Interactions of Pyrene Derivatives with Lipid Bilayers and with $(Ca^{2+}$–$Mg^{2+})$-ATPase†

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ABSTRACT: The intensities of fluorescence emission for pyrene and a number of its derivatives increase on binding to lipid bilayers and to the $(Ca^{2+}$–$Mg^{2+})$-ATPase purified from rabbit muscle sarcoplasmic reticulum. The effect is particularly marked for the less water-soluble derivatives. Changes in intensity for monomer and excimer emission as a function of lipid concentration can be fitted to a simple model to obtain binding parameters. The number of binding sites per lipid is 0.2–0.4. For the ATPase system, at least two classes of sites are necessary to fit the data, one corresponding to the lipid component and one to sites on the ATPase. Excimer emission from the postulated sites on the ATPase is less marked than that from lipid. Pyrenedodecanoic acid and pyreneundecyltrimethylammonium bromide, which bind to a large number of sites on the ATPase, cause marked inhibition of ATPase activity at high concentration. Pyrene and a number of water-soluble derivatives cause stimulation of the ATPase reconstituted with dimyristoleoylphosphatidylcholine and little inhibition and bind to a small number of sites on the ATPase. It is concluded that excimer emission from pyrene derivatives in systems containing proteins cannot be used to obtain reliable information about rates of diffusion in the lipid component of the membrane.

One approach to the study of membrane structure is through the use of hydrophobic spectroscopic probes which will partition into the membrane. Ideally, the spectroscopic properties of the probe should change on binding to the membrane to allow the extent of binding to be determined, and if the probe binds to both lipid and protein sites within the membrane, it should be possible to quantitate separately the binding to these two classes of sites. For structural studies, it is clearly an advantage to work with highly simplified membrane systems where the potential range of protein sites is reduced. In previous papers, we have shown that the $(Ca^{2+}$–$Mg^{2+})$-ATPase purified from muscle sarcoplasmic reticulum is suitable for such studies (East & Lee, 1982; Lee et al., 1982, 1983; Simmonds et al., 1982, 1984).

We have suggested that there are two classes of sites on the ATPase to which hydrophobic molecules can bind. The first class of sites (annular sites) is at the lipid–protein interface of the ATPase. Our concept of the nature of these sites is discussed at length elsewhere (East et al., 1985). Since the activity of the ATPase is markedly dependent on the structure of the surrounding, annular molecules with dioleoylphosphatidylcholine being close to optimum in structure, binding of nonphospholipid molecules at the annulus is likely to lead to reduction in activity. We have also detected a second set of sites (nonannular sites) to which hydrophobic molecules such as fatty acids and sterols can bind, overcoming the effects on activity of phospholipids of suboptimal structure (Simmonds et al., 1982, 1984). For example, the activity of the ATPase reconstituted into bilayers of 1,2-dimyristoleoylphosphatidylcholine (DMPC)[1] is about one-third of that for the ATPase reconstituted into bilayers of 1,2-dioleoylphosphatidylcholine (DOPC), and addition of cholesterol to the ATPase reconstituted with DMPC increases the activity of the ATPase to above that seen with the ATPase reconstituted with DOPC (Simmonds et al., 1982). Effects seen with oleic acid and cholesterol hemisuccinate, however, are more complex, with initial increases in activity being followed by inhibition at higher concentrations (Simmonds et al., 1982, 1984). We have suggested that initial activation follows from binding to nonannular sites with inhibition following from binding to annular sites at higher molar ratios of additive to phospholipid. Similar patterns of activation followed by inhibition are also seen with a variety of nonpolar molecules including alkanes (Johannsson et al., 1981; Jones et al., 1984) and hexachlorocyclohexanes (Jones et al., 1984). An alternative mechanism has been proposed for the effects of alkanes on the activity of ATPase reconstituted with DMPC. It has been suggested that membrane thickness is an important factor in determining ATPase activity and that addition of decane, for example, to DMPC initially thickens the bilayer toward the optimal thickness seen with DOPC but that too high a concentration of decane overthickens the bilayer (Johannsson et al., 1981).

One possible test of the proposed models is to study the effects of a variety of related hydrophobic molecules, some of which are free to partition anywhere within the membrane whereas others are anchored at the membrane–water interface by suitable polar groups. We have chosen a set of molecules based on the pyrene group because the fluorescence properties of the pyrene group can be used to quantitate binding and to provide information on the nature of the binding site(s) within the membrane.

Pyrene and its derivatives show significant fine structure (vibronic bands) in their fluorescence emission spectra, the relative intensities of the vibronic bands depending on solvent polarity (Nakajima, 1971; Mukhopadhyay & Georghiou, 1980). A second useful property of the pyrene group is excimer formation, observed at relatively high local concentrations

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[1] Abbreviations: DMPC, 1,2-dimyristoleoylphosphatidylcholine; DOPC, 1,2-dioleoylphosphatidylcholine; BRPC, 1,2-bis(9,10-dibromostearoyl)phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ESR, electron spin resonance; PC, phosphatidylcholine.

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(Birks, 1975a,b). Because excimer formation occurs by collision of excited and unexcited molecules, it is dependent on rates of diffusion and so has been used to study diffusion in lipid systems (Galla et al., 1979). Finally, we show here that, under suitable conditions, the intensity of fluorescence emission of pyrene and its derivatives increases markedly on binding to membrane systems and can be used to quantitate that binding.

Materials and Methods

Dioleoylphosphatidylcholine and egg yolk phosphatidylcholine were obtained from Lipid Products; pyrene was from Aldrich; pyrenebutyrate, pyrene-1-methanol, and pyreneundecyltrimethylammonium bromide were from Molecular Probes, and pyrenedodecanoic acid was from Calbiochem. The brominated analogue of DOPC was synthesized as described in East & Lee (1982). Dimyristoleoylphosphatidylcholine was prepared as in Jones et al. (1984). (Ca^{2+}–Mg^{2+})-ATPase was prepared from female rabbit (New Zealand White) hind leg muscle in the presence of dithiothreitol and phenylmethanesulfonyl fluoride as described (East & Lee, 1982). Lipid substitutions were carried out essentially as described in East & Lee (1982). Briefly, ATPase (0.5–1 mg) was incubated with a sonicated lipid suspension (4 mg) and cholate (2.25 mg) in buffer (180 μL; 250 mM sucrose, 1 M KCl, 5 mM MgATP, and 50 mM potassium phosphate, pH 8.0) for 20 min at room temperature, followed by 30 min at 0 °C. Before assay, samples were diluted 1000-fold. ATPase activity was measured by using a coupled enzyme assay in buffer (40 mM Hepes) containing Mg (5 mM), ATP (2.1 mM), and free calcium (1 μM) at 37 °C (Rooney & Lee, 1983).

