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Effect of Phospholipid:Protein Ratio on the State of Aggregation of the $(Ca^{2+}$-$Mg^{2+})$-ATPase†

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ABSTRACT: The organization of the $(Ca^{2+}$-$Mg^{2+})$-ATPase has been studied in reconstituted systems by fluorescence polarization of the ATPase labeled with fluorescein isothiocyanate (FITC) and resonance energy transfer between ATPase labeled with FITC and with eosin isothiocyanate (EITC). The fluorescence polarization of FITC-ATPase was found to decrease with increasing labeling ratio FITC:ATPase, indicating depolarization as a result of resonance energy transfer between ATPase molecules. Fluorescence polarization was, however, independent of the molar ratio of phospholipid to protein above a molar ratio of 50:1. Resonance energy transfer between FITC-ATPase and EITC-ATPase was also found to be independent of phospholipid:protein ratio. It is suggested therefore that the ATPase is not randomly distributed in the plane of the membrane but rather forms ordered clusters (probably rows of monomers or dimers) on the fluorescence time scale (nanoseconds) even in the presence of a large excess of phospholipid. This organization within the membrane is dependent both on the chemical structure of the phospholipid and on its physical phase.

Half the surface area of a typical biological membrane is occupied by protein so that interaction between the protein components in the membrane is likely. In a membrane where the protein diffusion coefficient is high, the rate of collision between membrane proteins will be high because of the small distance of separation of the proteins (Poo & Cone, 1974). Nevertheless, calculations of the expected distribution of phospholipid and protein molecules in a membrane show that, for random mixing of the components, the probability of protein–protein contact at any given instant in time is low at a molar ratio of phospholipid to protein of 100:1, typical of most membranes (East et al., 1985). Of course, in real systems, the mixing of phospholipid and protein molecules is unlikely to be ideal (random) and the distribution of molecules within the membrane will depend on the relative strengths of the phospholipid–phospholipid, phospholipid–protein, and protein–protein interactions. Currently, there is no single method to define such distributions. Rather, it is necessary to employ a variety of techniques, the results of each of which alone may be ambiguous, but which together may allow the drawing of an unambiguous conclusion.

The sarcoplasmic reticulum of skeletal muscle is particularly suited to studies of this kind, because approximately 80% of the protein in the membrane is one protein, the $(Ca^{2+}$-$Mg^{2+})$-ATPase, and the $(Ca^{2+}$-$Mg^{2+})$-ATPase can be purified and reconstituted in an active form into bilayer systems where the ATPase is the only species of protein (Warren et al., 1974). Electron microscopic studies of negatively stained membranes have shown that the ATPase in sarcoplasmic reticulum of rabbit skeletal muscle is present as extended rows of dimeric species in the presence of vanadate (Taylor et al., 1984); the ATPase in the sarcoplasmic reticulum of scallop muscle adopts an analogous structure even in the absence of vanadate (Ferguson et al., 1985). In the presence of Ca^{2+}, the ATPase in rabbit sarcoplasmic reticulum adopts a structure consisting of rows of monomeric species (Dux et al., 1985). These studies, of course, present a picture of the organization of the membrane with all motion frozen. A dynamic picture of the membrane, on a millisecond time scale, has been developed from saturation transfer ESR studies of spin-labeled ATPase (Thomas & Hidalgo, 1978; Lewis & Thomas, 1986; Napier et al., 1987). These studies have shown that the ATPase has considerable rotational freedom within the membrane, even under conditions where electron micrographs show the presence

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of ordered arrays. We interpreted these observations in terms of short-lived (on the millisecond time scale) protein–protein interactions, with the formation of ordered clusters of ATPase molecules that form and melt rapidly (Napier et al., 1987).

Here we report on the use of fluorescence methods to study the organization of the ATPase in the membrane and the effect of the molar ratio of phospholipid to protein on this organization. Because of the short time scale of the fluorescence method (nanoseconds), fluorescence spectroscopy will give a picture of the membrane in which motion is frozen, like electron microscopy. Both resonance energy transfer (Vanderkooi et al., 1977; Papp et al., 1987) and fluorescence polarization (Highsmith & Cohen, 1987) have been used previously to study the state of aggregation of the ATPase in sarcoplasmic reticulum.

