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MINI-REVIEW

Interactions of Lipids and Proteins: Some General Principles

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Abstract

Methods to describe the binding of phospholipids to membrane proteins are described. It is shown that it is difficult to obtain estimates of the number of phospholipids bound to the surface of a membrane protein from ESR experiments in which plots of free to bound spin label (y) vs. molar ratio of lipid to protein are extrapolated to $y = 0$. The relative advantages and disadvantages of ESR and fluorescence methods for measuring relative binding constants of phospholipids to membrane proteins are discussed. The particular problems associated with comparing binding constants of molecules of very different sizes (e.g., fatty acids and cardiolipin) are described and equations are presented to account for these problems. The possible effects of membrane viscosity and thickness on activity of membrane proteins are discussed, but it is concluded that effects of phospholipid structure on activity can only be understood in terms of a reasonably complete kinetic model for the protein.

Key Words: (Ca^{2+}–Mg^{2+})-ATPase; sarcoplasmic reticulum; phospholipids; fluorescence quenching.

Introduction

An important aspect of membrane structure is the nature of the interaction between phospholipids and proteins in the membrane. Our experience in other aspects of biochemistry makes it likely that, despite the wide variety of proteins found in biological membranes, similar rules will govern all phospholipid–protein interactions. The most powerful technique for studying these interactions is that of reconstitution, since this allows the insertion

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of purified membrane proteins into phospholipid bilayers of defined composition: native biological membranes are, in general, too complex to allow definitive studies at the molecular level. The technique of reconstitution is, of course, limited by the requirement for a purified membrane protein, and is most easily applied to membrane-bound enzymes, since assay of function is then much easier than for a protein such as a receptor. For these technical reasons, information is only currently available about a limited range of membrane proteins, but there seems no particular reason why these proteins should be in any way unusual in their properties. Here we will concentrate on one particular membrane protein, the $(Ca^{2+}-Mg^{2+})$-ATPase of sarcoplasmic reticulum (SR) of rabbit skeletal muscle.

How to Describe Binding of Phospholipids to a Membrane Protein

It now seems a reasonable assumption that the great majority of membrane proteins will span the phospholipid bilayer portion of the membrane, and that the membrane-penetrant part of the membrane protein will consist of one or more α-helices. It also seems likely that these α-helices will be composed of sequences of predominantly hydrophobic amino acid residues spanned at each end by a number of polar or charged residues (Eisenberg *et al.*, 1984). It is thus unlikely that membrane proteins will possess distinct binding sites for the bulk of the phospholipid molecules with which they are in contact in the membrane, although, of course, distinct binding sites might be present for a small number of particular phospholipids, as might be the case, for example, for cardiolipin on mitochondrial Complex 1 (Heron *et al.*, 1977). Rather, the bulk of the phospholipids are likely to interact with a hydrophobic surface of the membrane protein. Such a surface will be heterogeneous, both because of the molecular "roughness" of the amino acid residues making up the hydrophobic α-helices and because of the presence of charged amino acid residues close to the phospholipid–water interface of the membrane which could affect interactions with the charged head groups of the phospholipids. A full description of binding to such a heterogeneous surface is an impossibly difficult task, but, as long as the surface is not too heterogeneous, binding can reasonably be described in terms of a uniform surface. A number of equations have been presented to describe binding to surfaces (see Aveyard and Haydon, 1973), but the only one that is readily applicable is the Langmuir binding isotherm, which is equivalent to the conventional equation describing equilibrium binding to a number of distinct binding sites. That such an approach is not unreasonable can be seen by reference to Fig. 1. Figure 1 represents the hydrophobic surface of a membrane protein covered by the adjacent phospholipids (referred to as boundary

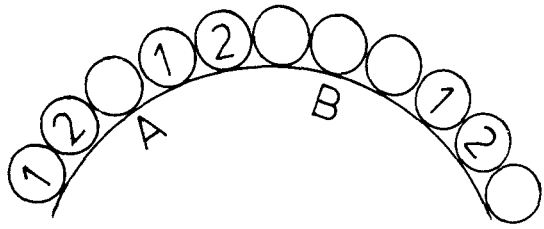

Fig. 1. Binding sites on the surface of a membrane protein, showing three phospholipids bound at random, indicating the sites occupied by the 1- and 2-fatty acyl chains.

or annular phospholipids). With total coverage of the surface by phospholipids, one phospholipid must leave the surface before another can enter. Although some rearrangement of phospholipids on the surface is possible during this process, a simple concerted exchange of two phospholipids on the surface seems more likely. In this sense, therefore, the process can be described by competitive binding of phospholipids at a number of "sites" on the protein surface. Binding of phospholipids to the surface of the protein can then be described as a series of displacement reactions of the type

$$PL + L^* \rightleftharpoons PL^* + L$$

where L and L* represent two classes of phospholipid and PL and PL* represent L and L* bound to the protein respectively. Such binding can then be described by an equilibrium constant K,

$$K = [PL^*][L]/[PL][L^*] \quad (1)$$

where the square brackets denote concentrations.

In developing such an analysis for phospholipid binding, it is necessary to give some thought to the structure of the phospholipid. For a phospholipid such as a phosphatidylcholine or a phosphatidylethanolamine with two fatty acyl chains, it is possible to picture modes of binding in which either only one fatty acyl chain interacts with the protein surface (edge-on binding of the phospholipid) or in which both fatty acyl chains interact with the surface (sideways binding of the phospholipid). For the following reasons, it seems most likely that sideways binding will be most likely (Lee, 1983). First, on binding to the protein surface, there will be a loss of translational and rotational entropy, and most likely a considerable loss of conformational entropy, and this will favor the binding of the minimum number of phospholipid molecules required to occupy the surface of the protein—to a first approximation, two phospholipids would have to bind in the edge-on mode to occupy the same surface area occupied by one phospholipid binding in the sideways mode. Second, the unbound fatty acyl chain of a phospholipid bound to the protein in the edge-on mode would be expected to have a

mobility intermediate between that for a phospholipid fully bound to a protein and that for a free phospholipid in the bilayer: no evidence for such an intermediate state has been reported (Lee, 1983). Although, in reality, the distinction between edge-on and sideways binding for a two-chain phospholipid may be rather indistinct, the differences would be particularly clear for a four-chain species such as cardiolipin.