Fluorescence spectra were recorded with a Perkin-Elmer MPF44A fluorometer. In studies of liposomes, lipid and probe were mixed in methanol solution, dried onto the sides of flasks, and resuspended in buffer. For studies of the ATPase, probes were added to the ATPase from concentrated solutions in methanol, the final methanol concentrations never exceeding 1%. Methanol at this concentration had no effect on ATPase activity. Samples were incubated for 2 h in the dark to allow equilibration. The buffer for fluorescence measurements was 40 mM Hepes–0.1 M NaCl–0.1 mM EGTA, pH 7.2. Where noted, solutions were degassed by bubbling with nitrogen gas.

Binding of water-soluble pyrene derivatives to liposomes and ATPase was measured directly by a centrifugation assay. Liposomes containing pyrenebutyrate or pyrene-1-methanol in a volume of 9 mL were centrifuged for 2 h at $108000g_{av}$ and 20 °C. The fluorescence intensity (λ_{em} = 395 nm; λ_{ex} = 342 nm) of the supernatant was then measured. Binding of pyrenebutyrate to the ATPase was determined in the same way.

Results

Interactions with Lipid Bilayers. The effect of phospholipid on the fluorescence emission spectrum of pyrene (1 μM) is illustrated in Figure 1. In buffer alone, pyrene, at 1 μM, showed predominantly monomer fluorescence emission. Addition of 100 μM egg yolk phosphatidylcholine enhanced the intensity of the monomer fluorescence emission and caused an increase in excimer intensity centered at ca. 480 nm. Higher concentrations of lipid caused a further increase in the intensity of monomer emission but a decrease in the intensity of excimer emission. These changes in intensity are shown in Figure 2 as a function of lipid concentration at a fixed pyrene concentration of 1 μM. As well as changes in fluorescence intensity, Figure 1 illustrates the expected changes in the relative intensities of the vibronic bands in the monomer

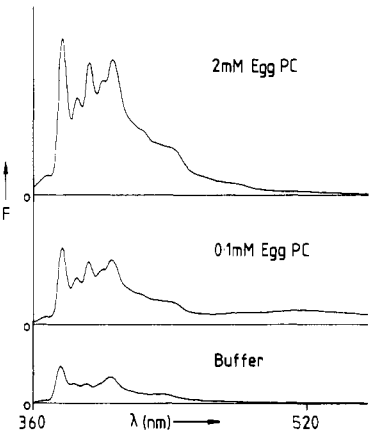

FIGURE 1: Fluorescence emission spectra of pyrene (1 μM) as a function of the concentration of egg yolk phosphatidylcholine. The sample was degassed with nitrogen.

Table I: Enhancement Ratios (R) for Pyrene Derivatives in Solvents and Liposomes

solvent	pyrene	pyrene-1-methanol	pyrene-dodecanoic acid
water	1.87	1.77	*a*
acetone	1.64	1.97	3.94
formamide	1.57	1.79	3.53
ethyl acetate	1.37	1.75	3.47
methanol	1.35	1.53	3.20
chloroform	1.25	1.55	3.60
ethanol	1.18	1.57	3.12
2-propanol	1.08	1.47	3.18
butanol	1.06	1.35	3.10
benzene	1.05	1.47	3.04
isobutyl alcohol	1.02	1.30	3.07
isopropyl ether	0.93	1.46	3.00
heptane	0.61	0.75	2.73
DOPC[b]	1.15	1.87	3.02
egg phosphatidylcholine [b]	1.18	1.87	3.02
(Ca^{2+}–Mg^{2+})-ATPase[c]	0.94		

[a] The large excimer emission made the R value impossible to measure. [b] Determined at a probe concentration of 1 μM and a lipid concentration of 1 mM. [c] Determined at a probe concentration of 1 μM and a protein concentration of 17 μM.

spectrum on binding to lipid. These changes have been expressed as an enhancement ratio or R value where R is the ratio of the height of the peak centered at ca. 373 nm to that at ca. 385 nm—the exact position of the peaks varies with the pyrene derivative. Values of R are listed in Table I. The R value observed for pyrene at very high lipid concentration corresponds to that seen in ethanol as solvent and is consistent with binding in the semipolar glycerol backbone region of the bilayer, as previously reported by others (Dorrance & Hunter, 1977; Lianos et al., 1980).

The marked dependence of fluorescence intensities on lipid concentration illustrated in Figures 1 and 2 is unexpected. In studies in a wide variety of solvents, it has been found that the intensity of the peak at ca. 385 nm changes very little with solvent, although the intensities of the other peaks change as a result of the Ham effect (Lianos et al., 1980). The effect seen in Figure 1 probably follows partly from the low aqueous solubility of pyrene, which has been measured as 0.8 μM (Davis et al., 1942) and ca. 0.3 μM (Nakajima, 1977). Further, Nakajima (1977) has shown that direct addition of solutions of pyrene in organic solution to water leads to the formation of microcrystals and that extensive sonication is necessary to break up the crystals and produce solution. Aggregates or microcrystals often have anomalously low fluorescence (see, for example, the fluorescent sterol choles-

