THE REACTION OF *N*-(1-PYRENE)MALEIMIDE WITH SARCOPLASMIC RETICULUM

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ABSTRACT The excimer fluorescence of the adduct of *N*-(1-pyrene)maleimide (PMI) with the Ca^{2+}-ATPase was proposed as a probe of ATPase-ATPase interactions in sarcoplasmic reticulum (Lüdi and Hasselbach, *Eur. J. Biochem.*, 1983, 130:5–8). We tested this proposition by analyzing the spectral properties and stoichiometry of the adducts of pyrenemaleimide with sarcoplasmic reticulum and with dithiothreitol and by comparing the effects of various detergents on the excimer fluorescence of the two adducts, with their influence on the sedimentation characteristics, ATPase activity, and light scattering of the pyrenemaleimide-labeled sarcoplasmic reticulum. These studies indicate that pyrenemaleimide reacts nearly randomly with several SH groups on the Ca^{2+}-ATPase, and suggest that the observed excimer fluorescence of pyrenemaleimide-labeled sarcoplasmic reticulum may reflect intramolecular phenomena rather than ATPase-ATPase interactions. Further work is required to establish the relative contribution of intra- and intermolecular mechanisms to the excimer fluorescence.

INTRODUCTION

Interaction between Ca^{2+} transport ATPase molecules in sarcoplasmic reticulum may contribute to the mechanism of Ca^{2+} translocation (Andersen et al., 1982; Ikemoto and Nelson, 1984; Yamamoto et al., 1984) and to the regulation of the permeability of the membrane (Jilka et al., 1975; Martonosi, 1984; Chiesi, 1984). The evidence in favor of such interactions is largely indirect (Møller et al., 1982; Martonosi and Beeler, 1983), and their physiological relevance is uncertain (Martin and Tanford, 1984; Martin et al., 1984). The existence of ATPase-ATPase interactions in reconstituted ATPase vesicles was suggested by fluorescence energy transfer experiments (Vanderkooi et al., 1977; Watanabe and Inesi, 1982; Yantorno et al., 1983; Yamamoto et al., 1984) but there is as yet no reliable technique that would permit the measurements of ATPase-ATPase interactions in the functional native membrane.

Lüdi and Hasselbach (1982, 1983) proposed that the excimer fluorescence of *N*-(1-pyrene)maleimide covalently bound to the Ca^{2+}-ATPase in native sarcoplasmic reticulum may serve as a probe of ATPase-ATPase interactions. The excimer fluorescence appears at labeling ratios as low as 0.3 mol pyrene-maleimide per mol of ATPase and completely disappears after solubilization of the sarcoplasmic reticulum membrane by detergents. These observations were taken to indicate that the excimer fluorescence arises from interactions between ATPase molecules that position the covalently attached fluorophores on two distinct ATPase molecules within the short distances (≃3 Å) required for the formation of excited-state dimers.

The validity of the PMI excimer fluorescence as an indicator of ATPase-ATPase interactions depends on two assumptions: (*a*) The selective labeling of a single SH group of the Ca^{2+}-ATPase by pyrenemaleimide. (*b*) The effect of detergents on excimer fluorescence is caused entirely by dissociation of the putative ATPase oligomers into monomers.

Based on these two assumptions, Lüdi and Hasselbach (1982, 1983) rationalized their observations in terms of an intermolecular excimer mechanism, in which the two pyrenes that form the excited state dimers are linked to two distinct ATPase molecules, constituting a dimer. There was no direct evidence provided against labeling of Ca^{2+}-ATPase molecules by pyrenemaleimide at multiple sites, that could give rise to intramolecular excimer fluorescence. Neither was an effort made to exclude direct effects of detergents on the formation of excited state dimers, or on the conformation of Ca^{2+}-ATPase molecules, that would account for the observed effects in terms of an intramolecular excimer mechanism.

In view of the radically different implications of the two possible mechanisms, we reinvestigated the reaction of *N*-(1-pyrene)maleimide (PMI) with sarcoplasmic reticulum. We are able to confirm the spectroscopic properties of

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PMI-labeled[1] sarcoplasmic reticulum as reported by Lüdi and Hasselbach (1982, 1983), but our observations indicate multiple labeling of ATPase molecules by PMI under conditions similar to those used in the earlier studies. Furthermore, the effect of detergents on excimer fluorescence was also observed using the adduct of pyrenemaleimide with dithiothreitol as a fluorophore, raising the possibility of a direct interaction between detergents and the pyrene that interferes with the formation of excited state dimers. Therefore our studies suggest that intramolecular excimer formation between PMI molecules attached to vicinal thiols within an ATPase molecule may contribute significantly to the observed excimer fluorescence.

Preliminary reports of these studies were presented (Kracke and Martonosi, 1984; Martonosi et al., 1985; Papp et al., 1985).

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were isolated from predominantly white rabbit muscles essentially as described by Nakamura et al. (1976). The preparations were usually stored before use in a medium of 0.3 M sucrose, 10 mM Tris-maleate buffer, pH 7.0 at a protein concentration of 25–40 mg/ml in polyethylene containers at −70°C. The biuret (Gornall et al., 1949) and the Lowry (Lowry et al., 1951) methods were used for determination of protein.

Fluorescence measurements were performed in an SLM 4800-S subnanosecond lifetime spectrofluorometer (SLM-Aminco Instrument Co., Urbana, IL). Temperature was maintained using a Lauda RM-6 circulating waterbath.

For analysis of the polarization of fluorescence, Glan-Thompson prisms were used and the emission monochromator was replaced by interference filters (Corion Corp., Holliston, MA) of 380 nm and 460 nm for measuring the monomer and excimer emissions, respectively.

Fluorescence lifetimes were measured at 6, 18, and 30 MHz, using glycogen solution as scattering reference. The emission monochromator was replaced, as above, with interference filters of 380 and 460 nm for the measurement of monomer and excimer fluorescence, respectively. Apparent phase (τp) and modulation (τm) lifetimes were obtained and analyzed for heterogeneity using the data collection and analysis routines supplied with the instrument. The emission and excitation spectra were uncorrected.

