Interaction of Fatty Acids with the Calcium–Magnesium Ion Dependent Adenosinetriphosphatase from Sarcoplasmic Reticulum†

A. G. Lee,* J. M. East, O. T. Jones, J. McWhirter, E. K. Rooney, and A. C. Simmonds

ABSTRACT: The fluorescence emission spectrum of dansylundecanoic acid is sensitive to the environment and appears at a lower wavelength when the fatty acid is bound to protein than when it is bound to phospholipid. When bound to the (Ca^{2+}–Mg^{2+})-ATPase of sarcoplasmic reticulum, the emission spectrum can be resolved into separate components assigned to fatty acid bound to protein and to lipid. Efficiency of energy transfer from the tryptophan residues of the ATPase to dansylundecanoic is higher for protein-bound probe than for lipid-bound probe. Fluorescence titrations are consistent with three fatty acid binding sites per ATPase with a K_d of 7 μM, and these sites are postulated to occur at the protein–protein interface in ATPase oligomers. Fatty acid incorporated into the lipid component of the membrane appears to be bound outside the lipid annulus around the protein.

An important question in membrane biochemistry concerns the nature of lipid–protein and protein–protein interactions in the membrane. It is known that the activity of a membrane protein such as the (Ca^{2+}–Mg^{2+})-ATPase of sarcoplasmic reticulum is sensitive to the nature of the surrounding lipid (Warren et al., 1974a; Bennett et al., 1978; Johannsson et al., 1981; East & Lee, 1982). However, the ATPase shows little selectivity between the various phospholipid classes, although it does show a relatively large preference for lipid in the liquid-crystalline phase over that in the gel phase (London & Feigenson, 1981; East & Lee, 1982). Elsewhere, we have shown that these selectivity measurements can be made conveniently by using the fluorescence quenching properties of brominated phospholipids (East & Lee, 1982). We have shown that brominated phospholipids are only weakly displaced from the annulus by fatty acids, so that presumably fatty acids tend to be excluded from the lipid–protein interface (A. C. Simmonds, J. M. East, O. T. Jones, J. McWhirter, and A. G. Lee, unpublished experiments). Nevertheless, we have observed that brominated and spin-labeled fatty acids can quench the tryptophan fluorescence of the ATPase, so that they can make contact with the protein at other types of site. These could be sites within the protein, or, more likely, sites between ATPase molecules if, as has been suggested, the ATPase is a dimer or higher oligomer within the membrane [see Ikemoto et al. (1981) and Moller et al. (1982)]. These fluorescence quenching studies are not readily quantitated in terms of numbers of binding sites. We have, therefore, developed an alternative fluorescence technique that allows further study of these fatty acid binding sites on the ATPase. We make use of the environmental sensitivity of the fluorescence of the dansyl fluorophor to distinguish between fatty acid bound to the phospholipid component of the membrane and fatty acid bound directly to the ATPase (Lee et al., 1981).

Materials and Methods

Lipids were obtained from Lipid Products and dansylundecanoic acid was obtained from Molecular Probes. (Ca^{2+}–Mg^{2+})-ATPase was prepared from female rabbit (New Zealand White) hind leg muscle as described in East & Lee (1982). Polyacrylamide gels showed the presence of essentially pure ATPase (>95%), and the protein/phospholipid ratio of 1/30 was in agreement with earlier reports (Warren et al., 1974b). ATPase activity was measured as outlined by Warren et al. (1974b) except that the buffer was 40 mM Hepes (pH 7.2) and the Ca^{2+} concentration was maintained at the optimal value (pCa^{2+} = 6.0) with 1.01 mM EGTA and 0.91 mM $CaCl_2$. Protein was estimated either by the biuret method or using the extinction coefficient given by Hardwicke & Green (1974). Fluorescence spectra were recorded on Spex Fluorolog and Perkin-Elmer MPF 44A fluorometers, digitized with a 12-bit A/D converter, accumulated, smoothed, and stored in a Z-80 based microcomputer system. Fluorescence probes were added as methanol solutions, the final methanol concentration never exceeding 1%. Methanol at this concentration had no effect on ATPase activity. The buffer for fluorescence measurements was 40 mM Hepes–0.1 M NaCl–0.1 mM EDTA, pH 7.2.

Fluorescence emission spectra for dansylundecanoic acid in a variety of organic solvents were found not to exhibit a simple Gaussian form, even when plotted on a wavenumber scale. Rather, the shape could be represented accurately by a skewed Gaussian of the form used previously to describe the shape of absorption bands (Siano & Metzler, 1969):

$$A = A_0 \exp[-(\ln 2)[\ln [1 + 2b(X - X_0)/\Delta X_{1/2}]^2/b]]$$

in the limit $2b(X - X_0)/\Delta X_{1/2} > -1$ and for $2b(X - X_0)/\Delta X_{1/2} < -1$, $A = 0$.