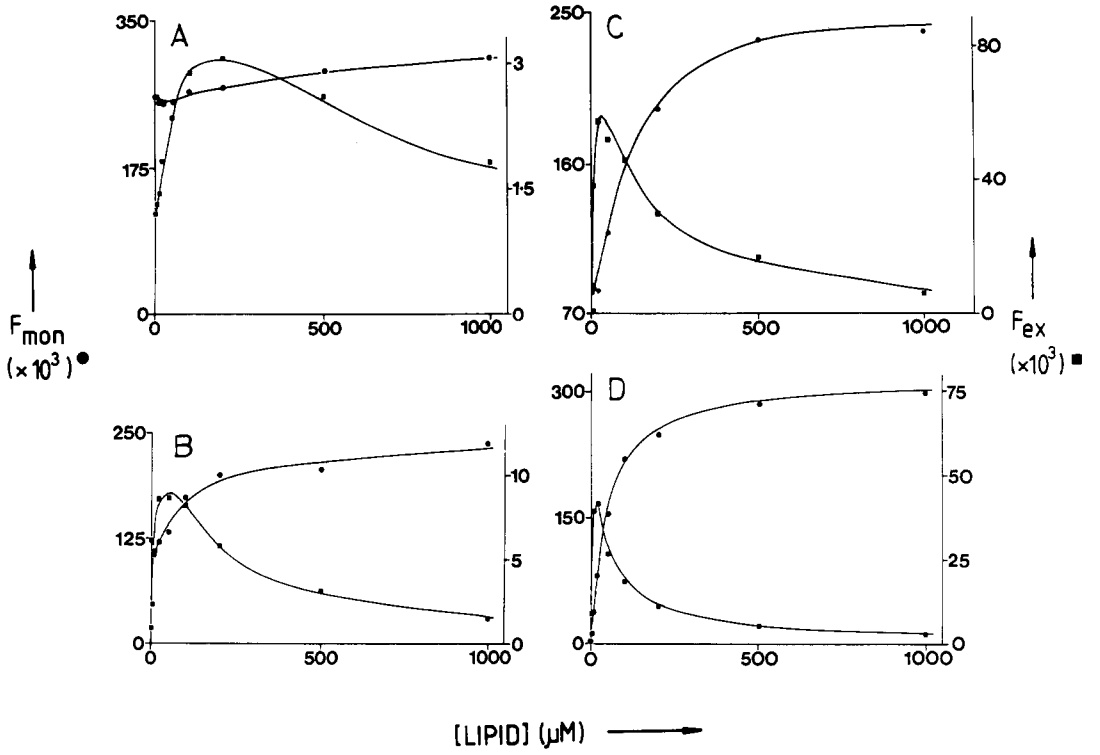

FIGURE 2: Dependence of the intensities of monomer (●) and excimer (■) emission on the concentration of egg yolk phosphatidylcholine at 20 °C for 1 μM (A) pyrenebutyrate, (B) pyrene-1-methanol, (C) pyrene, and (D) pyrenedodecanoic acid. Monomer and excimer intensities were measured at 395 and 480 nm, respectively. Solid lines are theoretical curves calculated with the parameters listed in Table II. The pyrene samples were degassed with nitrogen.

Table II: Binding and Fluorescence Parameters for Pyrene Derivatives in Lipid Systems at 20 °C

compound	phospholipid	K_d (μM)	N	$F_m'^l$	$F_{ex}'^l$	$x_{0.5}$	$F_m'^w$	$F_{ex}'^w$
pyrenebutyrate	egg PC	27.0	0.17	320	38	0.10	260	1.0
pyrene-1-methanol	egg PC	4.0	0.20	240	34	0.10	120	0.9
	DOPC	4.0	0.20	360	92	0.20	180	0.2
	BRPC	4.0	0.20	76	36	0.55	183	0.2
pyrene (degassed)	egg PC	2.0	0.20	260	138	0.08	80	2.5
pyrenedodecanoic acid	egg PC	1.0	0.40	320	70	0.06	4	1.3

ta-5,7,9-trien-3β-ol; Rogers et al., 1979). The majority of the pyrene is unlikely, however, to be present as simple suspensions of microcrystals since the fluorescence from crystals of pyrene is all excimer with no monomer emission (Birks, 1975b) whereas we observe predominantly monomer emission (Figure 1). A connection with aqueous solubility is also suggested by the observations with the derivatives of pyrene in Figure 2. For the relatively water-soluble pyrenebutyrate and pyrene-methanol at 1 μM, emission in buffer is predominantly monomer, and addition of lipid has relatively little effect on the intensity of monomer emission. For the less soluble pyrene-dodecanoic acid, however, excimer emission predominates in buffer, and addition of lipid causes large changes in monomer intensity.

For the pyrene experiments shown in Figure 2, oxygen was removed from the buffer by bubbling with nitrogen. Comparable profiles were, however, also obtained under non-deoxygenated conditions, and for the remainder of the experiments reported here, no attempts were made to remove oxygen.

It is shown in the Appendix that the fluorescence intensities of monomer and excimer emission for a fluorophore that partitions between lipid and aqueous phases can be written as

$$F_m^{obsd} = (P_t - P_b)F_m'^w/P_t + P_bF_m'^l/[P_t(1 + x/x')] \quad (1)$$

$$F_{ex}^{obsd} = (P_t - P_b)F_{ex}'^w/P_t + P_bF_{ex}'^l/[P_t(1 + x'/x)] \quad (2)$$

where P_t and P_b are the total and bound lipid concentrations of fluorophore, respectively, $F_m'^w$ and $F_{ex}'^w$ are the monomer and excimer intensities, respectively, observed in water in the absence of lipid, $F_m'^l$ and $F_{ex}'^l$ are the theoretical maximum monomer and excimer intensities, respectively, in the lipid phase, x is the mole ratio of fluorophore in the lipid phase to lipid sites, and x' is the mole ratio at which monomer fluorescence is half-quenched by excimer formation. The concentration of bound fluorophore, P_b, is calculated in terms of the dissociation constant for binding, K_d, and the number of binding sites per lipid, N (eq A3 and A4). As shown in Figure 2, the experimental data are well fitted by eq 1 and 2 with the parameters listed in Table II. It is difficult to estimate the accuracy of the derived parameters, but each parameter has a distinctive effect on the shape of the calculated plots, and variation of any parameter by more than a factor of 2 results in an obviously worse fit to the data.

