and in the presence of neutral and positively charged liposomes. ▲, 2 μg solubilized ATPase; ■, 2 μg solubilized ATPase and 0.38 mg phosphatidylcholine liposomes; ●, 2 μg solubilized ATPase and 0.38 mg phosphatidylcholine liposomes containing 10 % (w/w) stearylamine.

It appears from Figs 4a and 4b that there is little effect of ionic strength on the binding of the ATPase to the electrically neutral bilayers. However, the binding data replotted in a Scatchard diagram [35] in Fig. 5 showed that there was a systematic difference between the two ionic strengths. According to the general theory of multiple binding of ligands to macromolecules, with no interaction between sites, it is possible to interpret the slope of the linear Scatchard plot, $\bar{\nu}/c = k(n-\bar{\nu})$, as the apparent intrinsic association constant, k, for the binding reaction and the intercept on the $\bar{\nu}$ axis as the



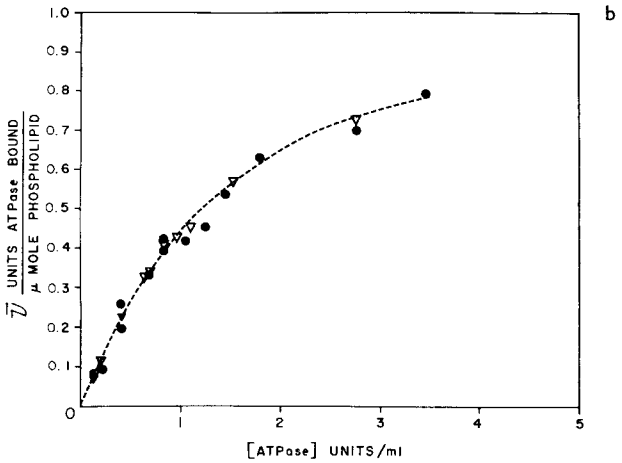


Fig. 4. ATPase-binding curves for phosphatidylcholine liposomes, as measured by differential ultracentrifugation of the liposomes–ATPase mixture. Each of the data points represents the mean of four separate determinations in which the experimental variation was within 10 %. The closed circles represent data obtained directly from ATPase assays of the pellet fractions, while the open symbols represent the calculated values based on the supernatant assays, assuming 100 % activity for the ATPase associate with the liposomes. The binding studies were carried out at 4 °C, pH 7.5, in 0.02 M Tris–0.01 M $MgCl_2$ for a, and in 0.02 M Tris–0.01 M $MgCl_2$ containing 0.1 M KCl for b.