Here A_0 is the maximum peak height, X_0 is the wavelength at the peak maximum, $\Delta X_{1/2}$ is the bandwidth at half-height, and b is the skew parameter, with positive values of b giving positively skewed Gaussians and negative values of b giving negatively skewed Gaussians.

Fluorescence emission spectra for dansylundecanoic acid bound to (Ca^{2+}–Mg^{2+})-ATPase were found not to correspond to a single emitting species but rather to a sum of two or three species. We fitted these spectra using nonlinear least-squares techniques, making use of our observation (see below) that the emission spectra for dansylundecanoic acid bound to lipids depend only slightly on the chemical nature of the lipid. Our procedure was as follows. First, we fitted the spectrum of dansylundecanoic acid in buffer alone to a single skewed Gaussian peak. Second, we fitted the spectrum of dansylundecanoic acid bound to lipids to the sum of two peaks, one corresponding to probe bound to lipid and the other to probe

† From the Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton, S09 3TU, U.K. *Received June 10, 1982.* The Science Research Council, the Medical Research Council, the Smith Kline & French Foundation, and the British Muscular Dystrophy Group provided financial support.

0006-2960/82/0421-6441$01.25/0

Table I: Fluorescence Properties for Dansylundecanoic Acid (4.6 μM) from Least-Squares Fits of Spectra

system	component	intensity	peak position (nm)	bandwidth (nm)	skew factor	SD of fit
buffer	water		557.5	99.0	0.18	0.013
dioleoylphosphatidylcholine (130 μM)	lipid	1.463	534.5	110.0	0.18	
	water	0.089	557.5	99.0	0.18	0.013
egg phosphatidylethanolamine	lipid	1.051	532.0	117.0	0.18	
(130 μM)	water	0.259	557.5	99.0	0.18	0.010
brain phosphatidylserine (130 μM)	lipid	0.929	522.1	130.1	0.18	
	water	0.168	557.5	99.0	0.18	0.020
dioleoylphosphatidylcholine +	lipid	0.971	536.0	113.0	0.18	
cholesterol (1:1) (130 μM)	water	0.222	557.5	99.0	0.18	0.010
bovine serum albumin (2 mg/mL)	protein	2.802	508.4	105.0	0.20	
	water	0.286	557.5	99.0	0.18	0.017
(Ca^{2+}-Mg^{2+})-ATPase, excited at 280	protein	1.534	476.0	89.0	0.4	
nm (0.2 mg/mL)	lipid	0.146	534.5	111.0	0.18	0.012
(Ca^{2+}-Mg^{2+})-ATPase, excited at 347	protein	0.77	476.0	89.0	0.4	
nm (0.2 mg/mL)	lipid	0.30	534.5	111.0	0.18	0.012
	water	0.20	557.5	99.0	0.18	
sarcoplasmic reticulum, excited at	protein	1.257	476.0	89.0	0.4	
280 nm (0.2 mg/mL)	lipid	0.390	534.5	111.0	0.18	0.012
sarcoplasmic reticulum, excited at	protein	0.868	476.0	89.0	0.4	
347 nm (0.2 mg/mL)	lipid	1.004	534.5	111.0	0.18	0.017

free in buffer, keeping the parameters for the height, width, and skew of the spectrum for probe free in buffer fixed at the previously determined values. Last, we fitted the spectrum of dansylundecanoic acid bound to (Ca^{2+}–Mg^{2+})-ATPase to the sum of three peaks. Parameters for two of these peaks were fixed at the previously determined values for probe in lipid and water, with the parameters for the third peak, assumed to be due to probe bound to sites on the protein, free. The correctness of the fitted values was checked both by their constancy throughout titrations with dansylundecanoic acid and by their invariance when fluorescence was excited directly (at 347 nm) or indirectly through the tryptophan of the ATPase (at 280 nm).

Our analysis shows that the fluorescence emission spectrum for dansylundecanoic acid bound to (Ca^{2+}–Mg^{2+})-ATPase when excited at 280 nm corresponds largely to a single species, assumed to be protein-bound probe (see below), so that fluorescence intensity at 480 nm can be used to monitor probe binding to the ATPase. The fluorescence titration data were fitted directly to

$$F = \alpha([L_0] - [EL]) + \beta[EL] \quad (1)$$

where α and β are coefficients describing the fluorescence intensity of probe in aqueous solution and bound to the ATPase, respectively, $[L_0]$ is the total probe concentration, and [EL] is the concentration of bound probe, given by

$$[EL] = A - (A^2 - 4n[E_0][L_0])^{1/2}/2 \quad (2)$$

with

$$A = K_d + n[E_0] + [L_0] \quad (3)$$

Here K_d is the dissociation constant, n is the number of binding sites, and $[E_0]$ is the enzyme concentration. The data were fixed by a derivative-free nonlinear least-squares technique (Dixon & Brown, 1979).