It seems intrinsically unlikely that the number of molecules required to give complete coverage of the hydrophobic surface of a membrane protein will be the same for a single-chain molecule such as a fatty acid, for a two-chain molecule such as phosphatidylcholine, and for a four-chain molecule such as cardiolipin. It is more reasonable to propose that what is constant is the total number of fatty acyl chains required to cover the surface, so that the numbers of fatty acid, phosphatidylcholine, and cardiolipin molecules binding to the surface will be in the ratio 4:2:1 respectively (Lee, 1983). Even when this approach is criticized (Marsh, 1985, page 155) it is implicit in the methods of analysis actually employed (Marsh, 1985, page 152). For such a model, however, binding can no longer be described by a simple Langmuir binding isotherm [Eq. (1)]. The problem can be understood by considering Fig. 1 for the case where all sites are initially occupied by fatty acids. If now a phospholipid is added that occupies two sites on the protein surface, then the situation illustrated in Fig. 1 could develop. Random binding of the phospholipids has resulted in a single site being left unoccupied between two bound phospholipid molecules (at A) and it is not possible for a phospholipid to bind to this single site. Again, random binding of phospholipid molecules could create three consecutive sites occupied by fatty acid (as at B) and only one phospholipid molecule could bind at these three sites. For statistical reasons of this type it becomes difficult to completely saturate the sites with a multivalent ligand such as a phospholipid. These statistical problems can be solved readily (Lee, 1983).

First, binding to the surface of the protein should be generalized to a sequence of displacement reactions of the type

$$PL_n + L^* \rightleftharpoons PL^* + nL$$

where L is a monovalent ligand and L* is a multivalent ligand occupying n sites on the protein surface: binding of one molecule of L* to the surface will therefore result in displacement of n molecules of L. The equilibrium constant for the reaction can be written as

$$K = [PL^*][L]^n/[PL_n][L^*] \quad (2)$$

Here square brackets denote concentrations expressed as mole fraction in the membrane, since this is the most natural unit. The mole fraction in the membrane is easily related to the more usual concentration unit of moles of

ligand per liter of medium: thus, for example, the mole fraction of free L in the bilayer is given by

$$[\mathrm{L}] = \{\mathrm{L}\}/(\{\mathrm{L}\} + \{\mathrm{L}^*\}) \tag{3}$$

where the brackets { } denote concentrations expressed as moles/liter. The equilibrium constant of Eq. (2) can then be rewritten as

$$K = \{\mathrm{L}_\mathrm{b}^*\}\{\mathrm{L}\}^n/(n\{\mathrm{L}_\mathrm{b}\}\{\mathrm{L}^*\}(\{\mathrm{L}\} + \{\mathrm{L}^*\})^{n-1}) \tag{4}$$

where subscripts b denote concentrations of L and L* bound to the protein. If the number of fatty acyl chain binding sites on the protein is N and if the protein concentration is P, then the concentrations of bound L and L* are related by

$$n\{\mathrm{L}_\mathrm{b}^*\} + \{\mathrm{L}_\mathrm{b}\} = NP \tag{5}$$

The concentration of L* bound to the protein can then be derived from Eq. (4) as

$$\{\mathrm{L}_\mathrm{b}^*\} = nK\{\mathrm{L}^*\}(\{\mathrm{L}\} + \{\mathrm{L}^*\})^{n-1}(NP - n\{\mathrm{L}_\mathrm{b}^*\})/\{\mathrm{L}\}^n \tag{6}$$

In their analysis of binding to DNA, McGhee and von Hippel (1974) showed that to describe the binding of a multivalent ligand, the free-site concentration had to be multiplied by a factor P_n, giving the probability that a free site is followed by at least $n - 1$ other free sites, thus allowing binding of a ligand of valence n. Thus for a multivalent ligand, Eq. (6) should be modified to give

$$\{\mathrm{L}_\mathrm{b}^*\} = nK\{\mathrm{L}^*\}(\{\mathrm{L}\} + \{\mathrm{L}^*\})^{n-1}(NP - n\{\mathrm{L}_\mathrm{b}^*\})/\{\mathrm{L}\}^n P_n \tag{7}$$

where the probability factor P_n is given by (Lee, 1983)

$$P_n = ((NP - n\{\mathrm{L}_\mathrm{b}^*\})/(NP - (n-1)\{\mathrm{L}_\mathrm{b}^*\}))^{n-1} \tag{8}$$

Equation (8) assumes "stereochemical" binding of the multivalent lipid: that is, that the lipid can bind only one way round to the site. If this requirement is relaxed, then it can be shown that P_n is unaltered but that K should be replaced by $2K$ to allow for the two statistical ways of binding. The equations are readily solved by the method of bisection with $\{\mathrm{L}_\mathrm{b}^*\}$ as the variable.

The expected pattern of binding for mixtures of single-chain and multichain phospholipids is illustrated in Fig. 2. As expected, it can be seen that the largest effects of the valence of L* on the binding of L to the protein occur at low mole fractions of L. The calculations shown in Fig. 2 were for stereochemical binding. The assumption of nonstereochemical binding reduces the differences between the binding of different L* so that, for example, the calculated binding curve for L* with $n = 4$ for nonstereochemical binding is similar to that shown in Fig. 2 for $n = 2$ with stereochemical

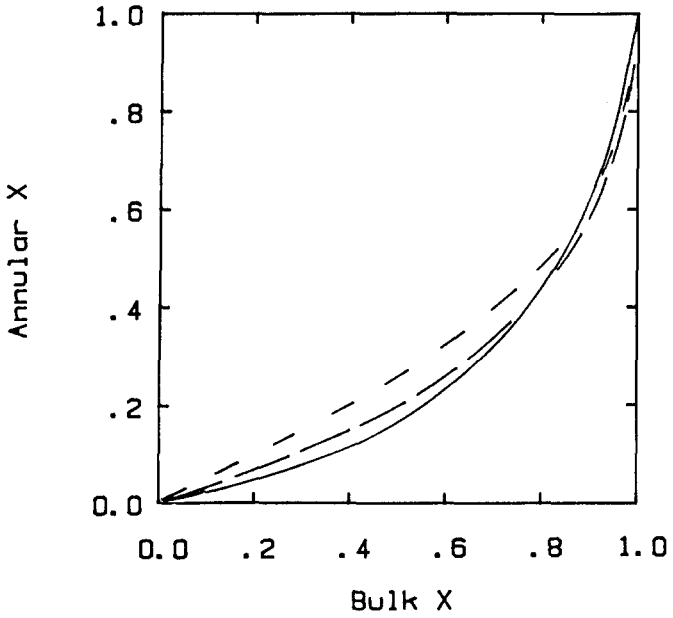

Fig. 2. Binding of mixtures of single- and multichain lipids to a membrane protein. Shown is the variation in the mole fraction X of bound (or annular) single-chain lipid as a function of the mole fraction X of the single-chain lipid in the bulk lipid bilayer for the case where the binding constant K of the multichain lipid relative to the single-chain lipid is 5. Shown are calculated curves for mixtures of a single-chain lipid L with another lipid L* with either a single fatty acyl chain (solid curve), with two chains (long dashed curve), or with four chains (short dashed curve). The calculations assume stereochemical binding.

binding. Figure 3 shows the effects at low mole fractions of L in more detail. It is clear that the effects of multivalence are large at low mole fractions of L. Since ESR experiments are usually performed at low mole fractions of spin-labelled phospholipid (typically 0.01), these effects must be taken into account before any meaningful comparison can be made between the derived relative binding constants K of phospholipids of different valence: failure to do so must result in misleading conclusions as to the thermodynamics of binding.