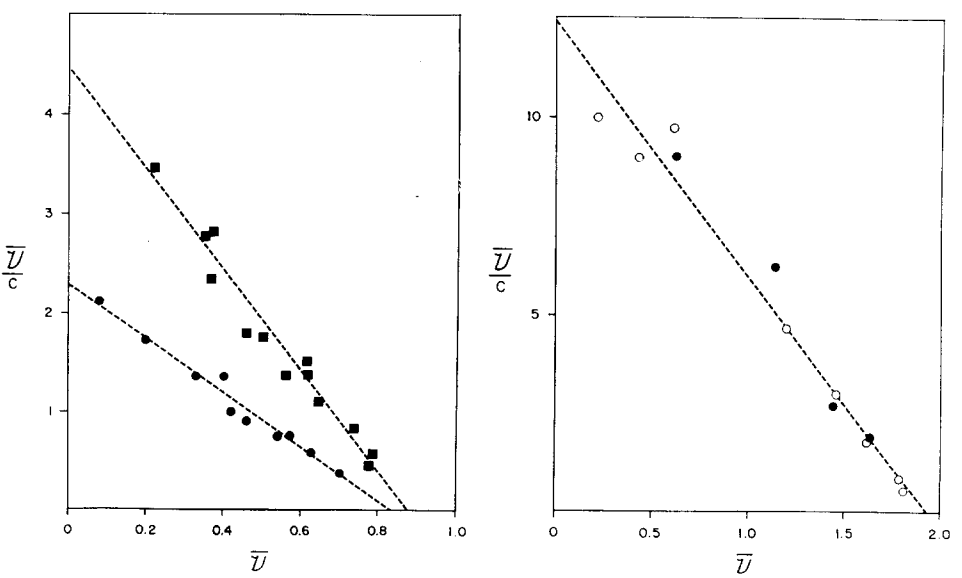


Fig. 5. Scatchard plots of binding of solubilized ATPase to phosphatidylcholine liposomes at 4 °C, pH 7.5, in 0.02 M Tris–0.01 M $MgCl_2$ (■) and 0.02 M Tris–0.01 M $MgCl_2$ containing 0.1 m KCl (●). Specific activity of ATPase was 20 units/mg.

Fig. 6. Scatchard plot of binding of solubilized ATPase to phosphatidylcholine liposomes at 4 °C, pH 7.5, in 0.02 M Tris–0.01 M $MgCl_2$. Specific activity of ATPase was 30 units/mg. ○ (open symbols) represent the binding data, determined from the supernatant fractions obtained by centrifugation of liposome–ATPase mixtures; ● (closed symbols) were determined from the supernatant fractions obtained by recentrifugation of the resuspended pellets from first binding study.

maximum number of ligand binding sites (n) on the macromolecule [36]. c is the concentration of free ligand and $\bar{\nu}$ the amount of ligand bound per mole of binding macromolecule, in this case, the phospholipid liposomes. Lines drawn through the data in Fig. 5 represent the least squares fit based on a linear regression analysis. The values of the slopes and intercepts on the $\bar{\nu}$ axis, listed in Table I, are expressed in molar quantities, assuming a molecular weight of 385 000 for the ATPase [25] and the value of 20 units/mg for the specific activity of the ATPase, determined for the preparation at the time of the binding experiments. Although there is a lower value of the slope, k, for the system containing 0.1 M KCl, the intercepts on the $\bar{\nu}$ axis are not significantly different for the two systems. The binding experiments were repeated with a freshly purified ATPase preparation, which had a specific activity of 30 units/mg. The Scatchard plot for this study is illustrated in Fig. 6. The data presented in the diagram were obtained from two successive binding experiments. The first experiment was carried out in the normal way, as described under Materials and Methods. The pellet fractions from this experiment were resuspended in fresh buffer, aliquots taken for ATPase analysis, and the remaining portion of the suspension was used as the starting mixture for the second experiment. The resuspended pellets were incubated for 1 h at 4 °C, centrifuged at $100\,000 \times g$ for 30 min, and the supernatant and pellet fractions assayed for ATPase. The results obtained in the second experiment correlated with the initial binding data as can be seen from Fig. 6, thus demonstrating that the formation of the liposome–ATPase complex is a reversible process. The binding parameters from this study are also listed in Table I.

TABLE I

BINDING PARAMETERS FOR THE FORMATION OF THE LIPOSOME–ATPase COMPLEX WITH PURIFIED PHOSPHATIDYLCHOLINE AT 4 °C IN TRIS–$MgCl_2$ BUFFER, pH 7.5

Ionic strength	ATPase Spec. act. (units/mg)	Linear correlation coefficient of Scatchard plots	$k \times 10^7$ $(\text{mole/l})^{-1}$	$n \times 10^4$ (moles ATPase/moles phospholipid)	Hill coefficient
0.05	20	−0.981	3.9±0.7*	1.07–1.24**	1.02
0.15	20	−0.985	2.1±0.4	0.99–1.21	0.98
0.05	30	−0.982	7.4±1.4	1.57–1.84	0.95

* Error bar is the range of 99 % confidence from a student's "t" test on Scatchard data.

