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ANNULAR AND NON-ANNULAR BINDING SITES ON THE (Ca^{2+} + Mg^{2+})-ATPase

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Quenching of the fluorescence of the (Ca^{2+} + Mg^{2+})-ATPase purified from muscle sarcoplasmic reticulum can be used to measure relative binding constants of hydrophobic compounds to the phospholipid-protein interface. We show that the binding constant for cholesterol is considerably less than that for phosphatidylcholine, so that cholesterol is effectively excluded from the phospholipid annulus around the ATPase. However, dibromocholestan-3β-ol causes quenching of the fluorescence of the ATPase, and so has access to other, non-annular sites. We suggest that these non-annular sites could be at protein/protein interfaces in ATPase oligomers. Oleic acid can bind at the phospholipid/protein interface, although its binding constant is less than that for a phosphatidylcholine, and it can also bind at the postulated non-annular sites. The effects of these compounds on the activity of the ATPase depend on the structure of the phospholipid present in the systems.

Introduction

The normal environment for most membrane proteins is a phospholipid bilayer. It is important to know whether this bilayer simply provides an inert, structural support for proteins allowing directional fluxes in and out of a cell or cell organelle, or whether there are discrete structural requirements for optimal interaction between a membrane protein and its surrounding lipids. Since the phospholipid composition of natural membranes is complex, we need to know the relative affinities of membrane proteins for the phospholipids in the membrane, as the composition of the phospholipid annulus around the protein could differ from the bulk phospholipid composition of the membrane. Further, we need to know relative affinities for other hydrophobic compounds such as cholesterol, fatty acids and drug molecules, since, by binding to the protein at the lipid/protein interface, they could displace phospholipids from the annulus around the protein, and thus affect protein activity.

It is known that the activity of a membrane protein such as the (Ca^{2+} + Mg^{2+})-ATPase from sarcoplasmic reticulum of muscle is sensitive to the chemical structure of the surrounding phospholipid (both fatty acyl chain and headgroup) and to the phase of the lipid, liquid-crystalline or gel [1–5]. Nevertheless, it has been shown that binding constants for phospholipids to the ATPase are largely independent of the chemical structure of the phospholipid, although the binding constant for lipid in the gel phase is about 30-times less than that for lipid in the liquid-crystalline phase [4,5]. If the relative lack of selectivity of the ATPase towards phospholipids also extended to other hy-

Abbreviations: BRPC, 1,2-bis(9,10-dibromooleoyl)-*sn*-glycerol-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine; DMPC, 1,2-dimyristoleoyl-*sn*-glycerol-3-phosphocholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

drophobic compounds, then the composition of the annulus around the ATPase would be highly dependent on the concentration of hydrophobic compounds that happened to be present in the environment surrounding the sarcoplasmic reticulum, with possible large fluctuations in ATPase activity.

We have developed a technique employing the fluorescence quenching properties of brominated phospholipids that allows us to detect displacement of phospholipids from the annulus around the ATPase. Bromination of dioleoylphosphatidylcholine (DOPC) yields 1,2-bis(9,10-dibromooleoyl)phosphatidylcholine (BRPC) which has physical properties very similar to dioleoylphosphatidylcholine [4]. The $(Ca^{2+} + Mg^{2+})$-ATPase contains a large number of tryptophan residues in hydrophobic regions of the protein structure whose fluorescence can be quenched by hydrophobic compounds [6]. When the ATPase is reconstituted with a mixture of BRPC and DOPC, the intensity of the tryptophan fluorescence observed depends on the mole fraction of BRPC in the lipid mixture. The extent of fluorescence quenching can be described as:

$$F/F_o = 0.4 + 0.6(1 - x_{BRPC})^{1.6} \quad (1)$$

where F_o is the fluorescence intensity of the ATPase in DOPC alone, and F is that in a mixture containing a mole fraction, x_{BRPC}, of BRPC (Fig. 1). We can use these fluorescence quenching plots to measure affinities of other hydrophobic compounds for the ATPase, relative to the affinity of the ATPase for DOPC. If the ATPase is reconstituted with a mixture of BRPC and a second component, A, whose affinity for the ATPase is less than that of DOPC, then the observed fluorescence quenching will be greater for the mixture with A than for that with DOPC at any given mole fraction of BRPC, since DOPC will have a greater tendency to bind to the ATPase than has A. The relative binding constant, $K_{A/DOPC}$, for component A and DOPC is given by

$$K_{A/DOPC} = \frac{K_A}{K_{DOPC}} \quad (2)$$

where K_A and K_{DOPC} are intrinsic binding constants to the ATPase. The relative binding constant can be calculated from the fluorescence quenching plots employing the relationship derived by London and Feigenson [5]:

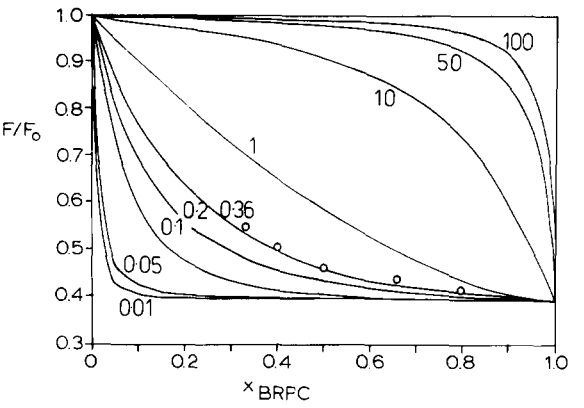


Fig. 1. Calculated fluorescence quenching plots (F/F_o) for ATPase reconstituted with mixtures containing the given mole fraction of BRPC, as a function of the relative binding constant, $K_{A/DOPC}$. The curve for $K_{A/DOPC} = 1.0$ corresponds to the experimentally determined fluorescence quenching plot for mixtures of DOPC and BRPC. The other curves were calculated for the given values of $K_{A/DOPC}$ as described in the text. Open circles represent experimental data for the oleic acid-BRPC-ATPase system.

$$K_{A/DOPC} = \frac{x_A(1 - x_{DOPC})}{(1 - x_A)x_{DOPC}} \quad (3)$$

where x_A and x_{DOPC} are the mole fractions of A and DOPC, respectively, which give the same level of fluorescence quenching in mixtures with BRPC. Fig. 1 illustrates the expected quenching profiles for various values of the relative binding constants.

Here we use the fluorescence quenching method to measure relative binding constants for cholesterol and oleic acid to the phospholipid/protein interface of the ATPase. We also use the fluorescence quenching properties of brominated derivatives of cholesterol and oleic acid to test for possible binding to the ATPase at sites other than the phospholipid/protein interface.

Materials and Methods

Lipids were obtained from Lipid Products and spin labels from Syva. Bromination of di-

oleoylphosphatidylcholine was performed as described [4]. Cholesterol was brominated in diethyl ether/glacial acetic acid (2 : 1, v/v) with bromine in the presence of sodium acetate. Crystals of dibromocholesterol formed were filtered and washed with cold methanol.

The specific optical rotation, α_D, of the product was -43.4, showing it to be the planar *trans*-diaxial ($5\alpha,6\beta$) dibromocholestan-3β-ol (literature value $-44°$, compared to $\alpha_D = +47°$ for the non-planar, *trans*-diequatorial ($5\beta,6\alpha$) form [7]).

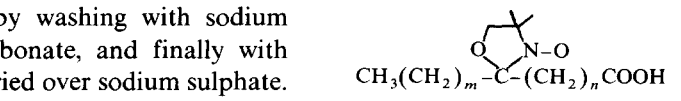
Oleic acid was brominated in chloroform solution in the presence of excess bromine. Unreacted bromine was removed by washing with sodium dithionite, sodium bicarbonate, and finally with water. The sample was dried over sodium sulphate.

Compounds were characterized by infrared spectroscopy and bromine analysis and gave single spots by TLC.