ATPase activities were measured at 25°C in the following three media: (*a*) Ca^{2+}-free medium: 80 mM KCl, 20 mM K-MOPS, 5 mM $MgCl_2$, 5 mM ATP, and 0.5 mM EGTA, pH 7.0. (*b*) Ca^{2+}-medium: same as the previous solution except that 0.45 mM $CaCl_2$ was also included. (*c*) Ca^{2+} + A23187 medium: same as Ca^{2+}-medium with 1 μM A23187.

The protein concentration was usually 0.1 mg/ml. The liberation of inorganic phosphate was measured by the method of Fiske and Subbarow (1925).

Polyacrylamide gel electrophoresis of sarcoplasmic reticulum proteins and their peptic hydrolysis products was performed according to the Laemmli (1970) or Swank and Munkres (1971) techniques. Cyanogen bromide cleaved myoglobin peptides of 2512, 6214, 8159, 14,404 and intact myoglobin of 16,949 molecular weight, supplied by LKB Instruments, Inc. (Gaithersburg, MD), were used as molecular weight markers for the peptic peptides.

Thin-layer chromatography of peptic peptides on silica gel (Sil G-25, Macherey-Nagel and Co., Brinkmann Instruments, Inc., Westbury, NY) or on cellulose MN 300 plates (Cel 300-10, Macherey-Nagel and Co.) was performed using the following solvent systems: (*a*) *n*-butanol: acetic acid: H_2O, 400:100:100 (vol/vol); (*b*) *n*-butanol: pyridine: acetic acid: H_2O, 244:378:75:302 (vol/vol); (*c*) isoamylalcohol: acetic acid: H_2O, 350:350:280 (vol/vol).

Preparation of Pyrenemaleimide-labeled Sarcoplasmic Reticulum Peptides for Thin-Layer Chromatography. Sarcoplasmic reticulum vesicles, stored frozen in 0.3 M sucrose, 10 mM Tris-maleate, pH 7.0, were thawed, washed twice by centrifugation at 50,000 *g* for 30 min, and suspended at a protein concentration of 2 mg/ml in 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.45 mM EGTA, and 20 mM MOPS, pH 7.0. Pyrenemaleimide was added from a freshly prepared 5 mM stock solution in acetone to a final concentration of 20 μM. The microsomes were incubated for 10 min on ice in the dark; the reaction was stopped with *N*-ethylmaleimide added to a final concentration of 1 mM. After digestion with pepsin (0.4 mg/ml) for 24 h at room temperature as described earlier (Martonosi, 1976), the samples were frozen in dry ice. In control experiments the microsomes were incubated either without pepsin or with pepsin that had been inactivated in boiling water for 10 min. Aliquots containing 100 μg protein were applied for thin-layer chromatography.

Materials

N-(1-pyrene)maleimide was the product of Molecular Probes, Inc., Junction City, OR. Bio-Rad Laboratories, Richmond, CA supplied acrylamide, *N,N'*-methylene-bis-acrylamide, *N,N,N',N'*-tetramethylethylenediamine, 2-mercaptoethanol, and Coomassie Brilliant Blue R-250. Zwittergents 3-08, 3-10, 3-12, 3-14, 3-16 and A23187 were obtained from Calbiochem-Boehring, Los Angeles, CA, octaethylene glycol dodecylether ($C_{12}E_8$) from the Kouyoh Trading Co., Tokyo, Japan, and the precoated glass plates for thin-layer chromatography were from Macherey-Nagel & Co., Brinkmann Instruments, Inc., Westbury, NY. Egg phosphatidylcholine was obtained from Makor Chemicals Ltd., Jerusalem, Israel. Sigma Chemical Co., St. Louis, MO supplied 3-[*N*-morpholino]-propanesulfonic acid, ethyleneglycol-bis (β-aminoethyl ether)*N,N'*-tetraacetic acid (EGTA), *N*-ethylmaleimide, trichloroacetic acid, 1-0-*n*-octyl-*p*-glycopyranoside, octyl phenoxy polyethoxyethanol (Triton X-100), lysophosphatidylcholine (egg yolk) and pepsin. Sodium dodecyl sulfate and guanidine HCl were obtained from Polysciences, Inc., Warrington, PA, *N*-ethyl[2,3-^{14}C]-maleimide from Amersham, Arlington Heights, IL, dimethylformamide, (Sequanol grade) from Pierce Chemical Co., Rockford, IL, sodium deoxycholate and bromophenol blue from Fisher Scientific Co., Fairlawn, NJ.

RESULTS

The Spectral Characteristics of PMI-labeled SR Vesicles

The reaction of *N*-(1-pyrene)maleimide with SH groups in sarcoplasmic reticulum is readily followed by an increase in fluorescence intensity (Fig. 1). The emission maxima at 375, 395, and 415 nm are attributed to the fluorescence of

[1] *Abbreviations used in this paper:* MOPS, 3-[*N*-morpholino]propane sulfonic acid; PMI, *N*-(1-pyrene)maleimide; NEM, *N*-ethylmaleimide; EGTA, ethyleneglycol bis(β-aminoethylether)*N,N,N'N'*-tetraacetic acid; Triton X-100, octylphenoxypolyethoxyethanol; SDS, sodium dodecylsulfate; $C_{12}E_8$, dodecyl octaoxyethylene glycolmonoether; Zwittergent 3-08 through 3-16, *N*-alkyl-*N,N'*-dimethyl-3-amino-1-propane sulfonate; $(PMI)_2$-DTT, the adduct of 2 mol of PMI with 1 mol of dithiothreitol (DTT).