The interaction of pyrene-1-methanol was also studied with 1,2-bis(9,10-dibromostearoyl)phosphatidylcholine (BRPC), a lipid which would be expected to quench the fluorescence of bound probe. As shown in Figure 3, binding of pyrene-1-methanol to BRPC indeed results in a decrease in monomer fluorescence intensity, in contrast to the increase in intensity seen on binding to DOPC. Importantly, the data can be fitted to the K_d and N values derived for binding to DOPC, although the value of $x_{0.5}$ (the mole ratio of probe at which the monomer intensity is half quenched due to excimer formation) is in-

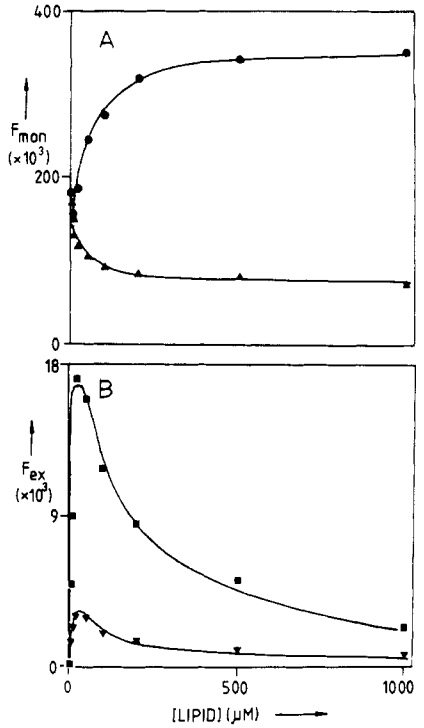

FIGURE 3: Comparison of the intensities of monomer (A) and excimer (B) emission for pyrene-1-methanol (1 μM) in DOPC (●, ■) and BRPC (▲, ▼) at 20 °C. (●, ▲) Monomer intensity measured at 395 nm; (■, ▼) excimer intensity measured at 480 nm. Solid lines are theoretical curves calculated with the parameters listed in Table II.

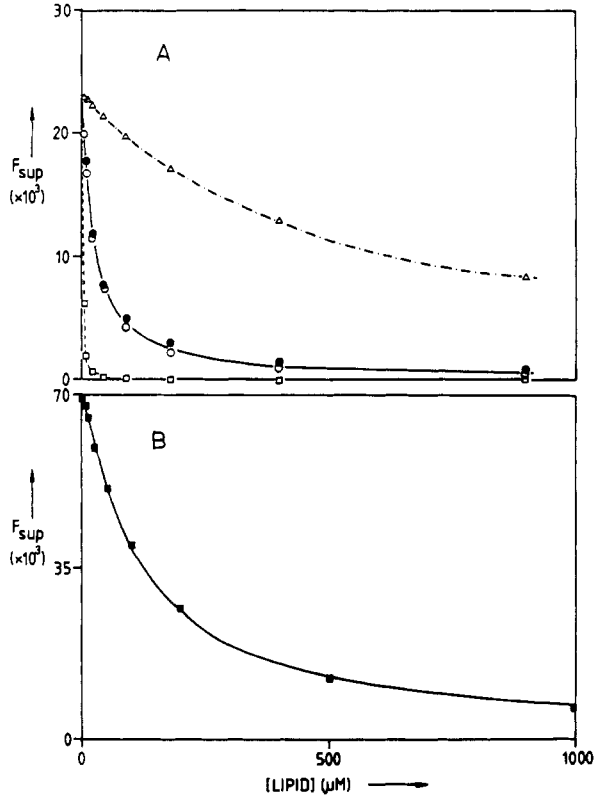

FIGURE 4: Binding of 1 μM pyrene-1-methanol (A) and pyrenebutyrate (B) to egg yolk phosphatidylcholine at 20 °C. Samples were centrifuged for 2 h at 108000g_{av}, and the fluorescence intensity of the supernatant was determined (λ_{ex} = 342 nm; λ_{em} = 395 nm). Experimental data (●, ■). (A) Calculated curves with N = 0.2 and K_d = 0.1 (---), 4.0 (—), and 100.0 (–·–) μM. (B) Calculated curve with N = 0.2 and K_d = 27 μM.

creased (Table II). The increase in the $x_{0.5}$ value is expected since quenching will reduce the probability of collisional en-