How to Quantitate Binding of Phospholipids to Membrane Proteins

In general, it is very difficult to measure the number of binding sites for a ligand on a protein when the number of binding sites is large. This is certainly true for studies of the binding of phospholipids to membrane proteins. Only one technique so far has given numbers of phospholipid

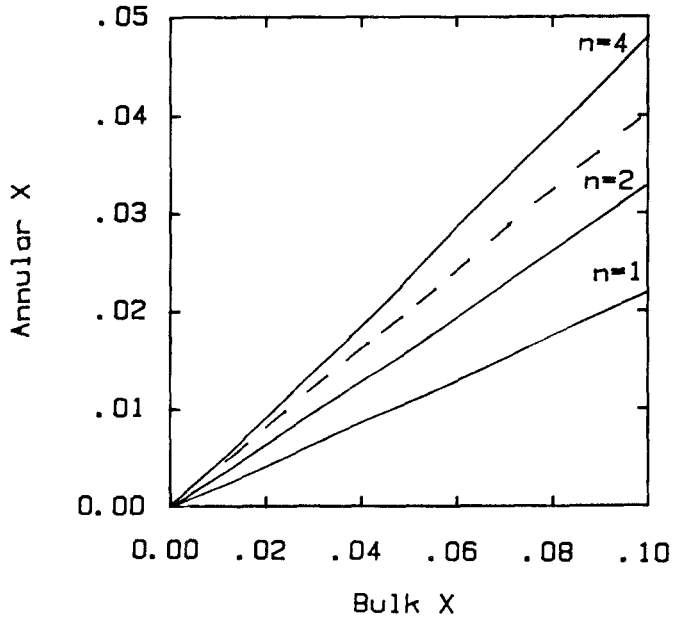

Fig. 3. Binding of mixtures of single- and multichain lipids to a membrane protein. The calculations were as in Fig. 2, and show results at low mole fractions of L in more detail. Solid lines show the results for stereochemical binding, and the broken line shows the results for nonstereochemical binding for $n = 4$.

binding sites and that is ESR. Relative binding constants have, however, been derived using both ESR and fluorescence methods. The ESR technique for measuring numbers of phospholipid binding sites relies on methods to quantitate the amounts of bulk (mobile) and annular (immobile) phospholipid in composite ESR spectra containing contributions from both bulk and annular phospholipids. The method of analysis most usually employed consists of guessing the shape of one component and then subtracting varying amounts of that component from the composite spectrum until what is left looks "reasonable" for the second component (Jost and Griffith, 1978). This procedure is, of course, rather subjective, and it is difficult to estimate the reliability of results obtained in this way. We have introduced a computerized procedure in which a library of spectra is scanned to give the best-fit pairs of components (East *et al.*, 1985). The procedure has the advantage of convenience and, more importantly, allows an objective comparison between alternative fits to the experimental data. From studies of the $(Ca^{2+}$–$Mg^{2+})$-ATPase we concluded that reasonably accurate fits to the data could be obtained from spectra obtained at lower temperatures (ca. 4°C) but that at higher temperatures a large number of alternative fits were possible, in part

because, at higher temperatures, an analysis in terms of two independent spectral components was invalidated by a rapid exchange between annular and bulk phospholipid, leading to partial averaging of the ESR spectra (East *et al.*, 1985).

Even when it is possible to obtain a reasonably accurate determination of the relative amounts of annular and bulk phospholipid from composite ESR spectra, there are serious limitations associated with the methods by which the number of binding sites is estimated. The usual experimental protocol is to measure the ratio of annular to bulk spin-labelled phospholipid as a function of the total phospholipid-to-protein ratio x. The data are then interpreted as a series of displacement reactions in which unlabelled and spin-labelled phospholipid compete for sites on the protein, as described by Eq. (1) (Brotherus *et al.*, 1981; Silvius *et al.*, 1984; Marsh, 1985). Unfortunately, this approach is only rigorously valid when the concentration of phospholipid is in large excess over the concentration of protein, since only then can the protein molecules be considered to be isolated entities within the membrane, each with its own independent shell of annular phospholipid. At lower molar ratios of phospholipid to protein, proteins can no longer be assumed to be isolated within the membrane, and the possibility of sharing phospholipid between the annular shells of two or more adjacent proteins and the possibility of protein–protein contact has to be considered. The relative significance of these effects depends, of course, on the energetics of the interactions between the various components present. If the interaction between protein molecules within the membrane is highly repulsive, then protein molecules can indeed be considered to be isolated: as long as there is sufficient phospholipid in the system to form a complete annular shell around each protein in the membrane, such shells will be formed since this will maximize the distance between protein molecules and so minimize the postulated unfavorable interaction between proteins. However, in the absence of such repulsive interactions, the distribution of protein molecules within the membrane will be closer to random, and calculations for random mixing of phospholipids and proteins show that extensive sharing of annular shells of phospholipids will occur (East *et al.*, 1985).

Unfortunately, the analysis presented by East *et al.* (1985) does not allow calculation of the actual numbers of annular phospholipids expected in the case of random mixing. Hoffmann *et al.* (1981), however, have presented a lattice model for the bilayer which gives the fraction f of annular phospholipid as

$$f = (1 - x)^N \tag{9}$$

where x is the mole fraction of protein in the membrane and N is the number of phospholipid binding sites on the protein. Although such a lattice model

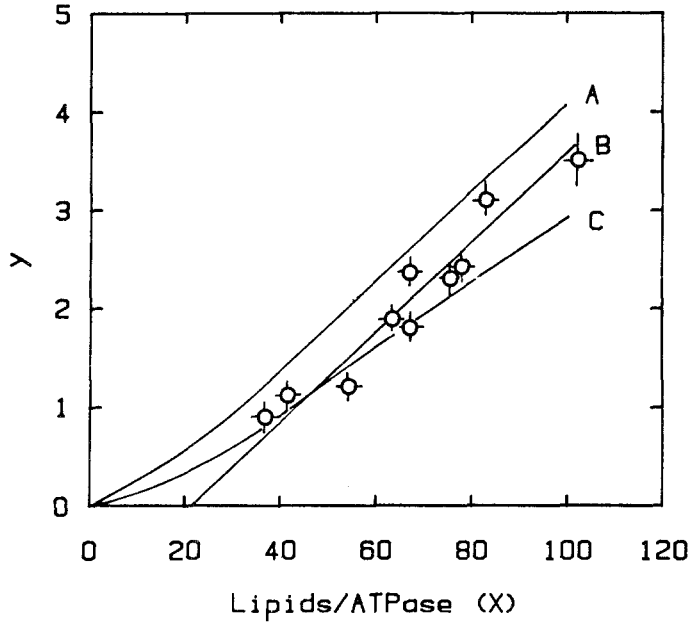

Fig. 4. Simulated binding curves for the interaction of spin-labelled phosphatidylcholine with the (Ca^{2+}–Mg^{2+})-ATPase. The ratio of bulk to annular spin-labelled phospholipid (y) is plotted against the molar ratio of total phospholipid to ATPase (x). The experimental data are from Silvius *et al.* (1984). (A) and (C) are theoretical curves calculated from Eq. (9) as described in the text with $N = 22$ and 30 respectively, and (B) is the curve calculated according to the approach of Silvius *et al.* (1984) with $N = 22$. All curves were calculated assuming equal binding of spin-labelled and non-spin-labelled phospholipids to the ATPase.