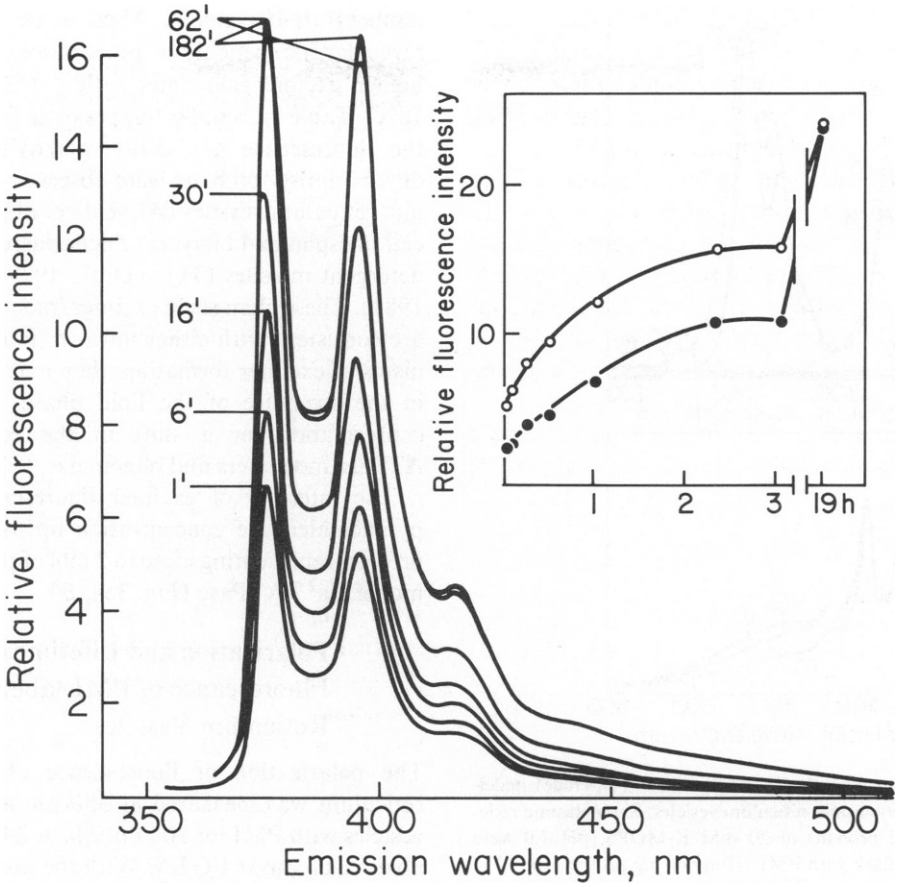

FIGURE 1 Kinetics of the reaction of *N*-(1-pyrene)maleimide with sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles (2 mg protein per ml) were reacted with pyrenemaleimide (10 nmols per mg protein) in a medium of 20 mM K-MOPS, pH 7.0 at 2°C. Samples (50 μl) were taken for the measurement of fluorescence intensity after reaction times of 1, 6, 16, 30, 62, and 182 min, as indicated. The samples were diluted in 1.95 ml 20 mM K-MOPS buffer, pH 7.0 and the emission spectra were recorded using light of 340 nm for excitation. The ratio of 460/375 (excimer/monomer) emission was 0.029 after 1 min and 0.063 after 62 min reaction time. In the insert the changes in emission intensity at 375 nm (monomer, 0) and 460 nm (excimer, ●) are given vs. reaction time. The excimer emission intensity was multiplied by 10.

pyrene monomer, while the broad shoulder at 460 nm represents the excimer fluorescence. At a PMI concentration of 10 nmol per mg protein, the reaction approaches completion after about 19 h of incubation at 2°C. During the progression of the reaction the ratio of the amplitudes of excimer (460 nm)/monomer (375 nm) fluorescence increases (see also Table I).

The excitation maximum of the monomer emission (measured at 375 nm) is at 343 nm (Fig. 2 *A*). The excitation maximum of the excimer band (measured at 460 nm) shows a red shift of about 3 nm with respect to the monomer, and it is slightly broadened (Fig. 2 *A*). A similar red shift and broadening was observed in PMI-labeled tropomyosin by Lehrer et al. (1981).

The rate of PMI reaction with sarcoplasmic reticulum is two to three times faster in the presence of 0.1–1 mM EGTA, than in its absence (Papp et al., 1985). In addition to this increase in the rate of reaction, EGTA also causes a large, temperature-dependent change in the ratio of the amplitudes of excimer/monomer (460/375) emissions, that is shown in Fig. 2 *B* and Table I. The spectra were normalized with respect to the monomer peak at 375 nm. With 0.1 mM or 1 mM EGTA present during the reaction of microsomes with PMI, the ratio of excimer/monomer fluorescence measured at 25°C is significantly increased, compared with microsomes that were labeled with PMI in

TABLE I
THE RATIOS OF EXCIMER/MONOMER FLUORESCENCE INTENSITIES

	F460/F375		
	10°	25°	37°
No EGTA	0.099	0.123	0.118
0.1 mM EGTA	0.177	0.198	0.237
1.0 mM EGTA	0.206	0.246	0.250

The ratios of the fluorescence intensities measured at 460 and 375 nm were determined in experiments of the type shown in Fig. 2 *B*. The microsomes were reacted with 10 nmol of PMI per mg protein without or with 0.1 or 1 mM EGTA for 12 h at 2°C. The measurement of fluorescence was performed at 10, 25, and 37°C using 340 nm light for excitation.