Materials and Methods

Dioleoylphosphatidylcholine (DOPC),[1] dimyristoleoylphosphatidylcholine (DMPC), and dipalmitoylphosphatidylcholine (DPPC) were from Lipid Products, Avanti Polar Lipids, and Sigma, respectively. Fluorescein 5′-isothiocyanate (FITC) and eosin 5′-isothiocyanate (EITC) were obtained from Aldrich and Molecular Probes, respectively.

(Ca^{2+}-Mg^{2+})-ATPase was purified from the hind leg muscle of rabbit as described in East and Lee (1982). Polyacrylamide gels of the ATPase stained with Coomassie Blue have shown that the ATPase is essentially pure (>97%) (Gould et al., 1987). The preparation contained 30 phospholipid molecules per ATPase, assuming a protein M_r of 110000.

ATPase was labeled with FITC or EITC as described in Froud and Lee (1986). ATPase (1.0 mg) in buffer (40 μL; 50 mM potassium phosphate, 1 M KCl, and 0.25 M sucrose, pH 8) was incubated with the required amount of FITC or EITC added from a stock solution in dry dimethylformamide (5 mM). The reaction was left to stand at room temperature for 1 h. Unbound label was then removed by two passages through 5-mL columns of coarse-grade Sephadex G-50, equilibrated in 0.2 M sucrose/50 mM Tris-HCl buffer, pH 7.0, contained in 5-mL disposable plastic syringes plugged with disks of porous polyethylene sheet (pore size 70 μm) (Belart); the columns were spun in a bench-top centrifuge at full speed for 2 × 1 min. Final labeling ratios were estimated with the extinction coefficient for the ATPase given by Hardwicke and Green (1974) and, following solubilization of samples in 1% SDS and NaOH (0.1 M), with extinction coefficients of 80000 for fluorescein at 500 nm (Pick & Karlish, 1980) and 83000 for eosin at 522 nm (Papp et al., 1987). Under the conditions described, labeling was found to be close to quantitative.

Labeled ATPase was reconstituted with the desired amount of exogenous phospholipid by solubilization in detergent, followed by rapid dilution into buffer (East & Lee, 1982). Briefly, the required amount of phospholipid was mixed with buffer (40 μL; 50 mM potassium phosphate, 1 M KCl, and 0.25 M sucrose, pH 8.0) containing $MgSO_4$ (5 mM), ATP (6 mM), and potassium cholate and sonicated to clarity in a bath sonicator (Megason); the cholate concentration was calculated on the basis of 1 mg of cholate/mg of protein plus 0.5 mg of cholate/mg of exogenous phospholipid. ATPase (0.125 mg) was then added, and the mixture was incubated at 5 °C for 1 h or, for DPPC, incubated at 45 °C for 15 min followed by 30 min at room temperature. After incubation, samples were diluted with buffer (200 μL) and stored on ice until use.

Measurements of fluorescence polarization were made with an Aminco-Bowman Fluorometer equipped with quartz Polacoat filters. Fluorescence was excited at 450 nm and observed at 520 nm. Fluorescence polarization was calculated from the equation

$$P = \frac{I_{0,0} - I_{0,90}(I_{90,0}/I_{90,90})}{I_{0,0} + I_{0,90}(I_{90,0}/I_{90,90})} \quad (1)$$

where the $I_{n,m}$ are the fluorescence intensities with the excitation and emission polarizers at n and m degrees respectively from vertical. A correction for light scatter was made for each $I_{n,m}$ by subtracting the value obtained for unlabeled ATPase reconstituted under identical conditions. Samples contained 0.55 μM ATPase in 40 mM Hepes–KOH, 0.1 M KCl, 5 mM $MgSO_4$, and 0.1 mM EGTA, pH 7.0 at 25 °C.

Measurements of resonance energy transfer were made with a Perkin-Elmer fluorometer, interfaced to an IBM microcomputer for data accumulation. Fluorescence was excited at 475 nm. Fluorescence spectra were fitted by using a modification of the program described in Rooney and Lee (1986), based on standard shape functions (Fraser & Suzuki, 1973). ATPase was labeled with FITC and EITC to give 1:1 labeling ratios, and FITC-ATPase and EITC-ATPase were reconstituted in a 1:3 ratio as described above and diluted for fluorescence measurements to give a total protein concentration of 0.29 μM in buffer (40 mM Hepes–KOH, 0.1 M KCl, 5 mM $MgSO_4$, 0.1 mM EGTA, pH 7.0) at 25 °C.