Results

The fluorescence emission spectra of a variety of dansyl derivatives are sensitive to environment, emission maxima shifting to shorter wavelength with decreasing solvent polarity. Although the spectra cannot be described as simple Gaussian peaks, even when plotted on a wavelength scale, they can be accurately fitted to skewed Gaussians, with a reasonable correlation between the skew parameter b and the position of the emission maximum (A. G. Lee et al., unpublished observations). Fluorescence spectra for dansylundecanoic acid bound to a variety of biological systems can also be described by skewed Gaussian peaks. Figure 1 shows a fit of the spectrum of dansylundecanoic acid bound to liposomes of dioleoylphosphatidylcholine to two peaks, one characteristic of lipid and the other of water: the skew parameter, peak width, and peak position for dansylundecanoic acid in water were fixed at the values previously determined. The fit is good, and the value of the skew parameter b for the lipid-bound fatty acid fits the correlation found between skew parameter and emission maximum in organic solvent. The fluorescence emission observed for dansylundecanoic acid bound to liposomes is only slightly dependent on the chemical composition of the liposomes, spectra for probe bound to phosphatidylethanolamine, phosphatidylserine, or phosphatidylcholine in the presence of cholesterol all being very similar (Table I). However, a significantly different spectrum is observed for dansylundecanoic acid bound to bovine serum albumin (Table I). The skew parameters, peak widths, and peak positions that gave the best fit to the spectra were unaltered throughout the course of titrations with dansylundecanoic acid.

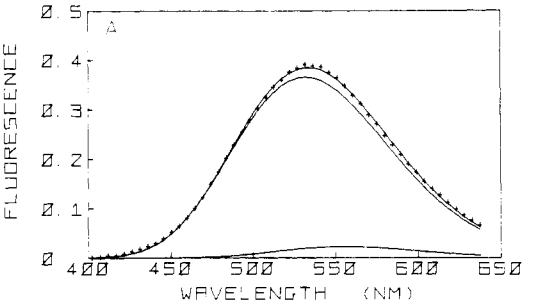

FIGURE 1: Fluorescence emission spectrum excited at 347 nm for dansylundecanoic acid (4.6 μM) in the presence of dioleoylphosphatidylcholine (130 μM): (+) experimental points. Solid lines: nonlinear least-squares best fit to the data and the two components of the fit.

As we prepare it (East & Lee, 1982), the (Ca^{2+}–Mg^{2+})-ATPase system has a lipid to protein ratio of 30 to 1. The fluorescence emission spectrum of dansylundecanoic acid bound to (Ca^{2+}–Mg^{2+})-ATPase is shown in Figure 2. When fluorescence is excited at 280 nm (where dansylundecanoic acid has no absorption peaks), fluorescence from the tryptophan residues of the ATPase is reduced, and fluorescence appears centered at ca. 480 nm, characteristic of the dansyl

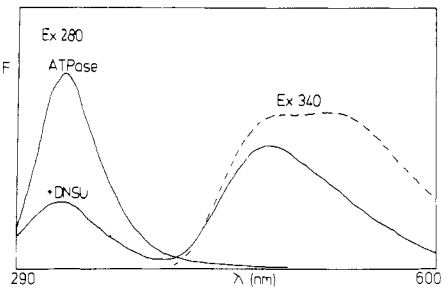

FIGURE 2: Fluorescence emission spectrum for dansylundecanoic acid bound to (Ca^{2+}–Mg^{2+})-ATPase. Dashed line: excited at 340 nm. Solid lines: fluorescence emission of tryptophan residues of (Ca^{2+}–Mg^{2+})-ATPase excited at 280 nm in the absence and presence of dansylundecanoic acid.

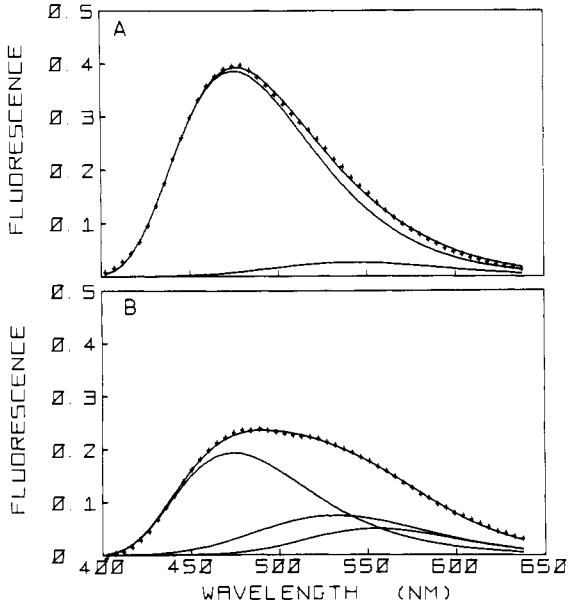

FIGURE 3: (A) Fluorescence emission spectrum excited at 280 nm for dansylundecanoic acid (4.6 μM) bound to (Ca^{2+}–Mg^{2+})-ATPase (0.2 mg/mL): (+) experimental points. Solid lines: best fit to the experimental data and the two components, with the skew parameter, peak width, and peak position of the lipid peak fixed. (B) Fluorescence emission spectrum excited at 347 nm for dansylundecanoic acid (4.6 μM) bound to (Ca^{2+}–Mg^{2+})-ATPase (0.2 mg/mL): (+) experimental points. Solids lines: best fit to the experimental data and the three components of the fit, characteristic of protein, lipid, and water, respectively. The skew parameters, peak widths, and peak positions of the lipid and water peaks were fixed.