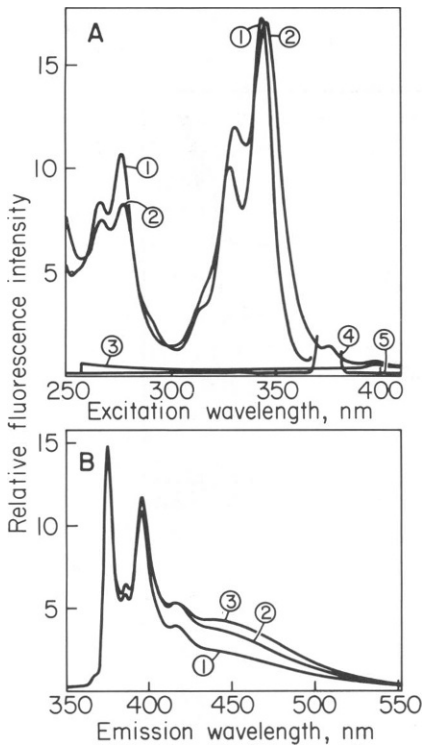

FIGURE 2 (*A*) Fluorescence excitation spectra of *N*-(1-pyrene) maleimide (PMI)-labeled sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles (2 mg/ml protein) in 20 mM K-MOPS, pH 7.0 were incubated at 2°C in the dark with PMI (10 nmol/mg protein) for 12 h. For measurements, 50 μl aliquots of PMI-labeled vesicles were diluted in 1.95 ml K-MOPS (20 mM, pH 7.0) to a final protein concentration of 50 μg/ml. The excitation spectra were recorded at 25°C on an SLM 4800 subnanosecond fluorometer. The emission monochromator was set at 375 nm and 460 nm for recording the monomer and excimer excitation spectra, respectively. Temperature was maintained constant by a Lauda RM6 circulator waterbath. *Line 1:* PMI-SR emission at 375 nm (monomer); *line 2:* PMI-SR emission at 460 nm (excimer); *line 3:* buffer baseline with 460 nm emission; *line 4:* unlabeled sarcoplasmic reticulum emission at 375 nm; *line 5:* buffer baseline with 375 nm emission. (*B*) Fluorescence emission spectra of *N*-(1-pyrene) maleimide (PMI)-labeled sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles (2 mg/ml protein) were labeled with PMI with or without 0.1 or 1 mM EGTA as described in Fig. 2 *A*. When present, EGTA was added 30 minutes prior to the addition of PMI. For fluorescence measurements, PMI-labeled SR vesicles were diluted with 20 mM K-MOPS, pH 7.0, to a final protein concentration of 50 μg/ml. The fluorescence spectra were recorded on an SLM 4800-S subnanosecond fluorometer using glycogen solution as scattering reference. Excitation wavelength was 340 nm. The measurements were performed at 10, 25, and 37°C (only the 25°C data are presented). *Sample 1:* without EGTA; *sample 2:* with 0.1 mM EGTA; *sample 3:* with 1 mM EGTA.

the absence of EGTA (Table I). A similar increase in excimer/monomer ratio was observed during the progression of the reaction of PMI with sarcoplasmic reticulum (Fig. 1). We assume that the effect of EGTA on the excimer/monomer ratio is attributable to a faster reaction of PMI with sarcoplasmic reticulum in the presence of EGTA.

The excimer/monomer fluorescence intensity ratio is temperature-dependent. Measurements on PMI-labeled sarcoplasmic reticulum preparations gave significantly higher excimer/monomer ratios at 25 or 37°C than at 10°C (Table I). Similar temperature dependent changes in the fluorescence of 1,3-di(1-pyrenyl)-propane and 1,3-di(α-naphthyl)propane were observed earlier in sarcoplasmic reticulum vesicles (Almeida et al., 1982, 1984), artificial phospholipid bilayers (Zachariasse et al., 1980) and in detergent micelles (Turro et al., 1979; Turro and Okubo, 1981). These changes in excimer/monomer intensity ratios are consistent with either intra- or intermolecular mechanisms of excimer formation; they may indicate transitions in the structure of the lipid phase, changes in protein conformation, or a shift in the equilibrium between ATPase monomers and oligomers.

The intensity of excimer fluorescence increases with pyrenemaleimide concentration up to ~20 nmol per mg protein, representing close to 2 mol of pyrenemaleimide per mol of Ca^{2+}-ATPase (Fig. 3 *A*, *B*).

Polarization and Lifetime of the Fluorescence of PMI-labeled Sarcoplasmic Reticulum Vesicles

The polarization of fluorescence of PMI-sarcoplasmic reticulum was measured at 380 nm after reaction of the vesicles with PMI for 10 min, 2 h, or 24 h in the presence or absence of 1 mM EGTA. With the progression of reaction in the absence of EGTA, the polarization of fluorescence decreased from 0.262 at 10 min to 0.243 after 24 h. The corresponding values of polarization in the presence of 1 mM EGTA were 0.245 at 10 min and 0.236 at 24 h. The polarization of excimer fluorescence (460 nm) was near zero, as expected from theory (Birks, 1973; Lakowicz, 1982; Steiner, 1983).

Lifetime data for the emission of the monomer (380 nm) and of the excimer (460 nm) show two components—one short, the other long (Table II). Longer reaction time of the sarcoplasmic reticulum with PMI tends to yield shorter lifetimes. In the absence of EGTA both excimer lifetimes are slightly shorter than the corresponding values for the monomer emission (Table II), but these differences are either not apparent or reversed in the presence of EGTA. The data on polarization and lifetime of fluorescence are in reasonable agreement with those reported by Lüdi and Hasselbach (1982).

Energy Transfer Between Protein Tryptophan and the Covalently Bound Pyrenemaleimide

Although the excitation maximum for the pyrenemaleimide adduct is at 343 nm (Fig. 2), excitation at the absorption maximum for tryptophan (285 nm), also causes significant pyrene emission (Fig. 3 *C*, *D*). This emission arises partly from direct excitation of pyrene at 285 nm

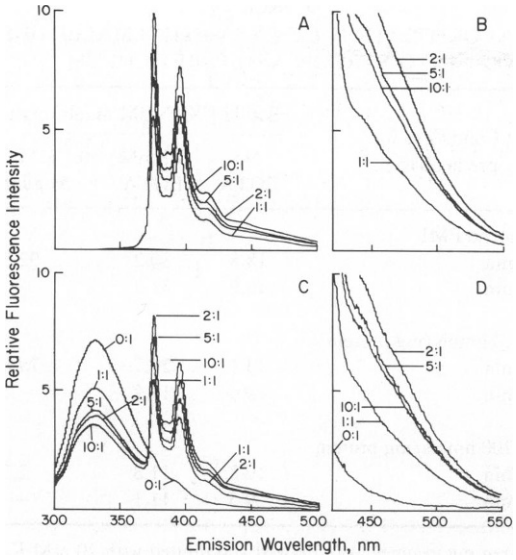

FIGURE 3 Effect of pyrenemaleimide: protein labeling ratios on the fluorescence emission spectra of sarcoplasmic reticulum membranes. SR (2.0 mg protein/ml) was labeled with 20 to 200 μM pyrenemaleimide in solution *A* containing 80 mM KCl, 20 mM K-MOPS, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.45 mM EGTA, pH 7.0 and incubated overnight in the dark, at 2°C, with stirring. Aliquots of the labeling mixture were diluted to 2.0 ml with solution *A* to give a final protein concentration of 50 μg/ml. Spectra were recorded at 23°C immediately after dilution. The labeling ratios that identify the spectral curves refer to the molar ratio of *N*-(1-pyrene) maleimide to the Ca^{2+}-ATPase, assuming that the protein content of SR consists entirely of Ca^{2+}-ATPase molecules of 100,000 D mol wt. The excitation wavelength was 342 nm, the excitation maximum of pyrenemaleimide, for *A* and *B*, and 285 nm in *C* and *D* for excitation of protein tryptophan. The emission spectra in panels *C-D* contain components arising from energy transfer from protein tryptophan and tyrosine groups to the pyrene fluorophore. Panels *B* and *D* represent 10-fold amplifications of the spectra between 400 and 550 nm. Excitation and emissions slits were 4 nm.