will obviously give the wrong values for f at low molar ratios of phospholipid to protein (since it ignores all problems associated with the very different sizes of phospholipid and protein molecules), it has been shown to provide a reasonable fit to the experimental data for a variety of membrane proteins (Hoffmann *et al.*, 1981) including data on the variation of f with varying phospholipid to protein ratio for the (Ca^{2+}–Mg^{2+})-ATPase (East *et al.*, 1985). This is the only approach which allows an estimation of the effects of probability on the number of annular phospholipids, and we will use it here for illustrative purposes. In Fig. 4 we show simulated binding plots calculated from Eq. (9) for $N = 22$ and $N = 30$ for the case where spin-labelled and non-spin-labelled phospholipid molecules bind equally to the ATPase, and a simulated binding plot calculated by the method of Silvius *et al.* (1984). It is clear from Fig. 4 that relaxing the unrealistic requirement for protein molecules to be completely isolated within the membrane is likely to lead to very different binding plots. Numbers of binding sites N are usually obtained from experimental data such as those shown in Fig. 4 by a straight line

extrapolation of the data to $y = 0$ and equating the intercept on the x axis with N (Brotherus *et al.*, 1981; Silvius *et al.*, 1984; Marsh, 1985). This procedure will be valid only for completely isolated proteins within the membrane: otherwise a linear extrapolation of the binding curves gives an intercept on the x axis which is very different from the number of binding sites. An accurate determination of the number of binding sites is theoretically only possible at a very high molar ratio of phospholipid to protein, x, since only then can the protein molecules be considered to be isolated: unfortunately, of course, at high molar ratios of phospholipid to protein, the ratio of bulk to annular phospholipid will also be very high, so that the ratio cannot be determined experimentally to the required accuracy. These problems would seem to be intrinsic to the ESR method, so that numbers of binding sites obtained from ESR studies must be considered to be only rough estimates.

Relative binding constants for a variety of spin-labelled molecules to membrane proteins have been obtained from the slopes of plots such as that shown in Fig. 4 (Brotherus *et al.*, 1981; Silvius *et al.*, 1984; Marsh, 1985; Devaux and Seigneuret, 1985). Again, the analysis used is only valid for isolated protein species, and the simulations shown in Fig. 4 suggest that the derived parameters might be quite inaccurate. As described above, problems may also arise when using the ESR method to measure the relative binding constants of lipids containing different numbers of fatty acyl chains. In ESR experiments, the spin-labelled species is added at low concentration (usually 1% or less of the total phospholipid) to avoid problems associated with spin–spin interactions. As shown in Fig. 3, it is at these low concentrations that statistical effects are likely to occur. Finally, in studies of the binding of single-chain molecules such as fatty acids to membrane proteins, it is usually assumed that binding only occurs to sites on the protein otherwise occupied by phospholipids (annular sites). This may not always be so: it is known that many membrane proteins occur in aggregated form in the membrane, thus presenting potential binding sites at protein–protein interfaces in such aggregates. Indeed, based on experiments with fluorescence probes, we have presented evidence for such binding both for the $(Ca^{2+}$–$Mg^{2+})$-ATPase and for bacteriorhodopsin (Lee *et al.*, 1982; Simmonds *et al.*, 1982, 1984; Froud *et al.*, 1986a, b; Rooney *et al.*, 1987). Despite all these problems, it should be stressed that the ESR method is one of the few methods available for studying phospholipid–protein interactions and that, within its limitations, useful information can be obtained.

Attempts to use NMR methods to quantitate the numbers and affinities of phospholipid binding sites on membrane proteins have generally not been successful, because the rate of exchange of bulk and annular phospholipid is fast on the NMR time scale, and only averaged spectra are obtained which

are difficult to analyze. An alternative spectroscopic technique, operating on a very short time scale, on which exchange of bulk and annular phospholipid is unlikely, is fluorescence spectroscopy. Potentially, fluorescence spectroscopy can be used in a number of ways to quantitate phospholipid–protein interactions, but in practice the most detailed information has come from quenching studies. A membrane protein such as the $(Ca^{2+}–Mg^{2+})$-ATPase contains a number of tryptophan residues concentrated in the hydrophobic, membrane-penetrant parts of the structure. The fluorescence of these tryptophan residues can be quenched by contact with a number of quenching species, including spin-labelled and brominated phospholipids (London and Feigenson, 1981; East and Lee, 1982). Since the fluorescence lifetime of the tryptophan group is just a few nanoseconds, whereas the time for two phospholipids to exchange position in the membrane is ca. 60 nsec (East *et al.*, 1985), quenching in the membrane will be static, and thus the extent of quenching will be proportional to the number of quenching phospholipids in the annular shell around the protein: unfortunately, the nature of this proportionality is not known *a priori*. Binding constants for a variety of phospholipids to the $(Ca^{2+}–Mg^{2+})$-ATPase, relative to that for dioleoylphosphatidylcholine (DOPC), have been obtained by reconstituting the ATPase into mixtures of quenching phospholipid and DOPC, and comparing the quenching profiles with those obtained for mixtures of quenching phospholipid with the second phospholipid. If it is assumed that equal degrees of quenching in the two different phospholipid mixtures reflect equal numbers of quenching phospholipids bound to the protein, then relative binding constants can be obtained from the quenching plots (London and Feigenson, 1981; Lee and East, 1982; Lee, 1987). The implicit assumption of a uniform distribution of quenchable tryptophan residues is clearly unrealistic, but for a protein containing a large number of tryptophan residues spaced close enough for efficient inter-residue energy transfer, the assumption is unlikely to have much effect on the derived relative binding constants (Lee, 1987). These methods have recently been reviewed at length (Lee, 1987), so that full details of the methods of analysis will not be repeated here.