The fluorescence emission spectrum of FITC-ATPase was corrected by using the procedures described in Rooney and Lee (1986). The overlap integral J between the absorption and emission spectra of FITC-ATPase was calculated to be 9.26×10^{-14} M^{-1} cm^3. The distance R_0 (Å) (Förster, 1959) was calculated as

$$R_0 = (9.765 \times 10^3)(K^2 \cdot J \cdot Q_D \cdot n^{-4})^{1/6} \quad (2)$$

where Q_D is the quantum yield (0.65), n is the refractive index of the medium (1.33), and K^2 is an orientation factor put equal to $^2/_3$, the isotropic value (Gutierrez-Merino et al., 1987).

Radiolabeled phosphatidylcholine was prepared from egg yolk phosphatidylethanolamine (Lipid Products) and [^{3}H]-methyl iodide (Amersham) by the method of Smith et al. (1977) and purified by preparative thin-layer chromatography. For studies of the mixing of phospholipid and protein in the reconstitution process, the ATPase was reconstituted as described above by using [^{3}H]phosphatidylcholine. The reconstituted ATPase system was then resolved by centrifugation on a linear 5–40% (w/v) sucrose gradient containing 20% (v/v) glycerol, 40 mH Hepes–KOH, 0.1 M KCl, 5 mM $MgSO_4$, and 0.1 mM EGTA, pH 7.0 at 4 °C, for 18 h at 93000g. Samples were analyzed for protein by measurement of tryptophan fluorescence and for phospholipid by counting in Labscint (Lablogic).

Results

The purified ATPase was reconstituted by solubilization in cholate with exogenous phospholipid followed by dilution into buffer. The homogeneity of the reconstituted membranes was judged by determination of their buoyant densities in sucrose gradients as described by Lentz et al. (1985). Specific densities of the reconstituted membranes were calculated by using values

[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; FITC, fluorescein 5′-isothiocyanate; EITC, eosin 5′-isothiocyanate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SR, sarcoplasmic reticulum; $C_{12}E_8$, dodecyl octaethylene glycol monoether.

Table I: Specific Densities of the Reconstituted ATPase Determined by Sucrose Gradient Centrifugation[a]

phospholipid:protein ratio in reconstitution	calcd specific density (g/cm^3)	obsd specific density (g/cm^3)
530:1	1.09	1.075–1.083
780:1	1.07	1.068–1.075

[a] The ATPase was reconstituted as described under Materials and Methods with phosphatidylcholine and resolved by centrifugation on a linear 5–40% (w/v) sucrose gradient.

Table II: Effect of $C_{12}E_8$ on the Fluorescence Polarization of FITC-Labeled ATPase[a]

molar ratio FITC:ATPase	polarization P		molar ratio FITC:ATPase	polarization P	
	$-C_{12}E_8$	$+C_{12}E_8$ (68 mM)		$-C_{12}E_8$	$+C_{12}E_8$ (68 mM)
0.12	0.34	0.34	0.68	0.22	0.31
0.30	0.27	0.32	0.72	0.18	0.30
0.34	0.20	0.30			

[a] Samples of the purified ATPase at a molar ratio of phospholipid:protein of 30:1 were labeled with FITC to the given molar ratio of FITC to ATPase. Fluorescence polarization was then determined in either the presence or absence of 68 mM $C_{12}E_8$.

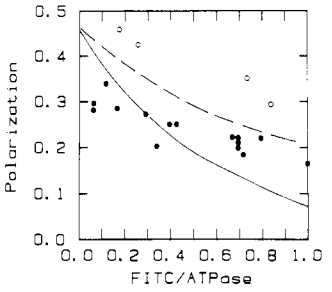

FIGURE 1: Polarization of purified FITC-ATPase (●) and FITC-SR (○) as a function of the molar ratio of bound FITC to ATPase at 25 °C. The lines show the dependence of fluorescence polarization on labeling ratio calculated for random mixing for FITC-ATPase (solid line) and FITC-SR (broken line).

of 1.03 g/cm^3 and 1.35 g/cm^3 for phospholipid and phospholipid-free ATPase, respectively (LeMaire et al., 1976) (Table I). Single symmetrical bands were observed on the gradients with no indication of the presence of any unincorporated ATPase.