$(Ca^{2+} + Mg^{2+})$-ATPase was prepared from female rabbit (New Zealand white) hind-leg muscle in the presence of dithiothreitol and phenylmethylsulphonyl fluoride as described [4]. Lipid substitutions were also carried out as described previously [4]. Briefly, ATPase (0.12–0.27 mg) was incubated with a sonicated lipid suspension (1–1.3 μmol) and cholate (0.4–0.5 mg) in buffer (40–50 μl; 250 mM sucrose/1 M KCl/5 mM MgATP/50 mM KPO_4, pH 8.0) for 1–1.5 h at 0°C. Before assay, samples were diluted at least 500-fold. ATPase activity was measured by the method of Warren et al. [8] except that the buffer was 40 mM Hepes and the optimal Ca^{2+} concentration (10^{-6} M) was maintained with EGTA (1.01 mM) and $CaCl_2$ (0.91 mM). For the dimyristoleoylphosphatidylcholine-ATPase system, some samples were diluted 5-fold before assay and incubated for 1.5 h at 37°C to ensure equilibration of lipid pools, followed by assay in the normal way.

Fluorescence measurements were made using either Spex Fluorolog or Perkin Elmer MPF 44A fluorimeters, exciting fluorescence at 285 nm and detecting fluorescence at 340 nm. Fluorescence experiments were generally carried out in parallel with measurements of ATPase activity. Thus, for experiments with cholesterol and dibromocholesterol, 0.125 mg ATPase was incubated with a sonicated lipid suspension plus sterol at the required molar ratio (1–1.3 μmol) and 0.4 mg cholate in the buffer given above (40 μl) either for 90 min at 0°C or for 15 min at 37°C followed by 75 min at 5°C. 36 μl of the mixture were diluted into 2 ml buffer (40 mM Hepes/100 mM NaCl/1 mM EGTA, pH 7.2) and the fluorescence intensity was measured.

ESR spectra were run on a Bruker ER 200D spectrometer interfaced to a Z-80 based microcomputer system. Effects of sterols on bilayer fluidity were characterized using the spin-labelled fatty acid probes [*m*, *n*]-nitroxide-labelled stearic acids:



incorporated into liposomes at a 100 : 1 phospholipid-to-probe molar ratio. Order parameters were calculated as described by Gaffney [12]. Fluorescence decay kinetics of the ATPase were measured on the cavity-dumped laser system described elsewhere [13], exciting fluorescence at 308 nm and detecting at 365 nm.

Protein was estimated either by the Biuret method or using the extinction coefficient given by Hardwicke and Green [9].

Results

Interaction with cholesterol

Warren et al. [10] have reported that cholesterol has no effect on the activity of the ATPase when reconstituted with a lipid such as DOPC, and thus they suggested that cholesterol is excluded from the annulus around the ATPase. This suggestion is supported by the data shown in Fig. 2A. We find that cholesterol decreases only slightly the activity of DOPC-ATPase. Fig. 2B shows that cholesterol also has no effect on the fluorescence of either the DOPC-ATPase or the BRPC-ATPase. This means that cholesterol can displace only negligible quantities of phospholipid from the annulus around the ATPase and that the binding constant of the ATPase for cholesterol compared to that for dioleoylphosphatidylcholine can be no greater than approx. 0.1.

Experiments with brominated derivatives of cholesterol are, however, not consistent with com-

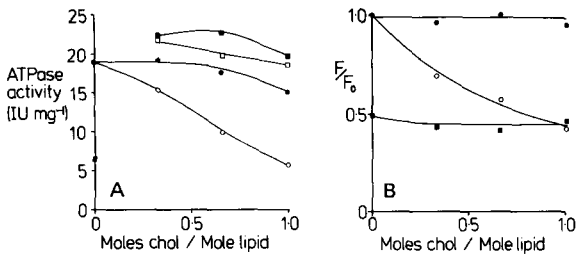


Fig. 2. A. The effect of cholesterol (chol) and dibromocholesterol on the activity of DOPC-ATPase and DMPC-ATPase at 37°C: cholesterol on DOPC-ATPase (●) and DMPC-ATPase (■); and dibromocholesterol on DOPC-ATPase (○) and DMPC-ATPase (□). B. The effect of cholesterol on the fluorescence of DOPC-ATPase (●) and BRPC-ATPase (■) and the effect of dibromocholesterol on the fluorescence of DOPC-ATPase (○).