and partly from energy transfer between protein side chain groups (tryptophan and tyrosine), and the covalent pyrenemaleimide adduct. Energy transfer is indicated by the decrease in tryptophan fluorescence at labeling ratios of 1–2 mol pyrene per mol of ATPase (Fig. 3 *C*), that is accompanied by proportional increase in the fluorescence of PMI. Based on a PMI extinction coefficient of 25,000 M^{-1} cm^{-1} at 285 nm, the PMI-SR emission spectra should be free of inner filter effects up to labeling ratios of 2 mol PMI per mol of ATPase. At labeling ratios higher than 2:1, self-quenching of PMI fluorescence occurs (Fig. 3 *C*), with only slight change in tryptophan fluorescence. These observations clearly establish that the observed changes in tryptophan fluorescence are due to energy transfer rather than to the filter effect of PMI. The relative amplitudes of the emission maxima of pyrene monomers and excimers are similar over a wide range of labeling ratios, whether 342 nm or 285 nm light was used for excitation. The energy transfer between protein tryptophan and the covalently bound pyrenemaleimide does not imply biologically significant structural relationship between the two chromophores, since energy transfer of comparable magnitude was also observed between tryptophan and an adduct of pyrenemaleimide with dithiothreitol after its incorporation into SR vesicles (see below in Fig. 10). The adduct cannot react covalently with the Ca^{2+}-ATPase and is presumably dissolved in the lipid phase of the membrane.

The Stoichiometry of PMI Reaction

The amount of pyrenemaleimide covalently bound to the sarcoplasmic reticulum proteins was determined by reaction of sarcoplasmic reticulum vesicles overnight with pyrenemaleimide at concentrations ranging from 10 to 100 nmols/mg protein, followed by extraction of the unreacted pyrenemaleimide with organic solvents (Table III). Based

TABLE II
LIFETIME DATA ON PMI-LABELED SARCOPLASMIC RETICULUM

	6 MHz				30 MHz				Lifetimes (heterogeneity)			
	380 nm		460 nm		380 nm		460 nm		380 nm		460 nm	
	τP	τM	τP	τM	τP	τM	τP	τM	τ1	τ2	τ1	τ2
PMI 2 h												
No EGTA	42.70	78.52	44.57	61.59	16.38	49.83	8.27	45.19	8.28 (0.12)	92.83 (0.88)	1.92 (0.04)	64.74 (0.96)
EGTA	46.20	81.35	50.60	60.24	15.49	50.52	12.90	59.28	7.65 (0.20)	75.86 (0.80)	2.85 (0.01)	67.77 (0.99)
PMI 24 h												
No EGTA	43.82	78.46	48.57	60.74	14.56	54.43	13.64	48.39	5.80 (0.26)	64.88 (0.74)	2.98 (0.05)	61.11 (0.95)
EGTA	43.95	81.88	45.14	58.75	14.60	57.48	8.24	45.33	5.38 (0.28)	62.88 (0.72)	1.59 (0.02)	64.85 (0.98)

Sarcoplasmic reticulum (SR) vesicles (2 mg protein/ml) were labeled with 10 nmols of PMI per mg protein in 20 mM K-MOPS, pH 7.0 at 2°C in the dark, in the presence or absence of 1 mM EGTA for 2 h or 24 h. EGTA was added 30 min prior to PMI addition. For lifetime measurements, 0.05 ml PMI-labeled SR vesicles (2 mg/ml protein) were added to 1.95 ml K-MOPS buffer (20 mM, pH 7.0); the final protein concentration was 50 μg/ml. The fluorescence lifetimes were measured 2 or 24 h after PMI addition as described under Methods, at 10°C, using an excitation wavelength of 340 nm. τP and τM are the phase and modulation lifetimes, respectively. τ1 and τ2 are the lifetimes obtained by heterogeneity analysis. The numbers in the parentheses represent the fraction of the emitting fluorophores corresponding to the τ1 and τ2 values.

TABLE III
THE STOICHIOMETRY OF PMI REACTION WITH SARCOPLASMIC RETICULUM

Total PMI nmols/mg protein	PMI bound to proteins nmols per mg protein	
	After extraction with acetone	After extraction with chloroform-methanol
10	9.1 (91%)	7.6 (76%)
20	16.6 (83%)	17.6 (88%)
50	35.5 (71%)	38.1 (76%)
100	57.5 (57%)	64.0 (64%)

Sarcoplasmic reticulum vesicles were incubated in a medium of 0.08 M KCl, 20 mM K-MOPS, pH 7.0, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.45 mM EGTA and pyrenemaleimide at concentrations of 10, 20, 50, and 100 nmol/mg protein overnight in the dark, at 2°C. For acetone extraction the vesicles were stirred with 10 vol of acetone for a few minutes at 25°C, followed by centrifugation. For chloroform-methanol extraction, the vesicle suspension was extracted with 20 vol of 2:1 mixture of chloroform/methanol. The absorption spectrum of the extracts was measured between 320 and 400 nm and the pyrenemaleimide concentration was calculated based on the following absorption coefficients: ϵ_{330} in 90% acetone = 34,200 $M^{-1}cm^{-1}$; ϵ_{342} in chloroform-methanol = 32,800 $M^{-1}cm^{-1}$.

on these data, at a PMI concentration of 20 nmols/mg protein, 83–88% of the PMI is covalently attached to proteins; therefore the noncovalently bound PMI (2.4–3.4 nmols/mg protein) is expected to make only slight contribution to the total fluorescence. The noncovalently bound PMI progressively loses its reactivity during overnight incubation by decomposition to the corresponding acid derivative. By increasing the PMI concentration to 100 nmol/mg protein, the amount of PMI bound can be readily increased to ≃60 nmol/mg protein. As most of the PMI reacts with the Ca^{2+}-ATPase, in agreement with Lüdi and Hasselbach (1982), the PMI-ATPase ratio may reach 6–8 mol of PMI per mol of ATPase. These observations clearly indicate that several SH groups are available for reaction with pyrenemaleimide.