group, consistent with fluorescence energy transfer between tryptophan and dansyl groups. The spectrum due to emission from the dansyl group can be fitted to the sum of two peaks, a major peak at short wavelengths and a minor peak corresponding to probe bound to lipid (Figure 3A). When fluorescence is excited directly at 340 nm, the spectrum is clearly composed of more than one major peak, with increased intensity at longer wavelengths (Figure 2). The spectrum can now be fitted to the sum of three peaks, one at short wavelength and two characteristic of probe bound to lipid and in the aqueous phase, respectively (Figure 3B). Importantly, the peak parameters that best fitted the spectra excited at 280 and 347 nm were the same (Table I), and these parameters did not vary during the course of titrations with dansylundecanoic acid. We attribute the peak at short wavelength to probe bound directly to the ATPase, since its intensity is considerably greater, relative to the lipid-bound component, when fluorescence is excited at 280 nm than when it is excited at 347 nm: fluorescence energy transfer is strongly dependent on distance. The skew parameter for this peak is again con-

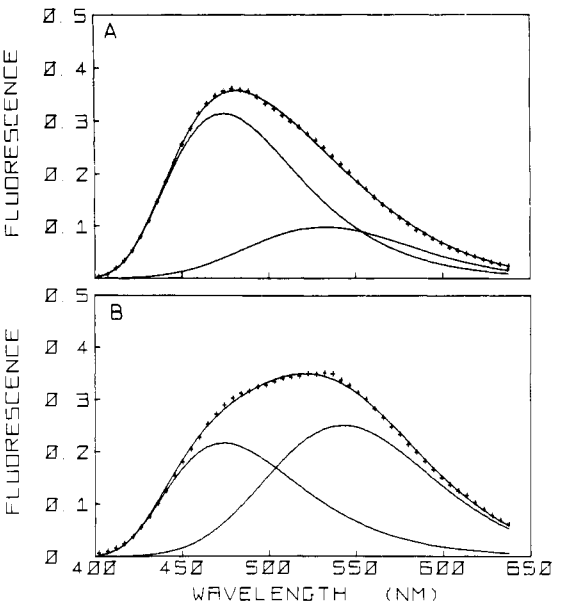

FIGURE 4: (A) Fluorescence emission spectrum excited at 280 nm for dansylundecanoic acid (4.6 μM) bound to sarcoplasmic reticulum (0.2 mg/mL): (+) experimental points. Solid lines: best fit to the experimental data and the two components of the fit, characteristic of protein and lipid, respectively. (B) Fluorescence emission spectrum excited at 347 nm for dansylundecanoic acid (4.6 μM) bound to sarcoplasmic reticulum (0.2 mg/mL): (+) experimental points. Solid lines: best fit to the experimental data and the two components of the fit, characteristic of protein and lipid, respectively.

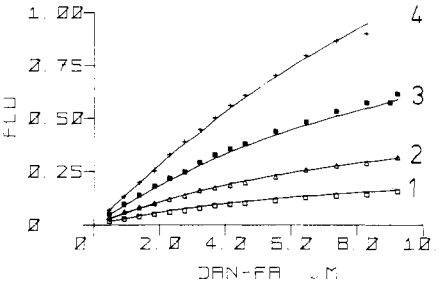

FIGURE 5: Fluorescence titration for dansylundecanoic acid with (Ca^{2+}–Mg^{2+})-ATPase at 23 °C, exciting at 280 nm and detecting fluorescence at 480 nm, in buffer (40 mM Hepes–0.1 M NaCl–0.1 mM EDTA, pH 7.2). Concentration of ATPase (based on a molecular weight of 119 000): 1, 0.28 μM; 2, 0.57 μM; 3, 1.14 μM; 4, 2.27 μM. Points: experimental. Solid lines: best fit to the binding equation, with parameters given in Table II.

sistent with the correlation established for organic solvents.

In the (Ca^{2+}–Mg^{2+})-ATPase preparation, the lipid/protein molar ratio is approximately 30/1, compared with a ratio of 90/1 in the original sarcoplasmic reticulum. Fluorescence emission spectra for dansylundecanoic acid bound to sarcoplasmic reticulum would therefore be expected to show a much larger lipid component in the spectrum than is observed for (Ca^{2+}–Mg^{2+})-ATPase. As shown in Figure 4 this is observed experimentally. Again, the same peak parameters provided an excellent fit to the data (Table I), lending further weight to our assignment of the spectral components.