** Values given show the range of 99 % confidence in the intercept on the ν axis of the Scatchard plots.

Binding of the ATPase to stearylamine–phosphatidylcholine liposomes

The concentration of free ATPase in equilibrium with the ATPase bound to stearylamine–phosphatidylcholine liposomes was considerably lower than for the corresponding phosphatidylcholine liposome–ATPase mixtures. The inhibition of ATPase in the stearylamine–phosphatidylcholine liposomes (Fig. 2) resulted in apparent values of $\bar{\nu}$ which underestimated the amount of protein bound by approximately 50 %. The binding data for this system (Fig. 7) clearly show that there was a marked decrease in the amount of ATPase bound with increasing ionic strength. These data provided linear double reciprocal plots, with a common intercept on the

$1/\bar{v}$ axis (see Fig. 8a). The corresponding binding data for the neutral phosphatidylcholine liposomes are plotted in Fig. 8b for comparison of the effect of ionic strength on the binding characteristics of the positively charged and neutral liposomes. A Scatchard plot for the stearylamine–phosphatidylcholine liposomes in Tris–$MgCl_2$ with 0.1 M KCl is shown in Fig. 9. The data were calculated from the ATPase concentrations of the supernatant fractions and the amount of ATPase in the starting mixtures. The calculation of $\bar{v}$ values therefore assumed 100 % activity in the stearylamine–phosphatidylcholine liposome–ATPase complex.

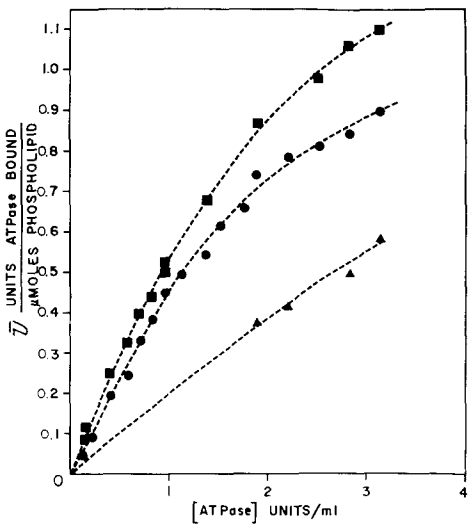
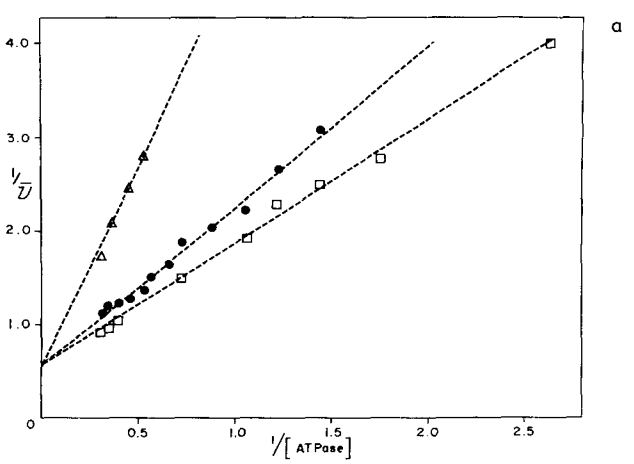


Fig. 7. ATPase binding to phosphatidylcholine liposomes containing 10 % (w/w) stearylamine. Each of the data points is the mean of two determinations in which the experimental variation was less than 5 %. The data were obtained by ATPase assay of the pellet fractions following differential centrifugation of the liposome–ATPase mixtures. The binding studies were carried out 4 °C, pH 7.5, in 0.02 M Tris–0.01 M $MgCl_2$ (■) and in the Tris–$MgCl_2$ buffer containing 0.1 M KCl (●) or 0.40 M KCl (▲).



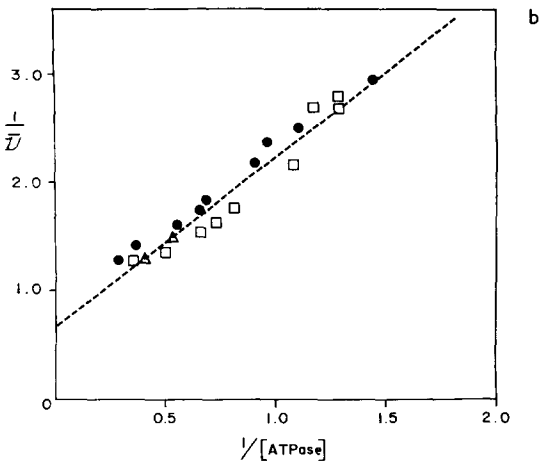


Fig. 8. Double reciprocal plots for the binding of solubilized ATPase to phosphatidylcholine liposomes containing 10% (w/w) stearylamine (a) and to pure phosphatidylcholine liposomes (b). Abscissa values are in (ATPase units/ml)$^{-1}$. Buffer systems are: □ ,Tris–$MgCl_2$, pH 7.5; ●, Tris–$MgCl_2$, pH 7.5, containing 0.1 M KCl; △, Tris–$MgCl_2$, pH 7.5, containing 0.4 M KCl.