Although both the ESR and the fluorescence methods have serious limitations, it is very encouraging that when both methods have been applied to the same membrane protein they have led to very similar conclusions. Thus, both methods suggest that binding constants of a variety of phosphatidylcholines, phosphatidylserines, and phosphatidylethanolamines for the $(Ca^{2+}–Mg^{2+})$-ATPase differ by no more than a factor of 2, as long as the phospholipids are in the liquid crystalline phase (London and Feigenson, 1981; East and Lee, 1982; Marsh, 1985; Froud *et al.*, 1986c), even for phospholipids which support very different ATPase activities (Caffrey and Feigenson,

1981; East and Lee, 1982; Froud *et al.*, 1986c). These results are also consistent with measurements of exchange rates of phosphatidylcholines between annular sites and the bulk phospholipid phase, which show that exchange is fast, consistent with a nonsticky interaction (East *et al.*, 1985). The phospholipid–protein interface of the ATPase is not without specificity, however, since phospholipids in the gel phase bind weakly to the annular sites as do long-chain alcohols and esters and sterols: a variety of amines bind strongly at the interface (Simmonds *et al.*, 1982, 1984; Lee *et al.*, 1982; Froud *et al.*, 1986b). From both fluorescence and kinetic experiments, it has also been suggested that a variety of hydrophobic molecules can bind to the ATPase at sites other than the annular sites at the phospholipid–protein interface. These other sites have been referred to as non-annular sites, and it has been suggested that they could occur at protein–protein interfaces in ATPase dimers (Simmonds *et al.*, 1982, 1984; Lee *et al.*, 1982; Froud *et al.*, 1986b; Jones and Lee, 1986; Rooney *et al.*, 1987; Lee, 1987). The presence of non-annular binding sites for fatty acids and sterols considerably complicates the interpretation of ESR studies with spin-labelled analogues, where it has generally been assumed that binding occurs only at annular sites on the protein.

To the extent that data are available for other membrane proteins, a low specificity for the phospholipid–protein interaction seems to be general. Thus binding constants for phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, and phosphatidic acids to myelin proteolipid (Brophy *et al.*, 1984), the acetylcholine receptor (Ellena *et al.*, 1983), $(Na^+–K^+)$-ATPase (Brotherus *et al.*, 1981; Esmann and Marsh, 1985), and rhodopsin (Watts *et al.*, 1979) differ by no more than a factor of 2. It has been reported that cardiolipin binds more strongly to $(Na^+–K^+)$-ATPase, $(Ca^{2+}–Mg^{2+})$-ATPase, and cytochrome oxidase than do the other phospholipids (Marsh, 1985), but for the ATPases this can be attributed, at least in part, to the four fatty acyl chains of cardiolipin compared to the two fatty acyl chains of the other phospholipids (see Fig. 2). For cytochrome oxidase, as for other mitochondrial membrane proteins, there is evidence for a specific interaction between cardiolipin and small number of sites on the protein, but stronger binding of cardiolipin to the ATPases cannot have any physiological significance since there is no cardiolipin in the membranes in which these proteins are found. Even if, as suggested here, phospholipids interact with an undifferentiated hydrophobic membrane surface, small differences in the binding of different phospholipids to membrane proteins would be expected. There is, however, no evidence that these small differences are exploited in any way to optimize or control the activity of the membrane proteins. Thus to refer to this differential binding as demonstrating "selectivity" is misleading: the word *select* is defined in the Oxford English Dictionary as meaning to pick out what is best or most suitable and there is no evidence that this is what is

happening in the membrane. It is much safer to describe differences in binding as demonstrating "specificity", since *specificity* is a neutral word with no connotation of fitness.

Effect of Phospholipids on Protein Function

Many studies of membrane function have involved measuring some property of the bulk phospholipid component of the membrane and have been predicted on the assumption that protein activity responds in a simple, and fairly passive way, to a change in the measured property. The property of the phospholipids that is thought to be important varies between studies and has included the phase of the phospholipid (liquid crystalline or gel), the fluidity or viscosity of the phospholipid, and the thickness of the phospholipid bilayer. Such an approach is likely to have meaning only for a protein with a single, unique rate-limiting step, as commonly found in the pages of elementary biochemistry textbooks. Thus, in a reaction sequence in which a substrate S binds to an enzyme E to give a complex ES which then reacts to give EP where P is the product of the reaction,

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$$

the rates of binding of S and dissociation of P are commonly assumed to be fast relative to the rate of the reaction ES $\rightarrow$ EP, so that this latter step is said to be rate-limiting. If such a reaction scheme were to apply to a membrane-bound enzyme, then for the phospholipid composition of the membrane to have any effect on enzyme activity, it would have to affect the ES $\rightarrow$ EP step in some way. Changes in phospholipid composition could affect the forward and backward rates for this step if binding constants for different phospholipids to ES and EP were different, because then changing phospholipid composition would change the equilibrium constant EP/ES, and the equilibrium constant is just the ratio of the forward to the backward rate. Changes in membrane thickness could affect the rate in the same way, since a change in membrane thickness would presumably arise from a change in the phospholipid composition of the membrane. The phase of the phospholipid could also affect activity in this way, since it has been shown that phospholipid in the liquid crystalline and gel phases interact differently with membrane proteins (East and Lee, 1982). The phospholipid phase could, however, affect activity in two other ways. In general, the miscibility of foreign compounds with phospholipids is greater in the liquid crystalline phase than in the gel phase, so that the degree of dispersal of membrane proteins is likely to decrease on transformation of the phospholipid into the gel phase (Lee, 1987): changes in the extent of protein–protein interactions following from an increase in the

local concentration of proteins is likely to result in changes in enzyme activity. It is also possible that the increase in "viscosity" of the phospholipid phase that will occur on transformation to the gel phase will increase the activation barrier for the changes between ES and EP and so result in a decrease in activity. Indeed, it has often been suggested that, even in the liquid crystalline phase, the exact "viscosity" of the membrane could be an important factor in determining the activity of a membrane protein. Thus, for example, Chong *et al.* (1985) have noted that increases in hydrostatic pressure and decreases in temperature lead to decreases in the fluidity of phospholipid bilayers and to decreases in the activity of a $(Na^+–K^+)$-ATPase and, because of this correlation, they suggested that membrane fluidity had a direct effect on enzyme activity. This idea has been tested for the $(Ca^{2+}–Mg^{2+})$-ATPase by reconstituting the ATPase into a series of phospholipid bilayers, and looking for a correlation between ATPase activity in a particular bilayer and the "viscosity" of that bilayer: no correlation was found (East *et al.*, 1984). Thinking more deeply about the problem, the viscosity of the bulk phospholipid phase is unlikely to have an effect on enzyme activity since the environment actually sensed by the membrane protein is not that of bulk phospholipid but is annular phospholipid. The ESR experiments described above show that the mobility of phospholipid fatty acyl chains in the annulus is inhibited by the presence of the relatively immobile protein surface: the mobility of the chains is determined predominantly by the nature of the protein surface, so that the "viscosity" of the annular phospholipid will be relatively constant and independent of the chemical structure of the phospholipids. Studies have also been reported in which membrane fluidity has been changed by addition of hydrophobic molecules such as alcohols to the membrane, and, again, observed changes in enzyme activity have been said to be caused by the resulting changes in membrane viscosity (see Almeida *et al.*, 1986). Studies with defined, reconstituted systems show that such conclusions are unsafe. Thus archetypal membrane "rigidifiers" and "fluidizers" such as cholesterol and alcohols either have no effect, or increase the activity, or decrease the activity of the $(Ca^{2+}–Mg^{2+})$-ATPase, depending on the structure of the phospholipid in the system: as described below, these effects have been explained in terms of direct binding to the ATPase (Froud *et al.*, 1986b).