If the fluorescence intensity at 480 nm for dansylundecanoic acid bound to (Ca^{2+}–Mg^{2+})-ATPase is predominantly due to protein-bound probe, when fluorescence is excited at 280 nm, then it can be used to monitor this binding. Figure 5 shows the results of a fluorescence titration with dansylundecanoic acid. A complication in the analysis of this data concerns the appreciable binding of dansylundecanoic acid to the lipid component of the membrane, which means that the concen-

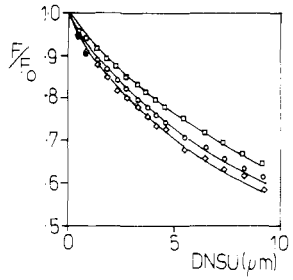

FIGURE 6: Quenching of tryptophan fluorescence of the ATPase by dansylundecanoic acid, at ATPase concentrations of (□) 2.27, (○) 1.29, and (◇) 0.64 μM. Solid lines: quenching curves calculated as described in the text.

tration of fatty acid free to bind to the ATPase will be less than the concentration of fatty acid added to the system. We have taken this factor into account as follows. First, we ignore depletion of the fatty acid by binding to lipid and simply fit the fluorescence data by a nonlinear least-squares procedure to the binding equation (eq 1). For a titration at 23 °C, for example, the parameters giving a best fit to the data are fluorescent enhancement factor $\beta = 0.277$, dissociation constant $K_d = 6.47$, and number of binding sites per ATPase $n = 3.38$. These approximate parameters show that up to 50% of the fatty acid will be bound to the ATPase at the highest protein concentration we use (2.27 μM) whereas only 10% will be bound at the lowest ATPase concentration (0.28 μM). We then assume that binding to the lipid component of the membrane is described by the same binding parameters as describe binding to dioleoylphosphatidylcholine (see below). By use of these parameters (E. K. Rooney, J. M. East, O. Jones, J. McWhirter, A. C. Simmonds, and A. G. Lee, unpublished experiments) at 2.27 μM ATPase (which at a 30/1 lipid/protein molar ratio is equivalent to 68 μM lipid) 13% of the free fatty acid will be bound to lipid, whereas at 0.28 μM ATPase only 2% will be so bound. Since at 2.27 μM ATPase up to 50% of the fatty acid will be bound to the ATPase, the net result is that 7% of the total fatty acid will be bound to lipid and about 1% at 0.28 μM ATPase. In this way, the concentrations of fatty acid are modified for lipid binding and the data refitted to eq 1. The effect was to increase the value of β and decrease n to 3.10 binding sites per ATPase, with an increase in K_d to 6.90. The residual sum of squares of the fit was not significantly altered. This iterative procedure was again repeated by using the new protein binding values to calculate new concentrations of fatty acid bound to ATPase and thus new values for fatty acid concentration bound to lipid, to give $n = 3.06$ and $K_d = 7.06$. Further iterations produced only insignificant changes. The estimated asymptotic correlation between n and K_d was only 0.16.

These same binding parameters can be used to describe the quenching of the tryptophan fluorescence of the ATPase caused by the binding of dansylundecanoic acid (Figure 6). It is assumed that the quenching of fluorescence by binding of dansylundecanoic acid is highly efficient, so that the extent of quenching is directly proportional to the number of occupied sites on the ATPase. Fluorescence quenching can then be described by

$$F/F_0 = x + (1 - x)f$$

where F and F_0 are fluorescence intensities in the presence and absence of dansylundecanoic acid, respectively, f is the fraction of unoccupied sites on the ATPase, and x is the fraction of the tryptophan fluorescence that is unquenched when all the sites are occupied. The fraction of occupied sites can be calculated directly from the parameters given in Table II. The only unknown is, therefore, x. As shown in Figure 6, excellent agreement is obtained with $x = 0.2$. Interestingly, London & Feigenson (1981) found that 80% of the tryptophan fluorescence of the ATPase could be quenched by spin-labeled phospholipids.