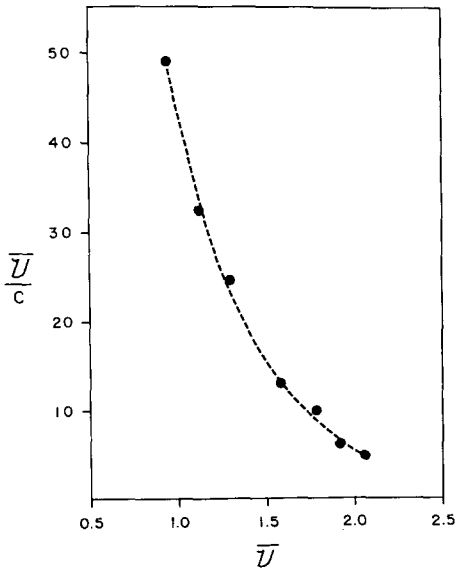


Fig. 9. Scatchard plot of the binding of solubilized ATPase to phosphatidylcholine liposomes containing 10% (w/w) stearylamine at 4 °C in Tris–$MgCl_2$ containing 0.1 M KCl, pH 7.5, The data were calculated from ATPase assays of the supernatant fractions obtained by centrifugation of the liposomes–ATPase mixtures.

Influence of cardiolipin, phosphatidylethanolamine, cholesterol, and acyl-chain fluidity

Cardiolipin (25 % by weight) was added to phosphatidylcholine in order to generate liposomes bearing a net negative charge in Tris–$MgCl_2$ buffer. Binding curves were obtained for the ATPase interaction with these negatively charged liposomes, and with pure phosphatidylcholine controls using a fresh ATPase preparation. The amounts of the ATPase bound to these liposomes at saturation are listed in Table II. There was no significant difference between the ATPase concentrations in the supernatant fractions for the two systems. Similar values were also obtained by direct ATPase assay on the pellet fractions, indicating that the presence of the cardiolipin did not affect the enzymatic activity of bound ATPase. ATPase assays on mixtures of the soluble ATPase and cardiolipin–phosphatidylcholine liposomes have confirmed that there is no influence of the negatively charged lipid on ATPase activity. Furthermore, the amount of ATPase bound to the cardiolipin–phosphatidylcholine liposomes was not significantly different in Tris–$MgCl_2$ buffer containing 0.1 M KCl. Since the cardiolipin molecule carries two negative charges at pH 7.5, it would appear that electrostatic forces are of minor importance in the interaction of the ATPase with this molecule. Similar binding characteristics were obtained for phosphatidylcholine liposomes containing phosphatidylethanolamine (33 % by weight). The incorporation of cholesterol (33 % by weight), however, caused a reduction in the ATPase bound at each ATPase concentration tested (Table II).

TABLE II

EFFECTS OF LIPID COMPOSITIONS AND TEMPERATURE ON THE AMOUNT OF SOLUBILIZED ATPase BOUND TO PHOSPHOLIPID LIPOSOMES

Liposome composition	Temperature (°C)	ATPase bound (mg protein/g total lipid)
Hen egg phosphatidylcholine	4	85±7*
Hen egg phosphatidylcholine–cardiolipin (3:1, w/w)	4	83±7
Hen egg phosphatidylcholine–phosphatidylethanolamine (2:1, w/w)	4	79±8
Hen egg phosphatidylcholine–cholesterol (2:1, w/w)	4	56±6
Synthetic dimyristoyl phosphatidylcholine	4	67±6
Hen egg phosphatidylcholine	30	87±8
Synthetic dimyristoyl phosphatidylcholine	30	91±7

* ±2 S.D.