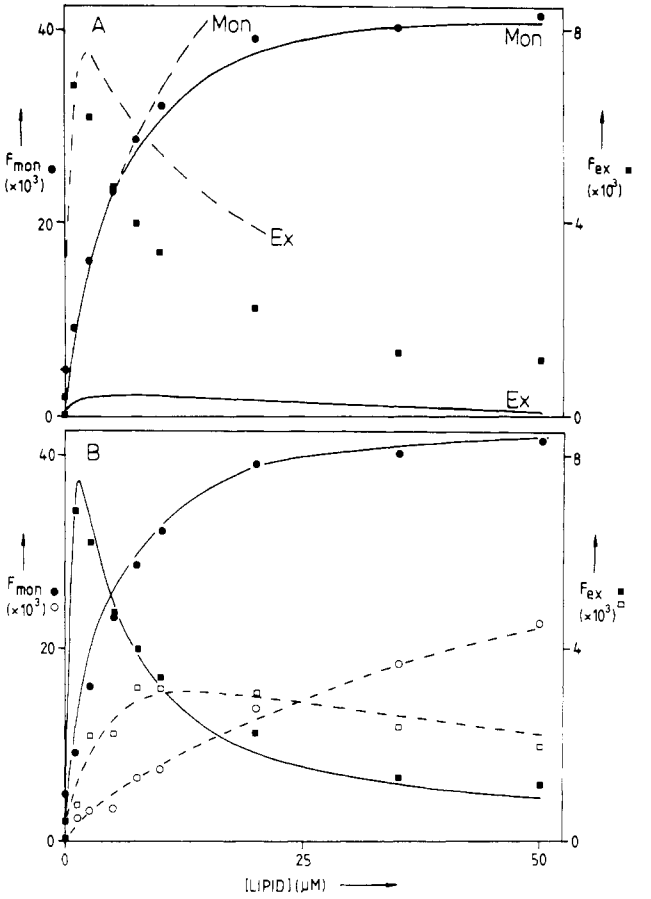

FIGURE 5: Dependence of the intensities of monomer (●, ○) and excimer (■, □) emission for 1 μM pyreneundecyltrimethylammonium bromide as a function of ATPase lipid concentration in the ATPase system (●, ■) compared to intensities in egg yolk phosphatidylcholine (○, □). Monomer and excimer intensities were measured at 395 and 480 nm, respectively. (A) (Solid line) Attempt to fit the ATPase data to lipid binding sites alone, with K_d = 0.7 μM, N = 0.3, $F_m'^w$ = 0.5, $F_{ex}'^w$ = 2.5, F'^l = 45, $F_{ex}'^l$ = 50; (broken line) attempt to fit to a two-site model with $F_m'^P$ = 80, $F_{ex}'^P$ = 10, and $x_{0.5}^P$ = 0.07. (B) Fit to the two-site model for the ATPase data (●, ■) and to the lipid data (○, □) with the parameters listed in Table III.

counters between excited and unexcited fluorophores. Similar results were obtained for pyrene binding to BRPC, with $x_{0.5}$ increased from 0.025 to 0.1.

For pyrene-1-methanol and pyrenebutyrate, the parameters obtained from the fluorescence analysis were checked by a direct binding assay. Bound and unbound fluorophores were separated by centrifugation, and the fluorescence intensity of the supernatant was determined. As shown in Figure 4, agreement with the parameters listed in Table II is good.

Interactions with (Ca^{2+}–Mg^{2+})-ATPase. As we prepare it, the (Ca^{2+}–Mg^{2+})-ATPase system has a lipid to protein ratio of 30 to 1. The spectrum of pyrene (1 μM) bound to 17 μM ATPase shows predominantly monomer emission with an R value of 0.94 (Table I). At such high protein concentrations, microcuvettes had to be employed to reduce the considerable light scatter. Under the conditions previously employed for fluorescence measurements, the maximum possible protein concentration that could be studied was 1.7 μM, corresponding to a lipid concentration of 50 μM. As with simple lipid bilayers, addition of increasing concentrations of the ATPase to pyrene and its derivatives led to distinctive changes in the intensities of monomer and excimer intensities, the excimer emission being particularly marked for pyrenedodecanoic acid and pyreneundecyltrimethylammonium bromide (Figures 5 and 6). Results for these two compounds were markedly

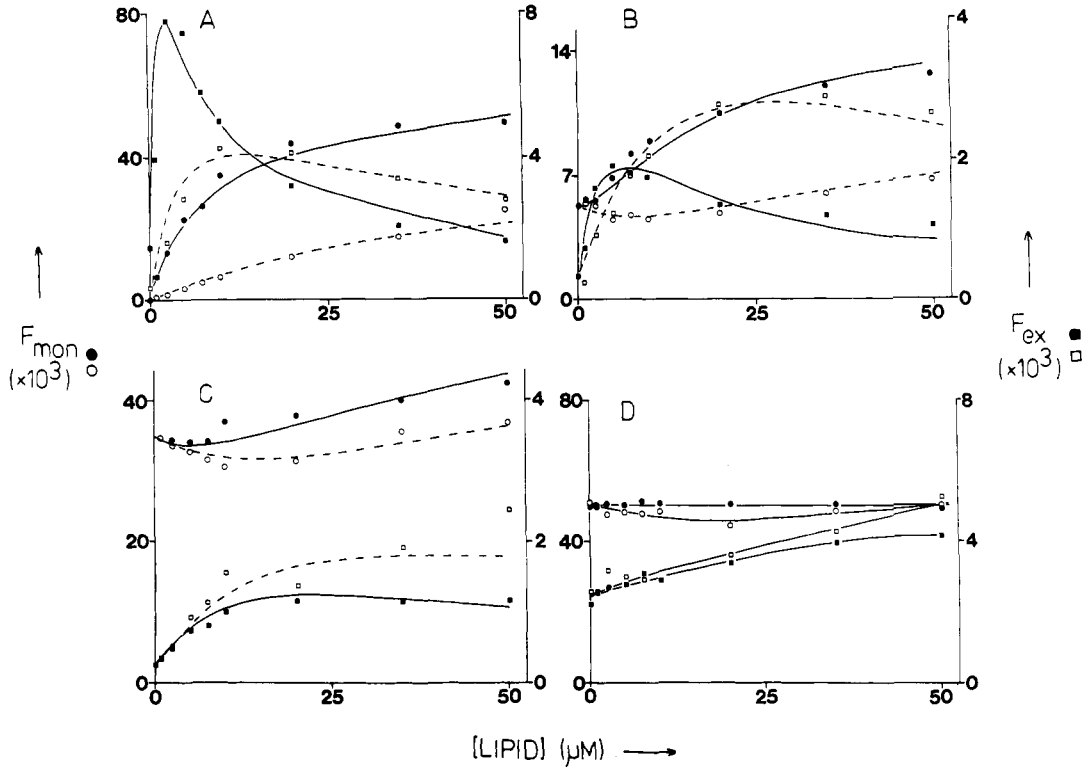

FIGURE 6: Dependence of the intensities of monomer (●, ○) and excimer (■, □) emission as a function of ATPase lipid concentration (●, ■) compared to intensities in egg yolk phosphatidylcholine (○, □) for 1 μM (A) pyrenedodecanoic acid, (B) pyrene, (C) pyrene-1-methanol, and (D) pyrenebutyrate. Monomer and excimer intensities were measured at 395 and 480 nm, respectively. For (A–C), the solid lines are theoretical fits to the ATPase data, and the broken lines are theoretical fits to the lipid data, with the parameters listed in Table III.