Table II: Binding Parameters for Dansylundecanoic Acid to (Ca^{2+}-Mg^{2+})-ATPase

temp (°C)	no. of binding sites per ATPase, n	dissociation constant, K_d (μM)	fluorescence enhancement, β
	Excitation Wavelength 280 nm		
23	3.06 ± 0.13	7.06 ± 0.63	0.334 ± 0.016
35	3.01 ± 0.14	6.41 ± 0.65	0.253 ± 0.014
	Excitation Wavelength 347 nm		
23	3.47 ± 0.21	7.15 ± 0.92	1.74 ± 0.11

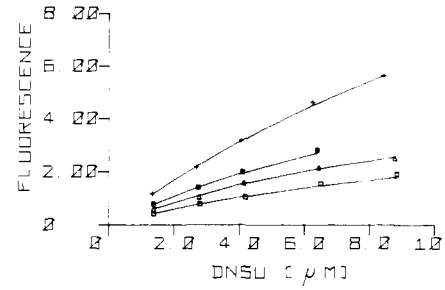

FIGURE 7: Fluorescence titration for dansylundecanoic acid with (Ca^{2+}–Mg^{2+})-ATPase at 23 °C, exciting at 347 nm and plotting the height of the peak attributed to protein-bound fatty acid obtained by nonlinear least-squares fitting. Concentration of ATPase: (+) 2.24 μM; (■) 1.12 μM; (▲) 0.84 μM; (□) 0.56 μM. Points: experimental. Solid lines: best fit to the binding equation, with parameters given in Table II.

Values of binding constants were also obtained from the fluorescence spectra excited at 347 nm. Now there is no part of the emission spectrum that can be attributed solely to the protein-bound component, and thus it is necessary to use the nonlinear least-squares technique to separate the components of the spectrum. Our procedure was as follows. We fitted the spectra to a sum of two components, one characteristic of the assumed protein-bound component with values of skew, width, and position fixed at the values given in Table I. The second component thus represents fatty acid both bound to lipid and free in the aqueous phase: because of the similar peak positions for lipid-bound and free fatty acid, a reliable and consistent separation of these two components could not always be obtained. Values of the peak height of the protein-bound component obtained in this way are shown in Figure 7. Again, the fluorescence data were fitted to the simple binding equation (eq 1) by assuming that binding to the lipid component of the membrane could be described by the same parameters that describe binding to dioleoylphosphatidylcholine. The best fit parameters are given in Table II. Agreement with the previous values is generally good, although the number of binding sites (3.47) is somewhat higher.

As described elsewhere (E. K. Rooney et al., unpublished experiments) it is not possible to obtain accurate parameters to describe the binding of fatty acids to lipid bilayers solely from fluorescence data. However, if the fluorescence data for the binding of dansylundecanoic acid to the lipid component of the ATPase system can be shown to be comparable to that for binding to dioleoylphosphatidylcholine, then it would be reasonable to assume that binding parameters were also similar. We can demonstrate that this is so from the spectra

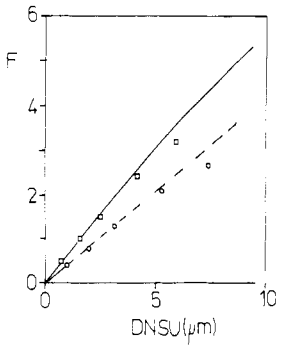

FIGURE 8: Fluorescence intensity at 530 nm for dansylundecanoic acid bound to the lipid component of the ATPase system plotted as a function of the concentration of available dansylundecanoic acid, unbound to the protein sites on the ATPase. ATPase concentrations and equivalent lipid concentrations: (□) 2.24 and 67 μM and (○) 1.12 and 34 μM, respectively. The solid lines represent fluorescence intensities of dansylundecanoic acid bound to dioleoylphosphatidylcholine at 67 and 34 μM, respectively.

excited at 347 nm. First, we subtract from the total, composite spectrum the component obtained by the fitting procedure assigned to fatty acid bound to protein. The resultant spectrum is then due to fatty acid both bound to lipid and free in buffer. The intensity of this resultant spectrum at 530 nm is plotted in Figure 8 as a function of the concentration of available fatty acid, for ATPase concentrations of 2.24 and 1.12 μM, corresponding to lipid concentrations of 67 and 34 μM, respectively. The concentration of available fatty acid is calculated as the total concentration minus that bound to the ATPase by using the binding parameters in Table II. These fluorescence values are compared in Figure 8 to the corresponding values observed for dansylundecanoic acid bound to dioleoylphosphatidylcholine (E. K. Rooney et al., unpublished experiments). Agreement is close.

Although detailed analysis has not been undertaken, the spectra for dansylundecanoic acid bound to sarcoplasmic reticulum (Figure 4) are consistent with similar binding parameters for this system and for purified (Ca^{2+}–Mg^{2+})-ATPase.

Discussion

We have shown elsewhere that although there is little selectivity in the binding of phospholipids to (Ca^{2+}–Mg^{2+})-ATPase, there is a marked selectivity for phospholipids over other hydrophobic molecules (East & Lee, 1982). Thus, at concentrations where the bilayer is stable, there is little displacement of phospholipids from the ATPase by a variety of fatty acids, esters, alkanes, and sterols. However, we have shown that although fatty acids and cholesterol tend not to bind at the annulus around the ATPase, they can bind to the ATPase (A. C. Simmonds et al., unpublished experiments). There are two obvious types of nonannular binding site for these hydrophobic molecules. If the structure of the ATPase is as suggested by MacLennan (1980) with eight passes of the polypeptide chain through the membrane, then hydrophobic compounds could bind between these polypeptide loops. Alternatively, it has been suggested that the ATPase is present in the membrane in an oligomeric form, possibly a dimer or a tetramer [see Moller et al. (1982)], and a second group of nonannular binding sites could then exist at protein–protein interfaces in these oligomers. Although we have no direct evidence, the latter possibility seems more likely.