Figure 1 shows the fluorescence polarization of FITC-labeled ATPase as a function of the molar ratio of FITC to protein. Figure 2 shows the fluorescence polarization for FITC-labeled ATPase reconstituted with DOPC as a function of the molar ratio of phospholipid to protein, at a variety of labeling ratios. For each labeling ratio the plot of polarization against phospholipid:protein ratio gave a good fit to a straight line as shown, with reduced χ^2 values of ca. 2×10^{-4} for all FITC labeling ratios except for the lowest (0.07:1) where it was 2×10^{-3}. The gradients (polarization/molar ratio of phospholipid to protein) of the best straight line fits varied between 0 and 2×10^{-4}. Addition of the detergent $C_{12}E_8$ to FITC-labeled ATPase increases fluorescence polarization at high labeling ratios, but has little effect at low labeling ratios.

As shown by Papp et al. (1987), both FITC and EITC label the ATPase at the ATP binding site and have excitation and emission spectra that make them a suitable pair of labels for use in energy transfer experiments. Figure 3 shows the fluorescence emission spectra of the ATPase labeled with FITC and EITC, excited at 475 nm. Unfortunately, as shown, the ATPase labeled with the fluorescence acceptor (EITC) shows significant fluorescence emission due to direct excitation at

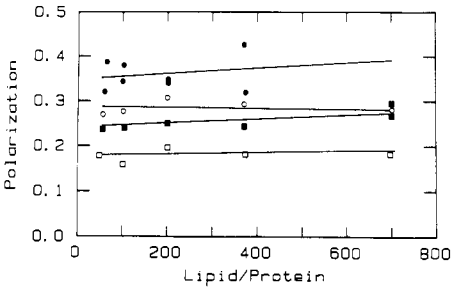

FIGURE 2: Polarization of FITC-labeled ATPase reconstituted in DOPC as a function of the molar ratio of phospholipid to protein. Molar ratios of bound FITC to ATPase: (●) 0.07; (○) 0.4; (■) 0.7; (□) 0.8. The straight lines represent best linear fits to the data.

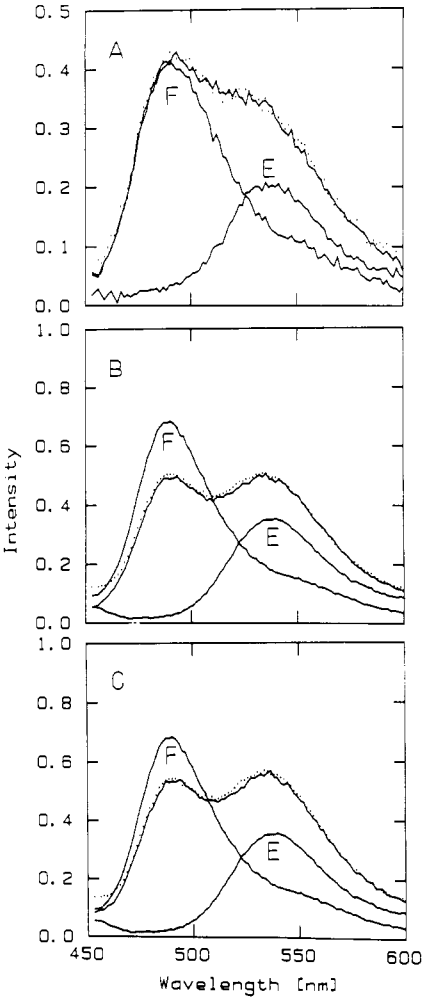

FIGURE 3: Fluorescence emission spectra excited at 475 nm for a 1:3 mixture of FITC-labeled and EITC-labeled ATPase, either mixed in the absence of cholate (A) or reconstituted in the presence of cholate and DOPC to give molar ratios of phospholipid to protein of 110:1 (B) or 880:1 (C). Shown are the emission spectra of the mixture and of samples containing FITC-labeled (F) and EITC-labeled ATPase (E) under identical conditions (solid lines) and the best fit to the data (dotted line) with the parameters given in Table III.