Table III: Binding and Fluorescence Parameters for Pyrene Derivatives in the (Ca^{2+}–Mg^{2+})-ATPase System at 20 °C

	protein component					lipid component		aqueous component	
compound	K_d (μM)	N	$x_{0.5}$	F_m'	F_{ex}'	F_m'	F_{ex}'	F_m'	F_{ex}'
pyrene-1-methanol[a]	2.0	3[d]	0.6	50	10.0	62	6.0	35	0.03
pyrene[b]	0.1	3[d]	0.6	18	3.5	11	7.5	5.0	0.2
pyrenedodecanoic acid[a]	0.1	30[e]	0.1	60	11.0	40	7.0	0.2	0.01
pyreneundecyltrimethylammonium bromide[c]	0.1	30[e]	0.3	45	14.0	45	5.0	0.5	0.25

[a] With N, K_d, and $x_{0.5}$ values for lipid binding as listed in Table II. [b] Undegassed, with N and K_d values for lipid binding as listed in Table II but $x_{0.5} = 0.025$. [c] With $N = 0.3$, $K_d = 0.7$, and $x_{0.5} = 0.07$ for lipid binding. [d] Range of 1–6 gives reasonable fits to the data. [e] Minimum number of sites 15; see text.

different for the ATPase and lipid systems, and it was not found to be possible to obtain a good fit to the experimental data for the ATPase system in terms of a single class of binding sites. The data were therefore analyzed in terms of two classes of binding sites. Monomer fluorescence and excimer fluorescence at both classes of sites were assumed to be given by equations analogous to eq A4 and A6 in the Appendix with the concentrations at protein sites being expressed as mole ratios of occupied sites to total sites. Binding to both classes of sites was described by binding equations analogous to eq A3 and A4. The total probe concentration is given by

$$P_t = P_f + P_b^1 + P_b^2$$

where P_f is the concentration of unbound probe and P_b^1 and P_b^2 are the concentrations bound to sites 1 and 2. The equations were solved numerically by using the method of bisection (McCormick & Salvadori, 1964). As shown in Figures 5 and 6, a good fit can be obtained to the experimental data for pyrenedodecanoic acid and pyreneundecyltrimethylammonium bromide, assuming that one set of sites is identical with those in simple lipid bilayers (Table II) and that there are a large number of other sites (>15) with higher affinity (Table III). Poor fits are obtained for 15 or less sites, but greater than this number of sites gives equally good fits to the data, as long as the ratio N/K_d is maintained constant.

For pyrene (Figure 6), profiles for the ATPase and lipid systems were again significantly different, but the extent of excimer formation was less than that observed for pyrenedodecanoic acid or pyreneundecyltrimethylammonium bromide. The data could now only be fitted in terms of binding to lipid sites and a relatively small number of sites of high affinity. Again, the fit is relatively insensitive to the exact number of sites, and reasonable fits were obtained in the range $N = 1$–6, as long as the ratio N/K_d was maintained constant. The experimental data for pyrene-1-methanol (Figure 6) were also fitted in terms of a small number of extra sites in the ATPase system (Table III). The effects on fluorescence intensities of the addition of ATPase to pyrenebutyrate were too small to allow an accurate determination of binding parameters, but the data are clearly more like those for pyrene-1-methanol than those for pyreneundecanoic acid.

Binding of pyrenebutyrate to the ATPase system was determined directly by a centrifugation assay. The fluorescence intensity of the supernatant is shown in Figure 7 and compared to the profile calculated for binding to the lipid component of the system alone with $K_d = 25$ μM and $N = 0.2$.

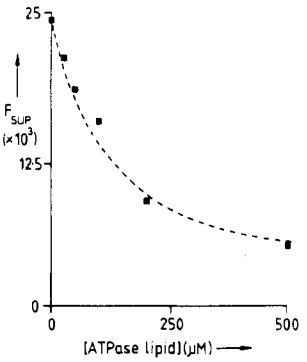

FIGURE 7: Binding of pyrenebutyrate to (Ca^{2+}–Mg^{2+})-ATPase as determined by centrifugation. (■) Fluorescence intensity of the supernatant; λ_{ex} = 342 nm, λ_{em} = 395 nm. Broken line, calculated curve for binding to lipid component alone with K_d = 25 μM and N = 0.2.

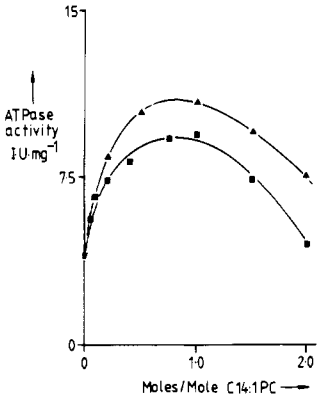

FIGURE 8: Effect of pyrene (▲) and pyrene-1-methanol (■) on the activity of the ATPase reconstituted into bilayers of DMPC at 37 °C.

Effects of pyrene and its derivatives on the activity of the ATPase reconstituted into bilayers of DMPC are shown in Figures 8 and 9. Effects were generally reversible except for pyrenetrimethylammonium bromide at molar ratios of amine to lipid greater than ca. 0.3 to 1.

Discussion

We have shown that pyrene and its derivatives can be used as fluorescence probes of the (Ca^{2+}–Mg^{2+})-ATPase system purified from sarcoplasmic reticulum. One useful property of the pyrene fluorophore is the "Ham effect", the enhancement of weakly allowed electronic transitions in polar solvents. It has been shown that the enhancement ratio R is unaffected by oxygen quenching (Glushko et al., 1981). In agreement with others (Dorrance & Hunter, 1977; Lianos et al., 1980), we find that the value of R for pyrene in bilayers of phosphatidylcholines is comparable to that in a protic solvent such as ethanol and thus that pyrene is located in a relative polar region of the bilayer, close to the bilayer surface. Data for the other pyrene derivatives are consistent with such an interpretation, with pyrene-1-methanol being situated in a very polar region of the bilayer and pyrenedodecanoic acid in an environment slightly less polar than that occupied by pyrene (Table I).