The major difficulty in trying to study the binding of hydrophobic molecules such as fatty acids to a membrane protein is the extensive binding to the lipid component of the membrane that can be expected. We have shown that this problem can be overcome using fluorescent derivatives of the fatty acids. Since the fluorescence emission properties of the dansyl group are sensitive to the nature of their environment, it is possible to use fluorescence emission spectra of compounds labeled with the dansyl group to distinguish between molecules bound to the lipid and to the protein components of the membrane (Moules et al., 1982). Fluorescence emission for dansylundecanoic acid bound to liposomes is relatively insensitive to the nature of the lipid. However, fluorescence emission for dansyl derivatives bound to proteins does vary with the protein system under study (unpublished observations). The fact that fluorescence emission spectra for dansylundecanoic acid bound to (Ca^{2+}–Mg^{2+})-ATPase fit well to a sum of three peaks (assigned to probe in buffer and bound to protein and lipid, respectively) therefore suggests a single type of protein binding site. If a range of protein binding sites were present in the system, then an undifferentiated total emission spectrum would be expected.

On excitation of fluorescence by energy transfer through the protein tryptophan residues rather than directly at 347 nm, there is a large increase in the ratio of the fluorescence intensity of the protein-bound component to that of the lipid-bound component. This indicates that the efficiency of energy transfer between protein tryptophan residues and lipid-bound fatty acid is relatively poor. If the lipid-bound fatty acid were in the lipid annulus around the protein, possibly at the lipid–protein interface, then efficiency of transfer would be relatively high. We therefore suggest that the lipid-bound fatty acid is bound outside the lipid annulus.

Analysis of the fluorescence emission spectra suggests that binding to the lipid component of the membrane is comparable to binding to liposomes of dioleoylphosphatidylcholine. An analysis of the binding to the ATPase suggests three binding sites per ATPase, with a dissociation constant of ca. 7 μM which is only slightly sensitive to temperature. Although we cannot rule out a range of sites of varying affinity, the observation that the fluorescence emission is consistent with a single class of sites suggests that this is unlikely. The difference in emission properties for lipid- and protein-bound dansylundecanoic acid cannot necessarily be attributed to differences in binding site polarity. We have shown that the dansyl group can show marked time-dependent behavior in biological systems and that differences in emission wavelength can be due to differences in the rates of solvent reorientation around the fluorescence probe (Ghiggino et al., 1981).

If the two types of binding site we find for (Ca^{2+}–Mg^{2+})-ATPase are a general feature of membrane proteins, then there are important consequences. Much use has been made of spin-labeled fatty acids to probe the fluidity of the lipid component of biological membranes. Extensive binding of spin-labeled fatty acids to proteins in the membrane would considerably complicate the interpretation of such experiments. Indeed, we have observed features in the ESR spectra of spin-labeled fatty acids bound to (Ca^{2+}–Mg^{2+})-ATPase which would be consistent with more than one class of binding site in this system (A. C. Simmonds and A. G. Lee, unpublished observations). Many drugs are hydrophobic and bind extensively to biological membranes. One possible site of action of such drugs could be at the lipid–protein interface (Lee et al., 1981), but, at least for (Ca^{2+}–Mg^{2+})-ATPase, this now seems less likely because of the relative specificity of these sites for phospholipids. Rather, binding to the nonannular sites on the ATPase should be considered. Binding at these nonannular sites does not necessarily affect activity. Neither dansyl-

undecanoic acid nor oleic acid has much effect on the ATPase activity of (Ca^{2+}-Mg^{2+})-ATPase, or of (Ca^{2+}-Mg^{2+})-ATPase reconstituted with dioleoylphosphatidylcholine, although oleic acid does cause a large stimulation of (Ca^{2+}-Mg^{2+})-ATPase reconstituted with the shorter chain phospholipid dimyristoleoylphosphatidylcholine (A. C. Simmonds et al., unpublished experiments).

References

Bennett, J. P., Smith, G. A., Houslay, M. D., Hesketh, T. R., Metcalfe, J. C., & Warren, G. B. (1978) *Biochim. Biophys. Acta 513*, 310-320.

Dixon, W. J., & Brown, M. B. (1979) *Biomedical Computer Programs, P Series*, University of California Press, Berkeley, CA.

East, J. M., & Lee, A. G. (1982) *Biochemistry 21*, 4144-4151.

Ghiggino, K. P., Lee, A. G., Meech, S. R., O'Connor, D. V., & Phillips, D. (1981) *Biochemistry 20*, 5381-5389.