plete exclusion of cholesterol from the ATPase. Bromination of cholesterol in diethyl ether/glacial acetic acid at 0°C gives the planar *trans*-diaxial (5α,6β)-dibromocholestan-3β-ol (dibromocholesterol). The interaction of dibromocholesterol with phospholipids is very similar to that of cholesterol, as shown by their ordering effect on spin-labelled fatty acids incorporated into phospholipid bilayers (Table I). Reconstitution of ATPase with mixtures of DOPC and dibromocholesterol results in fluorescence quenching, the extent of quenching observed at a 1 : 1 molar ratio of DOPC and dibromocholesterol being comparable to that observed with BRPC (Fig. 2B). This quenching requires incorporation of dibromocholesterol into the ATPase-membrane system: simple addition of dibromocholesterol to a preformed DOPC-ATPase complex had no effect on fluorescence emission.

TABLE I

EFFECT OF CHOLESTEROL ON SPIN-LABEL ORDER PARAMETERS

5-Doxyl stearic acid was incorporated into bilayers of egg yolk phosphatidylcholine containing sterol at the given molar ratio and at 18°C, and order parameters were calculated as described by Gaffney [12].

Sterol : lipid molar ratio	Order parameter, S	
	Cholesterol	Dibromocholestan-3β-ol
0	0.64	0.64
1 : 2	0.70	0.67
1 : 1	0.73	0.71

The effect of sterols on the activity of the ATPase depends markedly on the phospholipids present in the system. We find that although dibromocholesterol decreases the activity of the BRPC-ATPase system more markedly than does cholesterol, both cholesterol and dibromocholesterol increase the activity of the ATPase reconstituted with dimyristoleoylphosphatidylcholine (DMPC) from 6 to approx. 22 I.U. (Fig. 2A). It seems likely, therefore, that cholesterol and dibromocholesterol interact in an essentially similar manner with the ATPase. The fluorescence data then suggest two types of binding site on the ATPase: although cholesterol cannot bind at the phospholipid protein interface, it can bind at other, non-annular sites.

We observe a similar picture with the water-soluble cholesterol derivative, cholesterol hemisuccinate. Although cholesterol hemisuccinate has no effect on the fluorescence of a BRPC-ATPase complex at molar ratios of sterol to phospholipid of up to 4 : 1, dibromocholesterol hemisuccinate will quench protein fluorescence (Simmonds and Lee, unpublished observations).

Interactions with fatty acids

Two procedures were adopted for the reconstitution of the $(Ca^{2+} + Mg^{2+})$-ATPase with mixtures of fatty acids and lipid. Firstly, purified ATPase was incubated in buffer containing cholate with a large excess of a mixture of lipid and fatty acid, and then this mixture was diluted at least 500-fold into an assay mixture for determination of ATPase activity or into buffer for fluorescence measurements. Secondly, using the same procedure, the ATPase was reconstituted with lipid alone, and the fatty acid was then added to the lipid-ATPase complex as a concentrated solution in methanol. Comparable results were obtained using either procedure. Results are expressed in terms of the molar ratio of added fatty acid to phospholipid since it is not clear what the molar ratio will be in the membrane itself. Differential scanning calorimetry data suggest that up to two fatty acids per phospholipid can be incorporated