In the ATPase system, at a lipid:protein ratio of 30:1, the enhancement ratio R for pyrene is significantly less than that in bilayers of phosphatidylcholine and could indicate a less polar environment. However, it is possible that solvent reorientation is important in determining R values (Kalyanasundaram & Thomas, 1977): an environment of low mobility

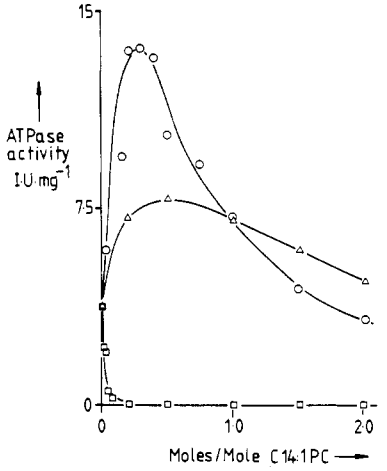

FIGURE 9: Effect of pyrenebutyrate (△), pyrenedodecanoic acid (○), and pyreneundecyltrimethylammonium bromide (□) on the activity of the ATPase reconstituted into bilayers of DMPC at 37 °C.

would then appear to be one of low polarity. It has been observed previously that the fluorescence emission spectra for hydrophobic molecules containing the dansyl group are consistent with a less polar environment for sites on the ATPase than for sites in the bulk lipid phase of the membrane, but here again, restricted solvent reorientation around the protein sites could account for the results (Ghiggino et al., 1981; Lee et al., 1982, 1983).

A more surprising observation was the marked increase in fluorescence intensity on addition of lipids to pyrene and a number of its derivatives. The effect is particularly marked for those derivatives which are expected to be the least soluble in water and so presumably follows from abnormally low fluorescence in water due possibly to the formation of aggregated species. Low fluorescence for aggregated species could follow from the reabsorption of fluorescence emission from the monomeric state prior to its escape from the aggregate and from formation of aggregates with incorrect packing for extensive excimer formation. The fluorescence profiles show an initial increase in the intensity of excimer fluorescence with increasing lipid concentration followed by a decrease, together with a steadily increasing intensity of monomer emission. The high excimer intensity at low lipid concentration follows from partition of the probe into the bilayer where its local concentration will be high, leading to extensive excimer emission. At higher lipid concentration, although the extent of binding to the lipid phase will be greater, the mole ratio of probe in the bilayer will be less, resulting in less excimer emission. The fluorescence intensities can be fitted to a binding model described in the Appendix to give the parameters listed in Table II. For pyrene and pyrene-1-methanol, the analysis was checked by comparison with binding to the lipid BRPC, where binding to the membrane results in quenching of fluorescence. Binding of pyrene-1-methanol and pyrenebutyrate to lipid bilayers was also checked directly by using a centrifugation procedure.

Elsewhere, we have shown that binding of fatty acids and other charged molecules can be limited by the charge that builds up on the liposomes as a result of the binding (Rooney et al., 1983) but such effects will be absent here because of the high ionic strengths employed. The binding constants for the amine and fatty acids listed in Table II will be effective binding constants as defined by Rooney et al. (1983), containing contributions from binding of both charged and uncharged forms. The limited stoichiometry of binding for the pyrene derivatives (0.2–0.4) is probably partly determined by

the pyrene group. It is known that a variety of other hydrophobic molecules with structures very different than those of the phospholipids exhibit limited miscibility with lipids [see Lee (1983)]. Thus, the properties of mixtures of phospholipids with benzene change markedly at a molar ratio of two phospholipids per benzene molecule (McDaniel et al., 1982), and phloretin binds with four lipids per site (Verkman & Solomon, 1982).

For pyrene, pyrenedodecanoic acid, and pyreneundecyltrimethylammonium bromide, the variations in intensities of monomer and excimer emission are very different for the ATPase and simple lipid bilayer systems when plotted as a function of lipid concentration calculated on the basis of a molar ratio of lipid to ATPase of 30 to 1 for the ATPase system. A number of possible explanations were considered. First, it has been shown by using ESR techniques that, in the ATPase system, ca. 18 lipids are immobilized at the lipid–protein interface with ca. 12 being in the bulk lipid phase (East et al., 1985). It is then possible that the pyrene derivatives partition only into the bulk lipid phase, but it is clear that replotting the ATPase data as a function of the concentration of the bulk lipid phase will not give agreement with the data for simple lipid bilayers. Second, it is possible that the probes are binding to the lipid component of the ATPase system with binding constants the same as those that characterize binding to simple lipid bilayers but that the mole ratio for half-quenching of monomer fluorescence, $x_{0.5}$, is altered due to the presence of protein. Since excimer formation depends on the rate of lateral diffusion (Birks, 1975), $x_{0.5}$ is likely to be higher in the ATPase system. As shown in Figure 5, it is possible to obtain a reasonable fit to the monomer fluorescence intensity in this way, but the calculated intensity of excimer fluorescence is much too low. We conclude, therefore, that it is necessary to postulate at least two classes of sites in the ATPase system to provide a fit to the data. The simplest postulate is that one set of sites corresponds to the lipid component of the system, with binding characteristics the same as for simple lipid bilayers, and that the second set of sites is on the ATPase. As shown in Figures 5 and 6, it is possible to obtain a good fit to the experimental data in terms of such a model. The data show that most of the probe must be bound to sites where the formation of excimers is less favorable than in simple lipid bilayers (excimer fluorescence intensities are considerably lower at high ATPase concentrations in the ATPase system than in simple lipid systems) but binding must be stronger to explain the marked excimer intensity at low ATPase concentrations (Table III). As illustrated in Figure 5, it is not possible to fit the data for the ATPase system with the same $x_{0.5}$ value for the protein sites as for the lipid sites. The larger $x_{0.5}$ value could follow partly from immobilization of the pyrene derivatives on the protein and partly from orientational factors which are unfavorable for overlap of pyrene rings on the protein.

For pyrenedodecanoic acid and pyreneundecyltrimethylammonium bromide, it is necessary to postulate a large number of binding sites on the protein, greater than ca. 15 in number. Since ESR studies have been interpreted in terms of an annular shell of ca. 30 phospholipids around the ATPase (East et al., 1985), it seems likely that these molecules are binding extensively at the annular sites. For pyrene itself, however, the data fit to a much smaller number of sites, between about one and six. For pyrene-1-methanol and pyrenebutyrate, changes in fluorescence intensity on binding to the ATPase are too small to allow proper estimates of numbers of binding sites, but the similarity of fluorescence emission in the ATPase and lipid systems suggests that the number of any protein binding sites must be small.