A number of membrane processes are known to be dependent on contact between initially separated molecules within the membrane, and such processes could, of course, be dependent on the viscosity of the lipid component of the membrane. However, even here it seems that in many cases the factor dominating diffusion in the membrane is not the viscosity of the lipid bilayer but interaction with microfilaments and microtubules within the cell. Bigelow *et al.* (1986) have suggested that rotational and lateral diffusion of the $(Ca^{2+}–Mg^{2+})$-ATPase in the SR membrane could be an important factor

in determining ATPase activity, presumably by controlling the formation of particular protein–protein interactions important for the reaction cycle. This seems rather unlikely. Indeed, we have found that a number of monoclonal antibodies raised to the ATPase will bind to the ATPase with no effect on ATPase activity, and yet attaching an entity as large as an antibody to a membrane protein must affect its rotational and translational motion within the membrane (East *et al.*, 1987).

We have found that effects of phospholipid on the activity of the (Ca^{2+}–Mg^{2+})-ATPase are complex. Thus in a series of phosphatidylcholines, ATPase activity increases with increasing chain length from C12 to C18 but then decreases with further increases in chain length (Froud *et al.*, 1986c; Lee, 1987). The activity of the ATPase reconstituted with a short-chain phospholipid can be increased by addition of, for example, oleyl alcohol, whereas for the ATPase reconstituted with DOPC, oleyl alcohol has only an inhibitory effect at high concentrations and, for the ATPase reconstituted with a long-chain phospholipid, it first inhibits and then activates (Froud *et al.*, 1986b): such effects are not readily interpreted in terms of a simple Michaelis–Menten scheme such as that discussed above! As a further example, the activity of the ATPase reconstituted with a phosphatidylethanolamine is lower than that for the ATPase reconstituted with the equivalent phosphatidylcholine, but the activity of the ATPase reconstituted with a mixture of phosphatidylcholine and phosphatidylethanolamine is higher than for either phospholipid alone (Gould, G. W., East, J. M., and Lee, A. G., unpublished results). These results point to a more complex reaction scheme for the ATPase than a simple Michaelis–Menten scheme with a single rate-controlling step.

Fortunately, a great deal of kinetic data is available for the (Ca^{2+}–Mg^{2+})-ATPase and this has been used to produce a kinetic model for the ATPase (Froud and Lee, 1986a, b; Gould *et al.*, 1986; Stefanova *et al.*, 1987; Lee, 1987). It is proposed that the ATPase exists in one of two conformations E1 or E2 (Fig. 5). In the E1 conformation, the two Ca^{2+} binding sites per ATPase are of high affinity and are exposed on the outer (cytoplasmic) side of the SR membrane. In the E1 conformation there is also a high-affinity binding site for MgATP. In the E2 conformation, the two Ca^{2+} binding sites are of low affinity and face the inside of the SR, and the MgATP binding site is of low affinity. The first two steps in the reaction sequence are the binding of Ca^{2+} and ATP to the high-affinity sites on E1. After phosphorylation and loss of MgADP (step 3) the ATPase undergoes a conformation change in which the Ca^{2+} binding sites become of low affinity and inward facing (step 4). Ca^{2+} is then lost from these sites, and, after hydrolytic cleavage of the phosphorylated intermediate, the enzyme recycles (step 8). The dependence of ATPase activity on the concentration of MgATP is complex (see Fig. 7) so that the presence of "catalytic" and "regulatory" sites for MgATP

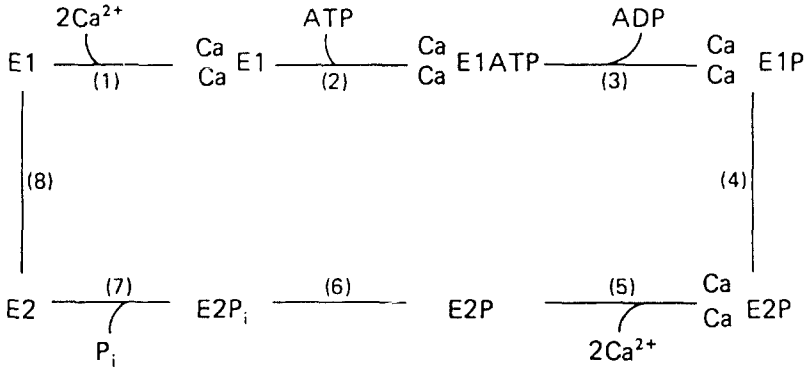

Fig. 5. Reaction scheme for the ATPase (Froud and Lee, 1986b).

of high (μM) and low (mM) affinity have been suggested. We have shown that the kinetics of the ATPase are consistent with the "catalytic" and "regulatory" sites being two states of the same site, and have suggested that the "regulatory" effects of MgATP follow from binding of MgATP to the adenine binding region of E1′PCa_2 after dissociation of MgADP. Recent studies with monoclonal antibodies which affect the ATP dependence of ATPase activity are also consistent with this suggestion (East *et al.*, 1987). The model has been described in detail elsewhere (Gould *et al.*, 1986; Stefanova *et al.*, 1987; Lee, 1987) so that little needs to be added here, except that some discussion of the role of Mg^{2+} may be useful since this seems a matter of contention and is still rather unclear.

The true substrate for the ATPase is MgATP rather than free ATP (Vianna, 1975; Gould *et al.*, 1986). Highsmith (1984) has presented evidence for a divalent metal ion binding site at the MgATP binding site, present in the absence of ATP. We have suggested that this is the same binding site that must be present for Mg^{2+} on E2 to explain the role of Mg in phosphorylation of the ATPase by inorganic phosphate (Froud and Lee, 1986b). Binding of MgATP to E1 and E2 will then be competitive with binding of Mg^{2+} (Gould *et al.*, 1986; Stefanova *et al.*, 1987). We have also suggested that MgATP binds to the phosphorylated form of the ATPase, E1′PCa_2, and increases the rate of the transition to E2′PCa_2 (Gould *et al.*, 1986). Simulations show that it has to be the MgATP complex that binds to E1′PCa_2 and not free ATP, because the concentration of free ATP present in the usual assay medium (typically 5 mM Mg^{2+}) will be very low—indeed, so low that the rate of binding of free ATP to E1′PCa_2, even with a maximal rate constant of 10^8, will be too slow to cause the required stimulation of the E1′PCa_2–E2′PCa_2 step. If then it is MgATP which binds E1′PCa_2, there are two possibilities