The influence of acyl chain fluidity on the ATPase binding was investigated using synthetic dimyristoyl phosphatidylcholine liposomes above and below the temperature of the gel–liquid crystalline phase transition which occurs around 24 °C [37]. Egg yolk phosphatidylcholine liposomes were employed as controls, and the results are presented in Table II. Below the phase transition there was less ATPase bound to the synthetic saturated phosphatidylcholine than to hen egg phosphatidyl-

choline liposomes. Above the phase transition, however, there was no significant difference in the ATPase binding to the two systems. ATPase binding to the hen phosphatidylcholine liposomes was not markedly temperature dependent.

DISCUSSION

Numerous studies of protein interaction with phospholipid liposomes have been reported, but this is the first in which the intrinsic association constant for protein binding has been determined. We have shown that the solubilized membrane ATPase from *S. fecalis* binds to phosphatidylcholine liposomes, and that no significant decrease in the maximum amount of ATPase bound occurred when the ionic strength was increased. The value of $k = 7.4 \cdot 10^7\ M^{-1}$ (Table I) corresponds to an apparent free energy change, $\Delta G'$, for the reaction of -9.9 kcal/mole of ATPase. The slightly lower k value obtained with the less active enzyme preparation (Table I) yields a $\Delta G'$ of -9.6 kcal/mole. Little change in the apparent intrinsic association constant was observed on increasing the temperature, for example, $k = 6.5 \cdot 10^7\ M^{-1}$ at 24 °C. The apparent enthalpy change $\Delta H'$ for the reaction is therefore small (c.a. -1 kcal/mole) while the corresponding entropy contribution, calculated from the Gibbs–Helmholtz equation is approximately $+30$ e.u./mole. This large entropy change provides the driving force for the formation of the liposome–ATPase complex and is indicative of hydrophobic interaction between the lipid and protein molecules [38, 39]. Table I shows that there was a small effect of ionic strength on the apparent intrinsic association constant, which suggests that there is a minor contribution from electrostatic forces to the complex formation. Estimates of the Hill coefficient [40] for the reaction were determined from the slope of $\log(\bar{v}/(n-\bar{v}))$ as a function of $\log c$. The values obtained were not significantly different from unity, therefore, we conclude that there is no form of cooperativity in the liposome–ATPase interaction and only one type of binding site in these membranes which is probably the hydrocarbon interior.

Addition of 10 % (w/w) stearylamine to phosphatidylcholine bilayers in Tris–$MgCl_2$, 0.1 M KCl increased the apparent intrinsic association constant for the liposome–ATPase reaction by at least an order of magnitude, as measured from the slope of the binding curve (Fig. 9). Attempts to straighten the Scatchard plot by correcting the ordinate $\bar{v}/c$ with the electrostatic interaction term e^{2wv} [41] were not successful. The curvature is, therefore, partly attributable to the existence of more than one type of binding site in the stearylamine–phosphatidylcholine membranes [42]. Incorporation of 10 % (w/w) stearylamine increased the maximum number of ATPase molecules bound to the mixed liposomes by approximately 2-fold. The marked decrease in the binding caused by increasing ionic strength suggested that electrostatic forces are responsible for the increased binding capacity. This result was expected since the ATPase is highly acidic [43] and possesses a net negative charge at pH 7.5, while the stearylamine carries a positive charge. At higher ionic strength, 0.45, it appeared that the amount of ATPase bound to the stearylamine-containing liposomes approached the level found for the pure phosphatidylcholine bilayers. The observation that incorporation of cardiolipin did not diminish the ATPase binding (Table II) supports the view that hydrophobic interactions are the principal forces stabilizing the liposome–ATPase complex. A possible alternative explanation for this

effect may be the presence of Mg^{2+} in the buffer solution [3].