The Involvement of Distinct Types of SH Groups in the Reaction of Sarcoplasmic Reticulum with PMI

Reaction of sarcoplasmic reticulum vesicles with [^{14}C]*N*-ethylmaleimide followed by peptic hydrolysis and the separation of the peptides by high voltage electrophoresis, permits the identification of several distinct SH peptides by autoradiography (Martonosi, 1976). This method offers an opportunity for the analysis of the effect of PMI on the reaction of [^{14}C]NEM with individual SH groups.

The reaction of sarcoplasmic reticulum SH groups with [^{14}C]NEM is promoted by EGTA (Table IV). After unfolding of the protein in 3 M guanidine, about one-half of the total SH groups react with NEM within 60 min at 2°C. Preincubation of microsomes with 10 nmol of pyrenemaleimide per mg protein causes significant inhibition of the [^{14}C]NEM reaction under all three conditions, and this inhibition becomes more pronounced at 200 nmol of PMI per mg protein (Table IV). Under these conditions the number of SH groups blocked by PMI, as given by the inhibition of [^{14}C]NEM incorporation, exceeds 1 mol per mol of ATPase.

TABLE IV
INCORPORATION OF [^{14}C]*N*-ETHYLMALEIMIDE INTO SARCOPLASMIC RETICULUM

Conditions for preincubation	Bound [^{14}C]NEM nmols/mg protein		
	No EGTA	0.1 mM EGTA	0.1 mM EGTA 3 M guanidine
Control, no PMI			
15 min	18.8	30.2	92.5
60 min	16.9	31.3	—
PMI, 10 nmols/mg protein			
15 min	13.1	20.7	79.8
60 min	12.6	19.2	—
PMI, 200 nmols/mg protein			
15 min	10.8	11.8	2.3
60 min	9.5	11.1	—

The frozen microsomes were thawed and diluted with 20 mM K-MOPS buffer, pH 7.0 to a protein concentration of ~2.5 mg/ml. After centrifugation at 55,000 *g* for 30 min the microsomes were resuspended in 20 mM K-MOPS, pH 7.0 to a final protein concentration of 10 mg/ml. The microsomes were preincubated in ice with or without 1 mM EGTA for 30 min, followed by the addition of pyrenemaleimide when indicated, at final concentrations of 10 and 200 nmols/mg protein, respectively. During preincubation some samples also contained guanidine (3 M) in addition to EGTA. After incubation with PMI for 15 to 60 min, 200 nmols [^{14}C]N-ethylmaleimide per mg protein (0.5 μCi per ml) were added and the reaction was allowed to proceed in ice for 60 min, as indicated. The reaction was stopped with 50-fold excess of unlabeled *N*-ethylmaleimide and the proteins were precipitated and washed with 2% TCA. After extraction with 90% acetone, dry acetone and ether, the dry powder was suspended in Lowry reagent A, containing 1% SDS, and after heating at 90°C for 1 h the protein was determined according to Lowry et al. (1951). Aliquots of the same samples were analyzed for radioactivity in a Beckman LS 7500 liquid scintillation counter using an Insta-Gel solution (Packard Instrument Company, Downers Grove, IL) as scintillator.

Autoradiograms of the peptide maps obtained from samples similar to those of Table IV, indicate that [^{14}C]NEM labels at least seven distinct peptide bands, and that preincubation of microsomes with either 10 or 200 nmol of PMI per mg protein decreases the [^{14}C]NEM radioactivity with slight selectivity differences in all labeled peptides (Fig. 4). These experiments, together with data provided earlier on the stoichiometry of PMI labeling of sarcoplasmic reticulum do not support the presumed selective labeling of a single SH group of the Ca^{2+}-ATPase by PMI. Rather, they indicate that the PMI reaction with the SH groups of the Ca^{2+}-ATPase is essentially random and therefore it is probable that each ATPase molecule is labeled by PMI at multiple sites.

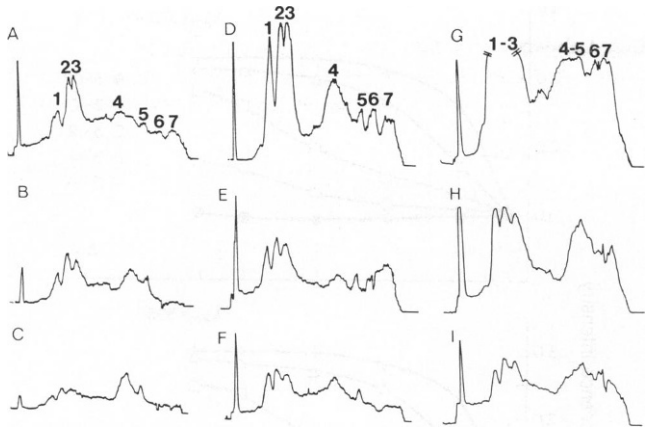

FIGURE 4 The effect of pyrenemaleimide on the reaction of [^{14}C]*N*-ethyl-maleimide with sarcoplasmic reticulum. The reaction of sarcoplasmic reticulum vesicles with PMI and [^{14}C]NEM was carried out as described under legend to Table IV, with the following modification. The acetone-ether dried sarcoplasmic reticulum powder was suspended in 0.06 N HCl and digested with pepsin at room temperature for 24 h, as described earlier (Martonosi, 1976). The peptides were separated by high voltage electrophoresis at pH 3.5 in a buffer solution of 2.2% acetic acid and 0.22% pyridine. The electrophorograms were exposed for autoradiography on Kodak X-OMat AR-5 x-ray film for 173 d at −70°C, followed by densitometry. In the densitograms the line of application is represented by the sharp peak on the left, that is attributable to insoluble aggregates. The majority of peptides moved from left to right toward the cathode. (*A*) control microsomes reacted with 200 nmol [^{14}C]NEM per mg protein for 60 min at 2°C. (*B*) same as *A* but pretreated with 10 nmols of PMI per mg protein at 2°C for 15 min. (*C*) same as *B* but pretreated with 200 nmol of PMI per mg protein (*D–F*) microsomes were exposed to 1 mM EGTA for 30 min prior to reaction with PMI or NEM. (*D*) EGTA treated microsomes reacted with 200 nmols of [^{14}C]NEM per mg protein at 2°C for 60 min. (*E*) same as *D* but after pretreatment with 10 nmol of PMI per mg protein. (*F*) same as *E* but after pretreatment with 200 nmol of PMI per mg protein. (*G, H, I*) microsomes were treated with 1 mM EGTA and 3 M guanidine for 30 min. (*G*) guanidine-EGTA treated microsomes reacted with 200 nmol of [^{14}C]NEM per mg protein for 60 min at 2°C. (*H*) same as *G*, but pretreated with 10 nmols of PMI per mg protein. (*I*) same as *H* but pretreated with 200 nmols of PMI per mg protein. For densitometry an LKB model 2202 laser densitometer and a Hewlett-Packard model 3390A integrator-plotter were used. Peaks *1–7* designate classes of SH peptides with distinct electrophoretic mobilities.