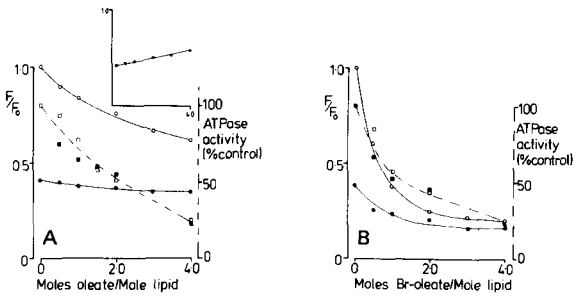


Fig. 3. A. The effect of oleic acid on the activity (broken line) of DOPC-ATPase (□) and BRPC-ATPase (■) and on the relative intensity of tryptophan fluorescence (solid line) of DOPC-ATPase (○) and BRPC-ATPase (●). The inset shows the ratio of fluorescence intensities oleic acid-BRPC-ATPase/oleic acid-DOPC-ATPase as a function of oleic acid concentration. B. The effect of dibromooleic acid on the activity (broken line) of DOPC-ATPase (□) and BRPC-ATPase (■) and on the relative intensity of tryptophan fluorescence (solid line) of DOPC-ATPase (○) and BRPC-ATPase (●).

into a lipid bilayer, but above that molar ratio there is some, undefined, change [11]. Assuming unlimited binding to the membrane, then using the measured binding constants for fatty acids to phospholipids (our unpublished results) we estimate that under these conditions approx. 96% of the oleic acid will be bound to the membrane.

As shown in Fig. 3, oleic acid inhibits ATPase reconstituted with either DOPC or BRPC. The effects can be reversed by addition of high concentrations of bovine serum albumin to complex the fatty acid. Addition of oleic acid to the DOPC-ATPase complex results in a decrease in fluorescence intensity for the tryptophan residues of the ATPase (Fig. 3A). Changes in fluorescence intensity for the ATPase have previously been observed on changing the phospholipid around the ATPase [4]. Importantly, however, addition of oleic acid to the BRPC-ATPase complex does not lead to an increase in fluorescence up to a molar ratio of fatty acid to phospholipid of 4 : 1, but, in fact, produces a 10% decrease in fluorescence. The net result is a small increase in the ratio of fluorescence intensities for the BRPC-oleic acid-ATPase system compared to the DOPC-oleic acid-ATPase system at any given molar ratio of oleic acid. For comparison of the relative fluorescence quenching curve with the simulated fluorescence curves of Fig. 1 it seems logical to express the mole fraction of fatty acid in the mixture in terms of total fatty acyl chains present, that is, mole fraction = (mol fatty acid)/(mol fatty acid + 2 × mol phospholipid). This gives a relative binding constant for the ATPase for oleic acid compared to that for dioleoyl phosphatidylcholine of no greater than approx. 0.36. Similar results are obtained using a spin-labelled phospholipid containing a [5,10]-nitroxide-labelled fatty acid as the quenching agent. As reported by London and Feigenson [5], this lipid causes a 67% quenching of the fluorescence of the ATPase, and addition of oleic acid up to a molar ratio of 3 : 1 causes no change in the extent of quenching.

TABLE II

EFFECT OF [1,14]-NITROXIDE-LABELLED STEARIC ACID ON THE FLUORESCENCE DECAY KINETICS OF THE (Ca^{2+} + Mg^{2+})-ATPase AT 365 nm

Concentration of fatty acid (μM)	Fitted function	A_1 [a]	τ_1 (ns)	A_2 [a]	τ_2 (ns)	X_u^2 [b]
0	Single exponential		4.36			8.4
	Double exponential	0.62	1.87	1.27	4.87	1.7
4	Single exponential		4.59			18.7
	Double exponential	0.99	2.68	0.64	6.14	1.1
14	Single exponential		4.37			17.1
	Double exponential	0.99	2.59	0.60	6.00	1.4

[a] Preexponential factor.
[b] Reduced x^2.

However, experiments with dibromooleic acid show a greater total fluorescence quenching than is observed with brominated phospholipids, suggesting that the dibromooleic acid has access to sites other than those available to the phospholipid (Fig. 3B). Similar quenching is caused by spin-labelled fatty acids [6].

Table II shows the effect of [1,14]-nitroxide-labelled stearic acid on the fluorescence decay kinetics of the ATPase. Although the decays for the ATPase are complex and fit badly to both single- and double-exponential functions, the kinetics are relatively little affected by addition of the nitroxide-labelled stearic acid, over a concentration range in which there is a marked reduction in fluorescence intensity [6]. This argues for quenching by a static mechanism.