The fact that the incorporation of phosphatidylethanolamine (33 %, w/w) into the phosphatidylcholine liposomes did not significantly change the binding characteristics was not surprising, because both natural phospholipids were obtained from the same source and probably contained similar fatty acid compositions, although this was not checked experimentally. The influence of cholesterol on the physical properties of phospholipid membrane systems has been studied in considerable detail [44, 45]. Chapman and Penkett [46] have reported that the incorporation of cholesterol into hen egg phosphatidylcholine liposomes reduces the molecular motion of the hydrocarbon chains of the phospholipid, as measured by high resolution proton magnetic resonance spectroscopy. The observed decrease in the ATPase binding to phosphatidylcholine liposomes containing 33 % (w/w) cholesterol is probably due to this effect of cholesterol on phospholipid membranes. A similar decrease in protein binding to liposomes caused by the incorporation of cholesterol has recently been reported by Papahadjopoulos et al. [47]. These authors observed a 30 % decrease in the binding of cytochrome *c* to phosphatidylserine vesicles after the incorporation of an equimolar amount of cholesterol. The binding experiments with the saturated dimyristoyl phosphatidylcholine (Table II) also demonstrate that acyl chain fluidity is a factor in determining the amount of ATPase which binds to the liposome. At 4 °C, the hydrocarbon chains of this phospholipid molecule are in a rigid configuration [37, 48] and the penetration of the ATPase into the gel phase is apparently restricted, whereas, above the gel–liquid crystalline phase transition, the penetration of the ATPase is facilitated by the fluid state of the hydrocarbon chains.

We have previously [24] shown that the solubilized ATPase combines with ultrasonicated phosphatidylcholine liposomes to generate multilamellar structures in which the lamellar thickness is 54 Å compared with 44 Å for pure phosphatidylcholine liposomes. The extent to which the structural characteristics of the liposomes change by the incorporation of the other lipids employed in binding studies has not yet been determined. The nature of the liposome–ATPase complex has not been elucidated, but penetration of the protein into the bilayer interior appears probable. Ultrasonication of the liposomes for 1 min did not significantly change the ATPase binding. Furthermore, it was not possible to increase the ATPase binding by allowing the lyophilized phosphatidylcholine to swell directly in the solubilized ATPase solution. The maximum binding observed was $1.8 \cdot 10^{-4}$ mole ATPase per mole phosphatidylcholine (Table I), which corresponds to a weight ratio of phospholipid to protein of approximately 11 : 1. We have calculated a value of approximately 13 : 1 (w/w) for ratio of total lipid to ATPase in the streptococcal membrane [49, 50]. Since the *S. fecalis* membrane does not contain phosphatidylcholine, the apparent agreement is coincidental. No requirement for lipid has been found with the lipid-free solubilized ATPase and the activity of the solubilized enzyme is similar to that in reconstituted streptococcal membranes as well as phosphatidylcholine liposomes [49]. This membrane-derived ATPase is, therefore, distinct from membrane-derived enzymes which require lipid factors for activity [3], a striking example being rat liver mitochondrial D-β-hydroxybutyrate dehydrogenase, which has an absolute requirement for phosphatidylcholine [51]. This latter group also includes the (Na^+-K^+)-ATPase of mammalian membranes [3]. The absence of a lipid requirement for enzyme activity does not preclude the existence of specific lipid–protein interactions

occurring in the biological membrane. The streptococcal membrane contains a significant proportion of cardiolipin [50], and the strong binding of ATPase to phosphatidylcholine liposomes containing 25 % (w/w) cardiolipin (Table II) may be relevant to the formation of the membrane–ATPase complex in the intact organism.

Nectin is a bacterial membrane protein which promotes binding of the solubilized ATPase to enzyme-depleted streptococcal membranes [52] and is found associated with the solubilized ATPase in Mg^{2+}-containing solutions. The protein is heat labile and is normally denatured in the routine ATPase purification by an initial heat treatment step. In one preparation carried out, this step was omitted and nectin activity in the ATPase fractions from the Agarose column was confirmed using the binding assay described by Baron and Abrams [52]. It was found that the presence of this low molecular weight (37 000) protein associated with ATPase did not alter the liposome binding characteristics. Baron and Abrams [52] have also reported that the nectin-dependent formation of the streptococcal membrane–ATPase complex restores the sensitivity of the enzyme to inhibition by dicyclohexylcarbodiimide. However, no effect of dicyclohexylcarbodiimide was observed on the ATPase activity of liposome–ATPase complexes with or without nectin, suggesting that the dicyclohexylcarbodiimide sensitivity may be conferred on the membrane bound nectin–ATPase complex by another component of the *S. fecalis* membrane. The streptococcal membranes are thought to contain a limited number of specific binding sites for the nectin–ATPase complex and reconstitution experiments probably involve the reinsertion of the complex into these sites [49, 52]. It is obvious that no specific receptor site for the ATPase exists in phosphatidylcholine liposomes, nevertheless, we have shown earlier [24] that Mg^{2+} plays a role in stabilizing the membrane–ATPase complexes in both the native and model membrane systems.