475 nm, which makes analysis of the efficiency of energy transfer between FITC-ATPase and EITC-ATPase more difficult. We have therefore chosen to analyze our results by fitting emission spectra of the composite system (FITC-ATPase plus EITC-ATPase) to linear sums of the emission spectra of FITC-labeled and EITC-labeled ATPase, according to

$$F(\lambda) = w_D D(\lambda) + w_A A(\lambda) \quad (3)$$

where $F(\lambda)$, $D(\lambda)$, and $A(\lambda)$ are the fluorescence intensities of the composite system, the donor, and the acceptor, re-

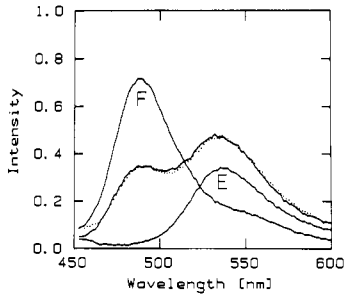

FIGURE 4: Fluorescence emission spectra excited at 475 nm for FITC-labeled (F) and EITC-labeled ATPase (E) reconstituted with DMPC at a molar ratio of phospholipid to protein of 110:1 and of a 1:3 mixture of FITC-labeled and EITC-labeled ATPase, also reconstituted at a molar ratio of phospholipid to protein of 110:1 (solid lines). The best fit to the data is shown by the dotted line, with the parameters given in Table III.

spectively, at the wavelength λ, and w_A and w_D are the weightings of acceptor and donor, respectively, that give the best fit to the composite spectrum. This procedure exploits maximally the information available in the spectra.

The quality of the fitting procedure is shown in Figure 3A. Here, 0.07 μM FITC-ATPase and 0.21 μM EITC-ATPase have been mixed in the absence of any detergent so that the resulting composite spectrum should be a simple sum of the spectra of 0.07 μM FITC-ATPase and 0.21 μM EITC-ATPase. As shown in Figure 3A, an excellent fit to the composite spectrum is obtained for a linear sum of the single-component spectra, the best fit values of w_D and w_A being 0.95 and 1.0, respectively. Panels B and C of Figure 3 show the emission spectra obtained when the same concentrations of FITC-ATPase and EITC-ATPase are reconstituted with DOPC at molar ratios of ATPase to phospholipid of 1:110 and 1:880, respectively. Under these conditions it is clear that the intensity of the FITC-ATPase emission is reduced and that of the EITC-ATPase emission is increased as a result of energy transfer. Good least-squares fits to sums of the two-component spectra are still obtained as shown in Figure 3B,C, with the parameters given in Table III. As shown in Figure 4, the fluorescence emission spectrum for the same concentrations of FITC-ATPase and EITC-ATPase reconstituted in unsaturated, short-chain phospholipid DMPC at a molar ratio of phospholipid to protein of 110:1 is significantly different from that obtained for reconstitution in DOPC (Figure 3B), with increased efficiency of resonance energy transfer; the best fit parameters are given in Table III. Spectra for the labeled ATPase reconstituted in DPPC are very similar to that shown in Figure 4; the best fit parameters are also given in Table III.

Discussion

The organization adopted by proteins in a biological membrane will depend on the relative strengths of the phospholipid–phospholipid, phospholipid–protein, and protein–protein interactions. Studies of a few relatively simple membranes have suggested that a nonrandom distribution of protein molecules may well be common. Thus, studies of sarcoplasmic reticulum using electron microscopy have shown that, under at least some conditions, the (Ca^{2+}-Mg^{2+})-ATPase adopts a structure consisting of rows of dimers, the rows being separated by phospholipid molecules (Ferguson et al., 1985; Taylor et al., 1984); a similar organization has been suggested for the (Na^+-K^+)-ATPase (Hebert et al., 1982) and for the acetylcholine receptor (Heuser & Salpeter, 1979). In a previous study we suggested that cholesterol also occurs in membranes as alternating rows of phospholipid molecules and phospholipid–cholesterol complexes (Rogers et al., 1979).