Discussion

It is likely that fluorescence quenching in membranes will be largely static. It has been estimated that the lateral diffusion coefficient for a fatty acid or sterol in a phospholipid bilayer in the liquid-crystalline phase is approx. 10^{-7}–10^{-8} $cm^2 \cdot s^{-1}$, corresponding to a hopping frequency of approx. $3 \cdot 10^6$ s^{-1} [14]. Further, the exchange of phospholipids between the annulus around the ATPase and the bulk lipid is slow on the ESR timescale ($V_{ex} < 10^7$ s^{-1}), although fast on the NMR timescale ($V_{ex} > 10^4$ s^{-1}) [15]. As shown in Table II, the fluorescence decay time for the tryptophans of the ATPase is approx. 4 ns, so that exchange of molecules between the annulus and the bulk phase of the membrane is likely to be very slow on the fluorescence timescale. This conclusion is supported by the data in Table II which show no significant effect of the fatty acid [1,14]-nitroxide-labelled stearic acid on the decay characteristics of the fluorescence of the tryptophan residues of the ATPase, over a concentration range at which it causes a large reduction in the intensity of fluorescence. This is characteristic of quenching by a static mechanism (see Ref. 16). To a good first approximation it is therefore possible to consider quenching in these systems to arise from quenching molecules already in contact with the protein, rather than due to collisional quenching between the quencher and excited tryptophan residues.

The simplest explanation for the results presented here is that cholesterol is excluded from the lipid/protein interface of the ATPase but can bind at a second set of sites from which phospholipids are excluded. Whilst oleic acid can bind at the lipid/protein interface, its binding constant is less than that for dioleoylphosphatidylcholine, and it can also bind at non-annular sites. If the ATPase exists as an oligomer in the membrane as suggested, for example, by Ikemoto et al. [17], then the non-annular sites could be sites at protein/protein interfaces. Since the ATPase can be solubilized in an active and monomeric form in detergent (see Ref. 18), it is likely that the protein/protein interfaces are hydrophobic, consistent with binding of fatty acids and sterols. An alternative possibility for the non-annular sites would be between loops of polypeptide within the protein (MacLennan [19] has suggested that the protein makes eight passes through the membrane), but the properties of such possible sites are unknown. This picture is consistent with results obtained (our unpublished data) on the binding of dansyl undecanoic acid to the ATPase, where three high affinity fatty acid binding sites were detected per ATPase molecule. One possible arrangement of binding sites is shown in Fig. 4. The ATPase is represented as an oligomer (probably a dimer or

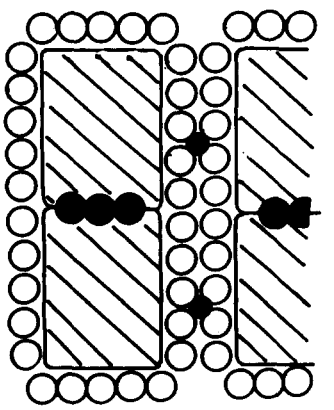


Fig. 4. Possible non-annular binding sites on the ATPase. For illustration, the ATPase is shown as a dimer. Binding sites (filled circles) for fatty acids and cholesterol are postulated to occur at the protein/protein interface and at sites between annular lipids. Binding at the lipid/protein interface is relatively unfavourable.

tetramer), surrounded by a phospholipid annulus of 30 phospholipid molecules per monomer, with the protein/protein interfaces providing a set of non-annular binding sites for hydrophobic compounds. If the loops of the ATPase passing through the membrane are α-helices, as suggested for bacteriorhodopsin [20], then it is possible that the non-annular sites occur at regions of intermonomer contact of the α-helices.