In an earlier report, we described the interaction of the purified, lipid-free, solubilized, ATPase with planar bilayer membranes [22]. Addition of the ATPase (10^{-9} M) to synthetic diphytanoyl phosphatidylcholine membranes increased the d.c. conductance 10^2–10^4-fold. The magnitude of the increase in membrane conductance was dependent on the presence of Mg^{2+}, and upon the Na^+ and K^+ concentrations in the range 10^{-2}–10^{-1} M. An additional 10-fold conductance increase was observed when ATP was added [21]. The zero-voltage conductance of the bilayer–ATPase complex exhibited a saturation effect above $5 \cdot 10^{-9}$ M ATPase in the bathing solution [22]. If the binding of the solubilized ATPase to phosphatidylcholine can be expected, a priori, to be similar in both planar bilayer and liposome membranes, then, at $5 \cdot 10^{-9}$ M ATPase the binding parameter $\bar{\nu}$ for the planar bilayer would be approximately $6 \cdot 10^{-5}$ mole ATPase per mole phospholipid (see data from Table I). Cook et al. [53] have determined that there are approximately $1.6 \cdot 10^{14}$ phosphatidylcholine molecules per cm^2 of film surface in planar bilayer membranes generated from hen egg phosphatidylcholine in *n*-decane. The number of ATPase molecules bound to the planar bilayer at the conductance saturation level is, therefore, expected to be on the order of 10^{10} molecules per cm^2. This value corresponds to a surface coverage of about 15 m^2/mg, which is considerably larger than the value of less than 1 m^2/mg found for many globular proteins adsorbed at oil–water interfaces [34, 55]. Interfacial tension measurements are being planned in order to determine the limiting area of adsorbed ATPase molecules at the decane–water and phosphatidylcholine–*n*-decane–water interfaces [53]. Preliminary studies at the air–

water interface (unpublished results) have shown that the solubilized ATPase is surface active and can penetrate phosphatidylcholine monolayers.

At concentrations of 10^{-10} M ATPase in the bathing solution, discrete conductance fluctuations were observed in the bilayer membrane [22]. These conductance fluctuations consisted of integral multiples of a unit channel approximately 10^{-10} Ω^{-1} in magnitude. If each channel represented the conductance contribution from a single ATPase molecule penetrating the bilayer [22], then the observed membrane conductance ($< 10^{-5}\ \Omega^{-1} \cdot cm^{-2}$) with $5 \cdot 10^{-9}$ M ATPase would correspond to a bilayer with about 10^5 conducting channels per cm^2, assuming the unmodified bilayer has a conductance of $10^{-9}\ \Omega^{-1} \cdot cm^{-2}$ [54]. This would mean that only 0.001 % of the bound ATPase was electrically active at low applied voltage; the proportion increased as the applied voltage was increased [22]. The voltage-dependent appearance of the conducting channels in the optically black films appears to be either a field-dependent penetration of surface adsorbed ATPase into the bilayer or a field-dependent transition between nonconducting and conducting conformations of the bilayer–ATPase complex. On the basis of the evidence, the latter case appears more likely. We plan to study the molecular nature of the liposome–ATPase complex using spectroscopic techniques including infrared spectroscopy, ORD, and CD. It is hoped that information relevant to the field-dependent conductance effects seen in the planar bilayer–ATPase system will be obtained by studying the influence of electric fields on the spectroscopic properties of the liposome–ATPase system [56].

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