The aim of the studies presented here is to determine whether the aggregational state of the (Ca^{2+}-Mg^{2+})-ATPase depends on phospholipid:protein ratio and, in particular, whether at high phospholipid:protein ratios the ATPase is present in the membrane as a random distribution of small units such as monomers or dimers. The studies involve the comparison of energy-transfer-dependent processes such as fluorescence depolarization or donor–acceptor transfer observed in reconstituted membranes with the results expected for a random distribution. Förster's theory of energy transfer from an excited donor to a neighboring acceptor in three dimensions was extended to the two-dimensional case by Tweet et al. (1964) and Wolber and Hudson (1979). They showed that, for example, the fluorescence intensity F for a donor species in the presence of an acceptor could be related to the fluorescence intensity F_0 of the donor in the absence of acceptor by

$$F/F_0 = \int_0^\infty \exp(-\lambda)\, \exp(-\epsilon C \lambda^{1/3})\, d\lambda \qquad (4)$$

where C is the concentration of acceptor in units of acceptors per R_0^2, that is

$$C = R_0^2 c \qquad (5)$$

where c is the concentration of acceptors per Å^2 of membrane and

$$\epsilon = 4.254\,09 \qquad (6)$$

The equation can be integrated by using a 15-point Laguerre procedure (Lee, 1982). In the calculation of c, areas occupied by ATPase and phospholipid molecules in the plane of the membrane were taken as 1963 and 80 Å^2, respectively (Gutierrez-Merino et al., 1987).

Förster (1951) also showed that resonance energy transfer between identical molecules will lead to fluorescence depolarization and that fluorescence depolarization will thus be proportional to the probability of transfer. The fundamental quantity for such calculations is fluorescence anisotropy r (Lakowicz, 1983) related to the polarization p by the equation

$$r = 2P/(3 - P) \qquad (7)$$

The ratio r/r_0 of fluorescence anisotropy in the presence and absence of energy transfer will therefore also be given by eq 4 above. r_0 was taken to be 0.36, corresponding to a p_0 of 0.46 (Gutierrez-Merino et al., 1987; Highsmith & Cohen, 1987)

Figure 1 shows the measured fluorescence polarization of FITC-labeled ATPase as a function of the labeling ratio. Also shown are data for FITC-labeled sarcoplasmic reticulum calculated by assuming that 75% of the protein in these SR samples is ATPase and that only the ATPase is labeled by FITC; the data agree well with that published by Highsmith and Cohen (1987). As shown in Figure 1, agreement between the experimental data and the fluorescence polarizations calculated by assuming a random distr ution of monomeric ATPase molecules in the membrane is poor; in these calculations the phospholipid:protein ratios were taken as 30:1 and 90:1 for the purified ATPase and SR, respectively. The poor agreement indicates that the distribution of ATPase molecules in the membrane is nonrandom. As described in detail by Highsmith and Cohen (1987), it is not possible to determine the nature of this nonrandom distribution from these experiments, but the data would be consistent with a structure consisting of rows of dimers as suggested by electron microscopic data (Highsmith and Cohen, 1987). The lower fluorescence polarizations observed for FITC-ATPase (in which the phospholipid:protein ratio is 30:1) than for FITC-SR

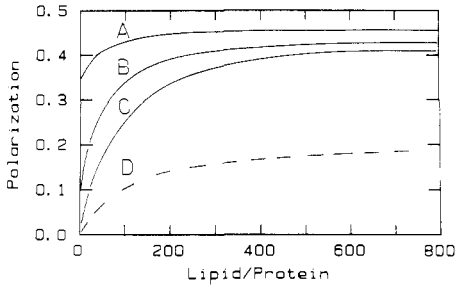

FIGURE 5: Fluorescence polarizations of FITC-ATPase as a function of phospholipid:protein ratio calculated assuming random mixing of monomeric ATPase and phospholipid (solid lines) or dimeric ATPase and phospholipid (broken line) for FITC:ATPase ratios of (A) 0.07, (B) 0.4, (C) 0.8, and (D) 0.8.