Unfortunately, it is not possible to provide a detailed model for the fluorescence quenching in these systems. Although quenching by bromines occurs by a contact mechanism, quenching of tryptophan residues not in contact with the bromines could occur by energy transfer to those that are. Since the distribution of tryptophans in the protein structure is unknown at the present time, detailed analysis is not possible. The main features of the quenching can, however, be represented by a simple but crude model. We consider the tryptophan residues in the ATPase to consist of two groups – group A quenched only by quenchers bound at the non-annular sites and group B quenched by quenchers bound at either the annular or the non-annular sites. When all the annular sites are occupied by brominated phospholipids, the relative fluorescence intensity is 0.4 [4], so that

$$F_{total} = 0.4F_A + 0.6F_B \quad (4)$$

where F_{total}, F_A and F_B are the total fluorescence intensity and fluorescence intensities from groups A and B, respectively. We assume that the quenching that results from binding at the non-annular sites is directly proportional to the degree of occupation of the sites, so that

$$F_A = 1 - \frac{x}{K_d + x} \quad (5)$$

Here K_d is the dissociation constant and x is the concentration of quencher expressed, for convenience, in terms of mole fraction of quencher in the membrane. Quenching that results from binding at the annular sites can be expressed from Eqns. 1–3 as

$$F' = [(1-x)/(1+x(K-1))]^n \quad (6)$$

where K is the relative binding constant at the lipid/protein interface and n is the number of quencher sites around an average tryptophan residue [4]. For a phospholipid with two fatty acyl chains we have found $n = 1.6$ so that for a fatty acid we can put $n \approx 3$. Quenching for tryptophans of group B will be the product of that arising from binding to both annular and non-annular sites, so that

$$F_{total} = 0.4F_A + 0.6F_A F' \quad (7)$$

For quenching by dibromooleic acid, we use the value for the relative binding constant obtained for oleic acid (0.36). This leaves one free parameter, K_d, in the simulation, Fig. 5 shows the simulated quenching curves obtained for $K_d = 0.7$ and agreement with the experimental data (Fig. 3B) is surprisingly good. Fig. 5 also shows a simulation of the quenching caused by dibromocholesterol, with no binding at the lipid/protein interface and with $K_d = 0.5$. Again, agreement is good.

It should be emphasized here that we are not suggesting a static model for the system. We have interpreted the fluorescence data in terms of an equilibrium distribution of molecules in the an-

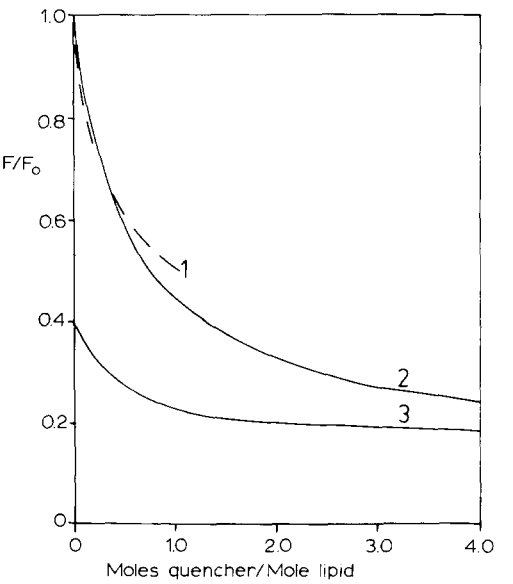


Fig. 5. Simulated fluorescence quenching plots. Simulations carried out as described in the text for: 1, dibromocholesterol on DOPC-ATPase; 2, dibromooleic acid on DOPC-ATPase; 3, dibromooleic acid on BRPC-ATPase.

nulus around the ATPase. The binding constant for cholesterol to the annular sites on the ATPase is considerably less than that for phosphatidylcholine, so that in a membrane containing a mixture of cholesterol and phosphatidylcholine, the majority of sites around the ATPase will, at any one time, be occupied by phosphatidylcholine. In this sense we say that cholesterol is excluded from the annulus. Differences in binding constant imply differences in on and off rate constants. Commonly, differences in off rate constants are greater than differences in on rate constants [21]. It is therefore possible that the relatively poor binding of cholesterol to the annulus of the ATPase predominantly reflects a higher off rate constant for cholesterol than for phosphatidylcholine.