Analysis of Peptic Peptides of PMI-labeled Sarcoplasmic Reticulum by Gel Electrophoresis and Thin-Layer Chromatography

PMI-labeled sarcoplasmic reticulum vesicles were subjected to complete peptic hydrolysis in 0.06 N HCl as described earlier (Martonosi, 1976), and the resulting peptide mixture was separated on polyacrylamide gels by the Laemmli (1970) or Swank and Munkres (1971) techniques. Inspection of the gels under ultraviolet light revealed that the pyrene fluorescence in undigested sarcoplasmic reticulum was associated nearly exclusively with the ≃100 KDa band of the Ca^{2+}-ATPase, in agreement with the observations of Lüdi and Hasselbach (1982). After exhaustive peptic digestion, the Ca^{2+}-ATPase band disappeared and the fluorescence migrated in a broad, heterogenous band, with approximate molecular weight in the range of 2,000–4,000 D.

Thin layer chromatography of the same peptide mixtures on silica gel with a solvent system of *n*-butanol/ acetic acid/ H_2O, 4:1:1 (vol/vol) yielded a total of 12 fluorescent peptide bands. Most of these bands contained significant autofluorescence that hindered the identification of PMI fluorescence. Since the undigested starting material contained intense fluorescence that was retained at the origin of the TLC plate, we assume that the PMI fluorescence was distributed after proteolysis among a large number of peptides, making their identification against the background of autofluorescent peptides unconvincing. Had the PMI reaction been confined to one or two SH groups, the detection of the corresponding one or two SH peptides could have been accomplished with certainty.

The Effect of Detergents on the Fluorescence of Pyrenemaleimide-labeled Sarcoplasmic Reticulum

It was argued (Lüdi and Hasselbach, 1982) that the excimer fluorescence of pyrenemaleimide-labeled sarcoplasmic reticulum indicates the presence of ATPase oligomers because nonsolubilizing concentration of detergents (myristoyl glycerophosphocholine or dodecyl octaoxyethylene glycol monoether) abolished the excimer fluorescence. This argument is flawed because the presumed effect of detergents on ATPase-ATPase interactions implies some conformational change that may also influence intramolecular excimer fluorescence. We attempted to clarify the mechanism of the effect of detergents on excimer fluorescence by comparing the effects of a variety of detergents on the emission spectra of pyrenemaleimide-labeled sarcoplasmic reticulum with their effects on the ATPase activity and light scattering of PMI-labeled SR suspensions.

Zwitterionic Detergents. Zwittergent detergents are sulfobetaines, having the following general structure:

$$C_nH^+_{2n+1}-\underset{\displaystyle CH_3}{\overset{\displaystyle CH_3}{\overset{|}{\underset{|}{N}}}}-(CH_2)_3-\overset{\displaystyle O}{\overset{\|}{\underset{\|}{S}}}-O^-,$$

Scheme I

where n = 8–16, corresponding to the 3-08 to 3-16 designation. Unlike other amphoteric surfactants, they retain their zwitterionic character over the entire pH range. This property is attributed to the presence of a strongly basic quaternary ammonium ion and an acidic sulfonate ion of equal strength. The ability of zwitterionic

detergents to abolish excimer fluorescence (EM = 460 nm) is a function of alkyl chain length (Figs. 5 and 6). Zwittergent 3-08 with an eight-carbon hydrophobic tail had no effect on the excimer fluorescence at concentrations up to 2 mg/ml, whereas Zwittergent 3-16, with a 16-carbon tail almost entirely abolished excimer fluorescence at a concentration of 0.03 mg/ml. The effectiveness of zwittergents of intermediate chain lengths fell in between (Fig. 5). The effectiveness of zwittergents to increase the monomer emission of PMI-labeled sarcoplasmic reticulum at 375 and 395 nm also increased with the length of the alkyl chain (Figs. 5 and 6).