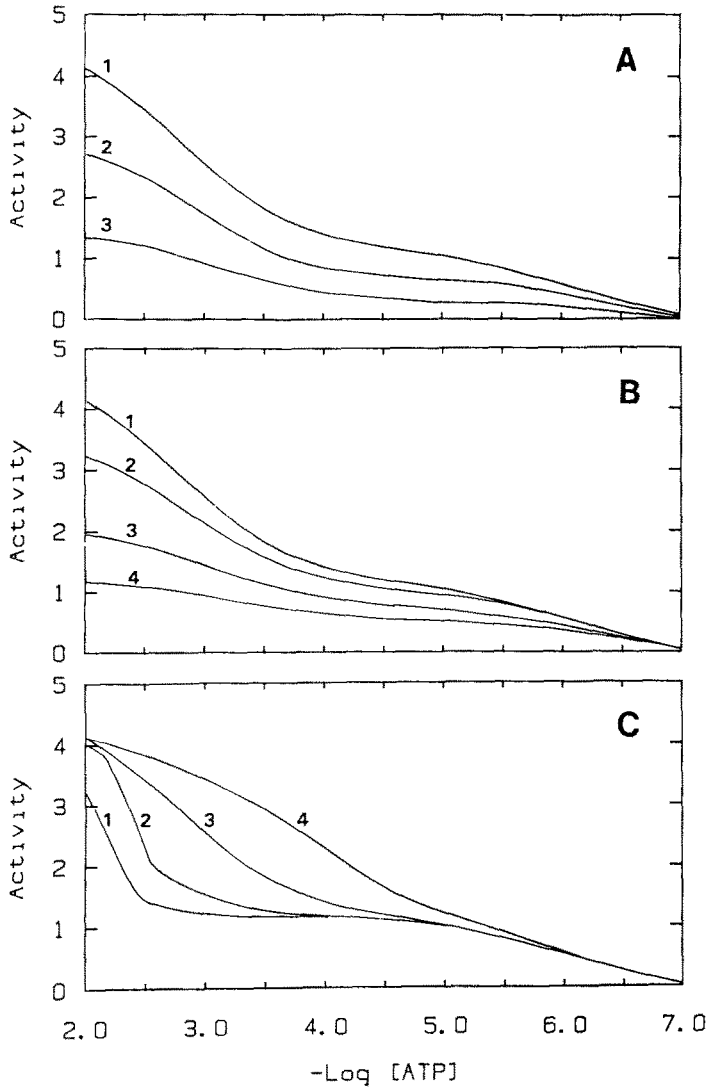

Fig. 6. Effects of rate parameters on simulated steady-state ATPase activities, calculated in terms of the Scheme shown in Fig. 5. Unless otherwise stated, the rate constants for the E1′PCa_2–E2′PCa_2 and E1′PMgATPCa_2–E2′PMgATPCa_2 transitions and the effective rate of dephosphorylation were set at 3.1, 15.4, and 22.5 respectively with a dissociation constant for binding of MgATP to E1′PCa_2 of 0.57 μM with an off-rate constant of 9.2. For all calculations, a dissociation constant of 8.9 μM with an off-rate constant of 36 was assumed for binding of MgATP to E1. The Mg^{2+} concentration was 5 mM, the K^+ concentration 12 mM, and the pH 7.2. All other rate parameters were as given in Lee (1987). (A) The effect of varying the rate of the E1′PCa_2–E2′PCa_2 and E1′-MgATPCa_2–E2′PMgATPCa_2 transitions: rates were (1) 3.1 and 15.4; (2) 1.5 and 7.5; (3) 0.6 and 3.0 respectively. (B) The effect of varying the rate of dephosphorylation. Effective rates of dephosphorylation under the given ionic conditions were: (1) 22.5; (2) 11.2; (3) 4.5; (4) 2.2. (C) The effect of varying the dissociation constant for binding of MgATP to E1′PCa_2. Dissociation constant (μM): (1) 57.0; (2) 5.7; (3) 0.57; (4) 0.057.

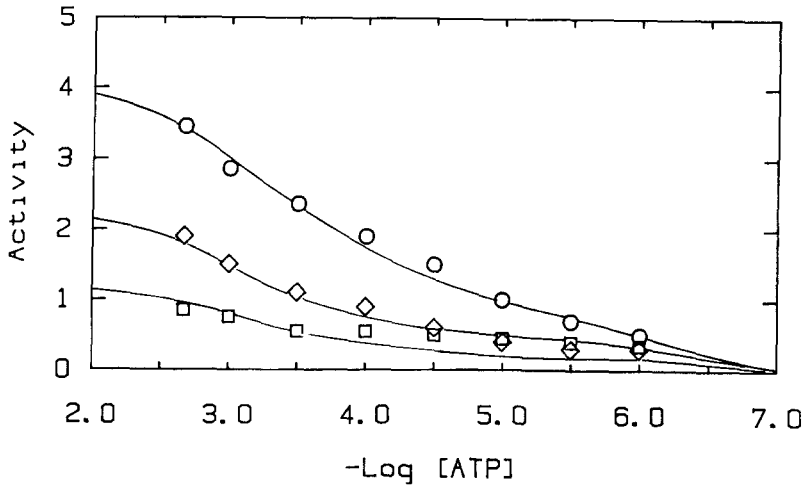

Fig. 7. Simulations of the effect of phospholipid structure on the ATPase activity of the (Ca^{2+}–Mg^{2+})-ATPase. The experimental points show ATPase activities at 25°C for the native ATPase (○) and for ATPase reconstituted with dimyristoleoylphosphatidylcholine (C14 : 1, □) or dierucoylphosphatidylcholine (C22 : 1, ◇). The solid lines show simulations with the parameters listed in Table I. Data from Froud *et al.* (1986b).

that need to be considered. In the first, following phosphorylation, MgADP is released from the ATPase so that MgATP can bind to the vacated site: as for binding to E1, free Mg^{2+} could bind in competition with the binding of MgATP. The second possibility is that free ADP is released from the ATPase following phosphorylation, leaving behind Mg^{2+} tightly bound on the ATPase, so that, on binding of MgATP, there will be a total of four divalent metal ions bound to the ATPase: two Ca^{2+} ions and two Mg^{2+} ions. Based on the observation that the level of phosphorylation of the ATPase by GTP in the presence of GDP increases with increasing Mg^{2+} concentration, Ronzani *et al.* (1979) concluded that the substrate for the ATPase had to be MgGTP, and that the product released from the ATPase had to be free GDP: for a reaction scheme such as that shown in Fig. 5, no such simple, definitive statement can be made. Although the idea that it is free ADP that is released from the ATPase is a popular one (see de Meis, 1981), for the following reasons we think that it is unlikely. First, it has been shown that CaATP can act as a substrate for the ATPase instead of MgATP, and that when CaATP is the substrate, a total of three Ca^{2+} ions can be bound to the ATPase, the extra Ca^{2+} ion presumably binding at the site usually occupied by Mg^{2+} (Dupont, 1980; Wakabayashi and Shigekawa, 1984): there have been no reports of four divalent metal ions binding under these conditions. It has been shown that the extra Ca^{2+} bound with CaATP as substrate can be removed