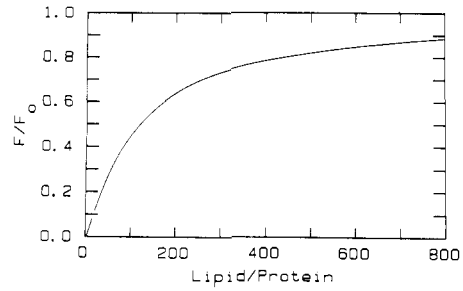

FIGURE 6: Fluorescence quenching F/F_0 for FITC-ATPase reconstituted with a 1:3 molar ratio of EITC-ATPase as a function of the phospholipid:protein ratio calculated assuming random mixing of monomeric ATPase and phospholipid.

(in which the phospholipid:protein ratio is 90:1) presumably follow from the lower phospholipid:protein ratios in the former, leading to increased energy transfer, as shown in the calculations for random mixing (Figure 1).

The fluorescence polarization of FITC-ATPase reconstituted into bilayers of DOPC is shown in Figure 2 as a function of phospholipid:protein ratio. The data show that above a phospholipid:protein ratio of 50:1, polarization is almost independent of phospholipid:protein ratio. Before this data can be interpreted, it is necessary to demonstrate that phospholipid and protein molecules are truly mixing in the reconstitution process. We therefore measured specific densities of the reconstituted systems, and as shown in Table I, the measured specific densities agree well with those calculated assuming complete mixing of phospholipid and protein.

The lack of effect on fluorescence polarization of dilution of FITC-ATPase with phospholipid shown in Figure 2 can be contrasted with the effect of the detergent $C_{12}E_8$ shown in Table II. Detergents have been shown to solubilize the ATPase in the form of small aggregates or monomers (Andersen et al., 1985; Silva & Verjovski-Almeida, 1985), and Highsmith and Cohen (1987) have shown that such solubilization increases fluorescence polarization for FITC-labeled SR. As shown in Table II, solubilization in $C_{12}E_8$ results in a large increase in fluorescence polarization for the ATPase labeled with a high molar ratio of FITC but no increase in polarization for the ATPase labeled at a low molar ratio. This is the result expected if fluorescence depolarization follows from energy transfer between neighboring FITC groups.

The lack of dependence of fluorescence polarization on phospholipid:protein ratio shown in Figure 2 can also be contrasted with that expected for random mixing of monomeric ATPase and phospholipid, as calculated from eq 4 (Figure 5). Another possibility worth considering is that of random mixing of dimeric ATPase species and phospholipid. The probabilities of only one, f_F, or both, f_{FF}, ATP sites on such a dimeric species being occupied by FITC are given by

$$f_F = 2y(1 - y) \quad (8)$$

and

$$f_{FF} = y^2 \quad (9)$$

where y is the fractional labeling of ATP sites. The fluorescence intensity of ATPase labeled with FITC increases linearly with the degree of labeling y (Froud & Lee, 1986) so that the fluorescence intensity of the doubly labeled dimeric species would have to be twice that of the singly labeled dimeric species. The fractions of fluorescence f_1 and f_2 coming from the singly and doubly labeled species respectively are then

$$f_1 = 1 - y \quad (10)$$

$$f_2 = y \quad (11)$$

The fluorescence anisotropy r of a mixture of isolated singly and doubly labeled dimers is then given by

$$r = r_1(1 - y) + r_2 y \quad (12)$$

where r_1 and r_2 are the fluorescence anisotropies of the singly and doubly labeled species, respectively. This equation can then be fitted to the experimental data at high phospholipid:protein ratios (700:1; Figure 2), after the latter has been corrected for the fluorescence depolarization due to energy transfer that occurs even at these high phospholipid:protein ratios, calculated from eq 4 to be 10%. The experimental data are fitted reasonably well with fluorescence anisotropies of 0.36 and 0.10 for the singly and doubly labeled species, giving calculated polarizations of 0.44, 0.34, 0.25, and 0.22 at labeling ratios (FITC:protein) of 0.07, 0.4, 0.7, and 0.8, respectively. At lower phospholipid:protein ratios, energy transfer between the postulated dimers will be more extensive and again should be given by eq 4. Figure 5 shows the expected dependence of fluorescence polarization on the phospholipid:protein ratio for the dimer model for a labeling ratio of 0.8. An identical profile would be obtained for random mixing of phospholipid and any other small aggregate of the ATPase, such as trimers or tetramers.