Binding to protein sites on the ATPase is stronger than to lipid sites. The relative order of binding constants for the pyrene derivatives, however, is the same for protein and lipid sites, suggesting that binding to protein sites is dominated by hydrophobic effects.

The fluorescence intensity profiles can be compared to effects on the activity of the ATPase reconstituted with the short-chain phospholipid DMPC (Figures 8 and 9). Pyreneundecyltrimethylammonium bromide causes a very marked decrease in activity for the ATPase, and pyrene-1-dodecanoic acid causes an initial increase in activity followed by inhibition, as seen previously with oleic acid (Simmonds et al., 1982). The biphasic activity profile was attributed to binding at a small number of nonannular sites, leading to stimulation, followed by binding at a large number of annular sites at higher concentration, leading to inhibition (Simmonds et al., 1982). The fluorescence experiments just described suggest binding of these two compounds to a large number of sites on the protein. For pyrene, pyrene-1-methanol, and pyrenebutyrate, stimulation of the ATPase is less, but there is little inhibition, and the fluorescence experiments suggest binding to a relatively small number of sites on the protein. Thus, although a detailed interpretation in terms of two classes of sites on the ATPase is not justified, the results are generally in agreement with such a model.

Finally, the results reported here emphasize the potential problems of using hydrophobic molecules as probes for the lipid bilayer portion of the membrane. Thus, excimer formation by pyrene and a number of its derivatives has been used to obtain diffusion coefficients for probe molecules in a number of membrane systems [for a review, see Lee (1982)]. If pyrene were to bind to other membrane proteins as strongly as it does to the (Ca^{2+}–Mg^{2+})-ATPase, then the measurements would be considerably complicated.

Appendix

Effect of Lipid Concentration on Fluorescence Intensity. At high concentrations in solution, fluorescence spectra of pyrene and related molecules show both monomer and excimer emission. The relative intensities of monomer (F_m) and excimer (F_{ex}) emission can be related to concentration (c) by (Birks, 1975a,b)

$$F_m = \phi_m/(1 + c/c') \quad \text{(A1)}$$

$$F_{ex} = \phi_{ex}/(1 + c'/c) \quad \text{(A2)}$$

where c' is the concentration of fluorophore at which monomer fluorescence is half quenched, ϕ_m is the intensity of monomer emission that would have been observed if no excimer had been formed, and ϕ_{ex} is the intensity of excimer emission that would have been observed if only excimer emission occurred.

When the fluorophore can partition between water and lipid phases, equations analogous to eq A1 and A2 have to be written for each phase. Binding to the lipid phase can be described in terms of a Langmuir adsorption isotherm, with binding to the lipid phase being limited by saturation of the lipid phase, the limit being expressed either in terms of the maximum number of molecules adsorbed per unit area of membrane or, equivalently, by the maximum number of molecules, N, adsorbed per phospholipid molecule (McLaughlin et al., 1976; Jones & Lee, 1984). Binding can then be expressed in terms of a "site" concentration given by NE_t where E_t is the total lipid concentration. The concentration of bound fluorophore, P_b, is given by

$$P_b = [A - (A^2 - 4NE_tP_t)^{1/2}]/2.0 \quad \text{(A3)}$$

where

$$A = K_d + NE_t + P_t \quad \text{(A4)}$$

and P_t is the total concentration of fluorophore and K_d is the dissociation constant for binding. Under conditions where P_t is maintained constant and the lipid concentration E_t is varied, the monomer, F_m^l, and excimer, F_{ex}^l, fluorescence intensities for fluorophore bound to lipid can be written as

$$F_m^l = P_b F_m'^l/[P_t(1 + x/x')] \quad \text{(A5)}$$

$$F_{ex}^l = P_b F_{ex}'^l/[P_t(1 + x'/x)] \quad \text{(A6)}$$

where $F_m'^l$ and $F_{ex}'^l$ are the monomer and excimer fluorescence intensities that the given concentration of fluorophore would exhibit in the lipid phase if all emission were monomer and excimer, respectively. The concentration of fluorophore in the membrane is expressed as the mole ratio x:

$$x = P_b/NE_t \quad \text{(A7)}$$

and x' is the mole ratio of fluorophore at which monomer fluorescence is half quenched.

The fluorescence intensities of monomer, F_m^w, and excimer, F_{ex}^w, from the fluorophore in the aqueous phase can be related approximately to the aqueous concentration $(P_t - P_b)$ by

$$F_m^w = (P_t - P_b)F_m'^w/P_t \quad \text{(A8)}$$

$$F_{ex}^w = (P_t - P_b)F_{ex}'^w/P_t \quad \text{(A9)}$$

where F_m^w and $F_{ex}'^w$ are the monomer and excimer intensities in the absence of lipid. These equations will be accurate at low fluorophore concentrations for fluorophores which are largely water soluble, where the intensities of monomer and excimer emission are close to being linearly related to concentration. For very water-insoluble probes such as pyrenedodecanoic acid, the fluorescence emission from the aqueous phase is so low compared to that from the lipid phase that more elaborate forms for eq A8 and A9 are not required.

When the concentration of fluorophore is greater than that giving aqueous saturation, P_t in eq A4 should be replaced by the saturating concentration P_s in the concentration range $P_t - P_b < P_s$. Simple calculation shows that, with a total concentration of 1 μM, no significant change in the calculated fluorescence profiles occurs down to $P_s = 0.3$ μM.

Registry No. DMPC, 18194-24-6; DOPC, 4235-95-4; BRPC, 61596-55-2; ATPase, 9000-83-3; pyrene, 129-00-0; pyrenebutyric acid, 3443-45-6; pyrene-1-methanol, 24463-15-8; pyrenedodecanoic acid, 73451-05-5; pyreneundecyltrimethylammonium bromide, 72185-37-6; water, 7732-18-5; acetone, 67-64-1; formamide, 75-12-7; ethyl acetate, 141-78-6; methanol, 67-56-1; chloroform, 67-66-3; ethanol, 64-17-5; 2-propanol, 2025-55-0; butanol, 71-36-3; benzene, 71-43-2; isobutyl alcohol, 78-83-1; isopropyl ether, 108-20-3; heptane, 142-82-5.

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