by EGTA, to give a metal ion-free phosphoenzyme (E2P), and that the extra bound Ca^{2+} can rapidly exchange with Ca^{2+} in the medium (Wakabayashi and Shigekawa, 1984). However, it appears that removal of Mg^{2+} from $E1'PCa_2$ following addition of EDTA may be relatively slow at low temperatures (Garrahan *et al.*, 1976; Kanazawa *et al.*, 1971), although it has been reported that Ca^{2+} and Mg^{2+} can exchange at this site at an appreciable rate (Yamada *et al.*, 1986). Finally, we have found that high concentrations of Mg^{2+} are inhibitory at both high and low concentrations of ATP: inhibition at low concentrations of ATP will follow from competition between Mg^{2+} and MgATP for binding to E1, and inhibition at high concentrations of ATP shows competition between binding of Mg^{2+} and MgATP to $E1'PCa_2$ (Stefanova *et al.*, 1987). The simplest explanation of all these results is, of course, the presence of a single Mg^{2+} binding site on $E1'PCa_2$ which can be occupied either by Mg^{2+} or by Mg^{2+} complexed to ATP. This is largely in agreement with the studies of Andersen and Moller (1985) who showed competition between the binding of MgATP and $MgVO_4$ to the ATPase. They, however, found some evidence that, although $MgVO_4$ reduced the affinity of the ATPase for MgATP, simultaneous binding of both MgATP and $MgVO_4$ was possible: the complexity of the experiment (with the concentrations of Mg^{2+}, ATP, and MgATP all being varied) rules out any definitive statement.

The Mg^{2+} binding site on $E1'PCa_2$ must be of high affinity, as shown by studies of the effect of Mg^{2+} concentration on the level of phosphorylation by inorganic phosphate of SR vesicles loaded with Ca^{2+} (Froud and Lee, 1986b; Stevanova *et al.*, 1987), so that, under the usual assay conditions, the site will always be occupied, either by free Mg^{2+} or by the MgATP complex. Because of the high affinity of the site for Mg^{2+}, the off-rate constant for dissociation will be relatively slow, but whether it will be slow enough to significantly affect the rate of the ATPase is unclear at present: in the absence of any definitive information, it has been assumed that binding of Mg^{2+} is fast enough to be treated by the quasi-equilibrium approach (Stefanova *et al.*, 1987). In a recent publication, Haynes and Mandveno (1987) have suggested that the maximal on-rate for binding of Mg^{2+} is ca. 10^6 (Haynes and Mandveno, 1987, page 274), which would necessarily result in a slow rate of dissociation of Mg^{2+} from $E1'PCa_2$, because of the large equilibrium binding constant (2×10^5; Stefanova *et al.*, 1987). However, the basis for the claim by Haynes and Mandveno (1987) is unclear: Diebler *et al.* (1969) have shown that the maximal first-order rate constant for the substitution of inner-sphere water around the Mg^{2+} aquo-ion is ca. 10^5, but what is of relevance here is the on-rate constant for binding, and this varies between ligands and is, for example, 1.3×10^7 for binding of Mg^{2+} to ATP^{4-} and 3×10^6 for binding to ADP^{3-}.

Table I. Kinetic Parameters Obtained by Simulation for the Reconstituted ATPase at 25°C, pH 7.2, $K^+ = 12\,mM$, $Mg^{2+} = 5\,mM$[a] [Rate Constants (s^{-1})]

Lipid	Dissociation constant MgATP to E1′PCa_2 (μM)	E1′PCa_2–E2′PCa_2	E1′$PMgATPCa_2$–E2′$PMgATPCa_2$	E2–E1	E2P dephosphorylation[b]
Native	0.05[c]	2.8	13.9	38.0	22.5
Dimyristoleoylphosphatidylcholine	0.57[d]	0.5	2.3	53.2	225.0
Dierucoylphosphatidylcholine	0.57[d]	1.4	6.9	38.0	11.2

[a]With K_d for binding of MgATP to E1 of 8.9 (μM) with an off-rate constant of $36\,s^{-1}$.
[b]Composite value for $K^+ = 12\,mM$, pH 7.2.
[c]With an off-rate constant of $2\,s^{-1}$.
[d]With an off-rate constant of $9.2\,s^{-1}$.

As shown in detail elsewhere, it is possible to interpret the effects of phospholipid on ATPase activity in terms of the above scheme (Froud *et al.*, 1986b; Lee, 1987). Since there is no single rate-controlling step in the reaction sequence, effects of phospholipids on activity can be complex, depending on which steps are affected. The three most important parameters in the simulations are the rates of the $E1'PCa_2$–$E2'PCa_2$ transition, the rate of dephosphorylation of E2P, and the affinity of $E1'PCa_2$ for MgATP. As shown in Fig. 6, each of these parameters has distinctive effects on simulations of ATPase activity. In particular, decreasing the rate of dephosphorylation decreases activities at high ATP concentrations proportionally more than at low concentrations of ATP whereas reducing the rate of the $E1'PCa_2$–$E2'PCa_2$ transition decreases the rates more evenly, and changing the affinity of $E1'PCa_2$ for MgATP markedly affects activities at intermediate concentrations of MgATP. Figure 7 compares experimental data for the effect of long- and short-chain phosphatidylcholines on the activity of the ATPase with the results of simulations using the rate constants given in Table I. Although at this stage, the kinetic model must be considered to be preliminary, it is clear that it is capable of explaining the observed results. The task now is to relate changes in the kinetic parameters with molecular changes on the ATPase. An initial step was taken by estimating the equilibrium constant E1/E2 from measurements of the fluorescence intensity of the ATPase labelled at the ATP binding site with fluorescein isothiocyanate (Froud *et al.*, 1986a). It was found that the equilibrium constant varied with phospholipid structure, indicating that the strengths of binding of phospholipids to E1 and E2 were slightly different. Changes in the equilibrium constant E1/E2 mean, of course, changes in one or both of the rates E2 to E1 or E1 to E2, but simulations suggest that the overall ATPase activity is relatively insensitive to changes in these rates. However, a change in an equilibrium constant at one part of the cycle must be exactly balanced by equal and opposite changes elsewhere in the cycle, since the overall cycle shown in Fig. 5 corresponds to the hydrolysis of MgATP: the set of parameters used in the simulation shown here (Lee, 1987) gives a free energy change ΔG^0 of -8.0 kcal/mole (at 1 M ATP, ADP, P_i, and Mg^{2+}, pH 7.2) which can be compared to the measured free energy change for the hydrolysis of ATP of -7.8 kcal/mole (Guynn and Veech, 1973). A suggestion, attractive because of its simplicity, is that a change in the equilibrium constant E1/E2 is balanced by a change in the equilibrium constant $E1'PCa_2/E2'PCa_2$, and simulations show that the rate of the ATPase is very sensitive to the rate of the $E1'PCa_2$–$E2'PCa_2$ transition. It has also been shown that the effects of hydrophobic additives such as sterols or alcohols can be understood in terms of changes in the rates of these same steps (Froud *et al.*, 1986b). The observation that solution of the ATPase in monomeric form in the detergent $C_{12}E_8$ gives activity profiles similar to that

shown in Fig. 6C for high-affinity binding of MgATP to $E1'PCa_2$ suggests that protein–protein interactions could play an important role in determining ATPase activities. However, the important point here is that effects of phospholipids on the activities of membrane proteins can only be understood in terms of a reasonably complete and accurate model for the kinetics of the protein.

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