The simplest explanation for the data shown in Figure 2 is thus that the ATPase exists in these membranes in the form of extended aggregates and that the state of aggregation is independent of the phospholipid:protein ratio above a phospholipid:protein ratio of 50:1. This is also consistent with the data on energy transfer between FITC-ATPase and EITC-ATPase (Table III). For random mixing of monomeric FITC-ATPase and EITC-ATPase, the efficiency of energy transfer (as given by the quenching of donor fluorescence) as a function of phospholipid:protein ratio should follow eq 4 as shown in Figure 6. In fact, the efficiency of energy transfer is independent of phospholipid:protein ratio at ratios of 140:1 and above (Table III).

It is known that the distribution of ATPase molecules between the two surfaces of the reconstituted membranes is random (Gould et al., 1987), unlike the unique orientation found in native sarcoplasmic reticulum. However, the similar fluorescence polarizations found in SR and in the reconstituted systems argue for similar structures, and Lentz et al. (1985) observed rows of ATPase molecules in reconstituted systems using freeze–fracture electron microscopy. Further, Dux and Martonosi (1983) observed extensive crystallization of the detergent-purified ATPase in the presence of vanadate, suggesting a possible selective association between ATPase molecules of uniform orientation (Martonosi, 1984).

The nature of these extended aggregates cannot be determined from the experiments reported here. However, the similarity between the fluorescence polarization values in SR

Table III: Resonance Energy Transfer between FITC-ATPase and EITC-ATPase in Reconstituted Systems Containing a 1:3 Molar Ratio of FITC-ATPase to EITC-ATPase at 25 °C[a]

phospholipid	molar ratio phospholipid:protein	F/F_0 fluorescein[b]	F/F_0 eosin[c]
DOPC[d]	140:1	0.70	1.12
	250:1	0.76	1.12
	470:1	0.74	1.15
	910:1	0.74	1.18
DMPC[d]	140:1	0.47	1.22
	250:1	0.43	1.28
	470:1	0.40	1.37
	910:1	0.44	1.44
DPPC[e]	140:1	0.49	1.30
	250:1	0.46	1.35
	470:1	0.54	1.46
	910:1	0.49	1.42

[a] Fluorescence excited at 475 nm. [b] F/F_0 is the fluorescence intensity of FITC-ATPase reconstituted with EITC-ATPase relative to that of FITC-ATPase reconstituted in the absence of EITC-ATPase. [c] F/F_0 is the fluorescence intensity of EITC-ATPase reconstituted with FITC-ATPase relative to that of EITC-ATPase reconstituted in the absence of FITC-ATPase. [d] In the liquid-crystalline phase. [e] In the gel phase.

and in the reconstituted systems (Figures 1 and 2) suggests that the structure adopted in the reconstituted system could consist of rows of ATPase molecules separated by rows of phospholipid molecules, the rows of ATPase molecules consisting of either monomers or dimers, as suggested for sarcoplasmic reticulum (Dux et al., 1985; Ferguson et al., 1985; Taylor et al., 1984). The results presented in Table III suggest that the detailed organization in the membrane is dependent on the phospholipid component of the membrane. The greater energy transfer observed between FITC-ATPase and EITC-ATPase in DPPC in the gel phase than in DOPC in the liquid-crystalline phase suggests either that more ordered rows of ATPase molecules are present in gel-phase phospholipid or that the rows are more closely spaced; the latter would be consistent with the observations from freeze–fracture electron microscopy that ATPase molecules are excluded from gel-phase phospholipid (Kleemann & McConnell, 1976; Lentz et al., 1985). The greater energy transfer observed for the ATPase reconstituted with the short-chain phospholipid DMPC would also suggest a more nonrandom distribution in bilayers of this phospholipid than in DOPC.

Finally, it should be emphasized that such structures will be dynamic on a time scale longer than the nanosecond time scale characteristic of the fluorescence technique. Indeed, measurements using saturation transfer ESR spectroscopy suggest that rotation of the ATPase molecules on a millisecond time scale, either as monomers or as small aggregates, is relatively free (Napier et al., 1987).

Registry No. ATPase, 9000-83-3; DOPC, 10015-85-7; DMPC, 13699-48-4; DPPC, 2644-64-6.

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