When considering the effects of hydrophobic compounds on the activity of the ATPase, three types of binding site need to be considered – binding to the bulk lipids in the membrane, binding at the lipid/protein interface (annular binding sites) and binding to the protein at the non-annular sites. Binding at the lipid/protein interface is potentially important, since the activity of the ATPase is known to be highly dependent on the nature of the annular phospholipid [1–5], so that displacement of phospholipid by a hydrophobic compound would be expected to produce a large change in activity. A high selectivity of the annular sites for phospholipid would therefore act to 'buffer' the ATPase activity against changes that would otherwise be produced by the fortuitous presence of hydrophobic compounds in the vicinity of the membrane. Cholesterol is excluded from the annulus and has little effect on the activity of DOPC-ATPase, whereas when the ATPase is reconstituted with a phospholipid of suboptimal chain length such as dimyristoleoylphosphatidylcholine cholesterol greatly increases the activity of the ATPase.

For oleic acid, there is inhibition of activity for the ATPase reconstituted with dioleoylphosphatidylcholine (Fig. 3A) whereas for the ATPase reconstituted with dimyristoleoylphosphatidylcholine activity first increases and then decreases (data not shown). It is possible, therefore, that binding at the phospholipid/protein interface leads to a decrease in activity, whereas binding at non-annular sites can lead to an increase in activity, depending on the phospholipid in the system.

Finally, before comparing the results presented above for the effects of cholesterol and fatty acid on the $(Ca^{2+} + Mg^{2+})$-ATPase with other results in the literature, it is necessary to be clear about the exact physical conditions of the system employed. Native vesicles of sarcoplasmic reticulum prepared in the presence of reducing agent (1 mM dithiothreitol) have a $(Ca^{2+} + Mg^{2+})$-ATPase activity tightly coupled to Ca^{2+} uptake, and activity is low because of the build-up of Ca^{2+} within the vesicles. Vesicles prepared in the absence of a reducing agent tend to be partially uncoupled and so show a higher ATPase activity. Finally, if the vesicles are treated with a detergent such as cholate, then ATPase activity is fully uncoupled from the accumulation of Ca^{2+}, and the full ATPase activity is exhibited. Our studies have been with the fully uncoupled ATPase.

Madden et al. [22] have claimed that cholesterol markedly inhibits $(Ca^{2+} + Mg^{2+})$-ATPase activity. However, their studies were with partially uncoupled vesicles prepared in the absence of reducing agent [23,24] and it has been shown that in such a system the major effect of cholesterol is on the leakage of Ca^{2+} out of the vesicles, with relatively little effect on the ATPase itself [23]. Cholesterol has no effect on tightly coupled vesicles prepared in the presence of reducing agent [23,24].

Similar comments apply to studies of the effects of fatty acids on the $(Ca^{2+} + Mg^{2+})$-ATPase. Our studies have been with the fully-uncoupled ATPase and so reflect direct effects of fatty acids on the ATPase. Effects of fatty acids on ATPase activity in vesicles reflect effects on both Ca^{2+} uptake and Ca^{2+} release, and the complexity of such effects is shown, for example, in the studies of Katz et al. [25]. The low ATPase activities we observe at high molar ratios of fatty acid to phospholipid are largely in agreement with the studies of Fiehn and Hasselbach [26]. They observed that addition of oleic acid to vesicles of sarcoplasmic reticulum that had been depleted of phospholipid by phospholipase treatment caused an increase in ATPase activity to a level comparable to that observed for fully coupled vesicles. However, it is likely that after phospholipase treatment, ATPase activity is fully uncoupled from accumulation of Ca^{2+}, so that the more meaningful comparison would be

with the ATPase activity in a fully uncoupled system: the activity observed by Fiehn and Hasselbach [26] in the presence of oleic acid is very much less than that expected for the fully uncoupled system, consistent with the inhibition we observe for the fully uncoupled state.

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