Hardwicke, P. M. D., & Green, N. M. (1974) *Eur. J. Biochem. 42*, 183-193.

Ikemoto, N., Myao, A., & Karobe, Y. (1981) *J. Biol. Chem. 256*, 10809-10814.

Johannsson, A., Keightley, C. A., Smith, G. A., Richards, C. D., Hesketh, T. R., & Metcalfe, J. C. (1981) *J. Biol. Chem. 256*, 1643-1650.

Lee, A. G., East, J. M., Rooney, E. K., Moules, I. K., & Simmonds, A. C. (1981) *Pediatr. Res. 15*, 1162-1167.

London, E., & Feigenson, G. W. (1981) *Biochemistry 20*, 1939-1948.

MacLennan, J. (1980) *Ann. N.Y. Acad. Sci. 358*, 149-155.

Moller, J. V., Andersen, J. P., & Le Maire, M. (1982) *Mol. Cell. Biochem. 42*, 83-107.

Moules, I. K., Rooney, E. K., & Lee, A. G. (1982) *FEBS Lett. 138*, 95-100.

Siano, P. B., & Metzler, D. E. (1969) *J. Chem. Phys. 51*, 1856.

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1974a) *Biochemistry 13*, 5501-5507.

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1974b) *Proc. Natl. Acad. Sci. U.S.A. 71*, 622-626.

Interactions between Phospholipid Head Groups at Membrane Interfaces: A Deuterium and Phosphorus Nuclear Magnetic Resonance and Spin-Label Electron Spin Resonance Study†

Frank Sixl and Anthony Watts*

ABSTRACT: The head group interactions in fully hydrated, mixed bilayers of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, specifically deuterated in the head groups at the α- and β-methylene and $N(CD_3)_3$ positions, have been investigated by deuterium and phosphorus-31 nuclear magnetic resonance (NMR) and 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) spin-label electron spin resonance (ESR) studies at pH 7.5. In binary lipid bilayers, the two lipids were found to be completely miscible by spin-label partitioning and phosphorus-31 NMR experiments. Also the phosphorus-31 NMR studies showed no evidence that the mean orientation of the phosphate group of either lipid was significantly altered in the binary system. In contrast, large changes in the deuterium residual quadrupole splittings for the deuterated head group segments were observed by deuterium NMR for both phosphatidylcholine and phosphatidylglycerol when one lipid was titrated against the other. The quadrupole splittings for the choline methyls decreased from 1.4 kHz in pure phosphatidylcholine bilayers to 0.4 kHz for bilayers containing 75 mol % of phosphatidylglycerol at 28 °C. A similar behavior was found for the head group β-CD_2 segment; the quadrupole splittings were reduced from 5.8 to 1.1 kHz in the same range of concentration and temperature. A converse observation was made for the α-CD_2 group in that the quadrupole splittings increased from 6.3 to about 10 kHz, and in mixed bilayers containing less than 50 mol % of phosphatidylcholine; magnetic inequivalence of the two deuterons gave two very well resolved NMR lines. In bilayers containing head group deuterated dimyristoylphosphatidylglycerol, the two α-CD_2 deuterons showed quadrupole splittings of 9.0 and 10.8 kHz, which increased to 10.2 and 11.3 kHz, respectively, on adding 75 mol % of phosphatidylcholine at 28 °C; for the β-CD the quadrupole splittings increased from 1.8 to 6.6 kHz for the same mixture ratio and temperature. The deuterium spin-lattice relaxation times T_1 remained essentially unchanged for the α-CD_2 and β-CD_2 groups of dimyristoylphosphatidylcholine but increased for the choline methyls by about 20% when dimyristoylphosphatidylglycerol and dimyristoylphosphatidylcholine were mixed in a 1:1 mole ratio. The results indicate that some reorientations in the lipid head groups and changes in their amplitudes of motion are induced in the two-component bilayers by the presence of one lipid on the other but their rates of motion remain rather similar.

The properties of biological and model membranes depend to a large extent on the head group structure of their phospholipid molecules. The binding of ions (Akutsu & Seelig, 1981) and antibiotics (Sixl & Galla, 1979, 1980), as well as the function of membrane-bound enzymes (Sanderman, 1978), is believed to be determined by the presence of structurally different phospholipid head groups and may be triggered by the surface charge of the lipid matrix (Watts et al., 1978). In particular, the choline head group of synthetic lecithins has been studied in detail by a variety of techniques, such as X-ray (Pearson & Pascher, 1979), proton NMR[1] (Hauser, 1981),

† From the Biochemistry Department, University of Oxford, Oxford, OX1 3QU United Kingdom. *Received May 20, 1982.* We acknowledge the Royal Society and the Deutsche Forschungsgemeinschaft for financial support to F.S. under Contract No. Si299-1 and the S.E.R.C. for consumables under Grant No. GR/B/69111.

0006-2960/82/0421-6446$01.25/0