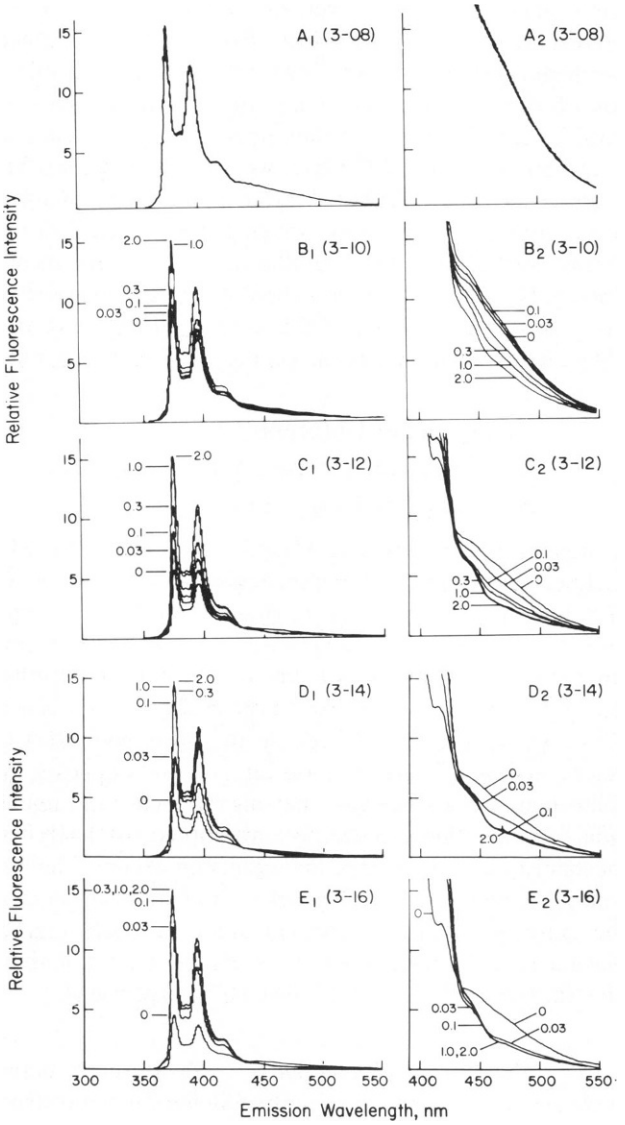

FIGURE 5 The effect of zwitterionic detergents (Zwittergents) on the fluorescence emission spectra of *N*-(1-pyrene)maleimide labeled sarcoplasmic reticulum. To pyrenemaleimide labeled SR suspended in 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.45 mM EGTA, and 20 mM MOPS buffer, pH 7.0, detergents were added from a 10 mg/ml stock solution to final concentrations ranging between 0.03 and 2 mg/ml and the spectra were recorded at 23°C. The final protein concentration was 0.050 mg/ml. The final detergent concentrations are indicated in panels *B* to *E*. In panel *A* all six detergent concentrations (0, 0.03, 0.1, 0.3, 1.0, and 2.0 mg/ml) gave the same spectra and were not identified individually. Data obtained with Zwittergents 3-08, 3-10, 3-12, 3-14, and 3-16 are represented in panels *A* to *E*, respectively. Panels A_1–E_1 give the emission spectra between 350 and 550 nm. In panels A_2–E_2 a 10-fold amplification of the portion of the spectra between 400 and 550 nm is presented that contains the excimer band. The emission spectra were measured at an excitation wavelength of 342 nm. Excitation and emission slits were both 4 nm and the temperature was 23°C.

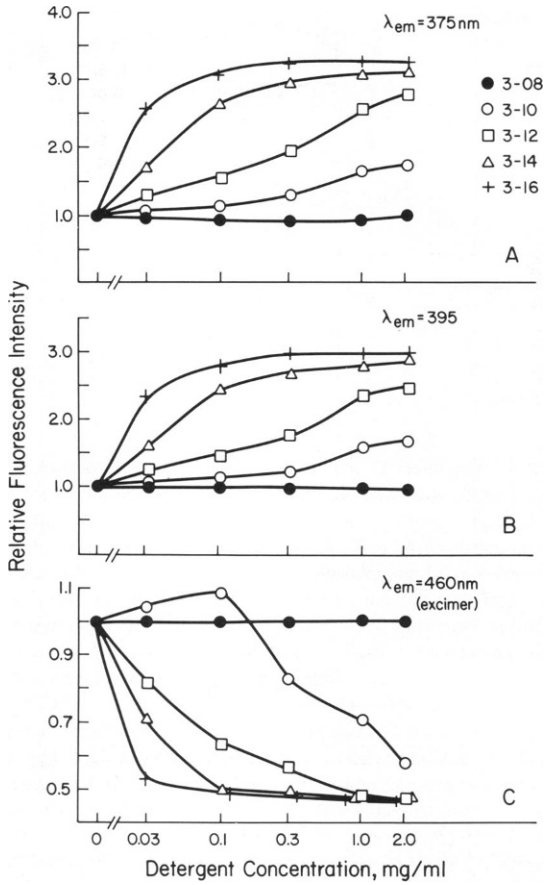

FIGURE 6 Effect of zwitterionic detergents on the monomer and excimer fluorescence of the *N*-(1-pyrene) maleimide labeled sarcoplasmic reticulum. The relative fluorescence intensities measured at 375 nm (*A*), 395 nm (*B*), and at 460 nm (*C*) in Fig. 5 were normalized to the corresponding spectra taken in the absence of detergent, and plotted against the final concentration of the detergent. Symbols: ●, 3-08, 0, 3-10; □, 3-12; △, 3-14; +, 3-16.

Other Detergents. Lysolecithin, sodium dodecylsulfate, deoxycholate and Triton X-100 decreased excimer fluorescence (460 nm) and produced a large increase in monomer fluorescence (375 and 395 nm) already at 0.1 mg/ml concentration (Fig. 7). Dodecyloctaoxyethyleneglycol monoether ($C_{12}E_8$), Brij-35 and octyl glucoside had only slight effects on the excimer fluorescence even at 1 mg/ml concentration, although they increased the fluorescence at 375 and 395 nm.

These observations indicate that the changes in excimer fluorescence induced by detergents are always paralleled by large changes in "monomer" fluorescence measured at 375 and 395 nm. A similar increase in the fluorescence

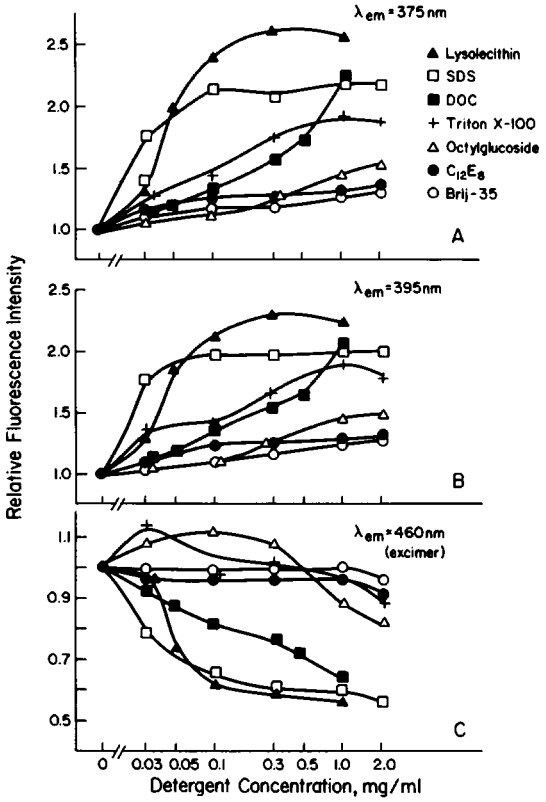

FIGURE 7 Effect of various detergents on the monomer and excimer fluorescence of *N*-(1-pyrene) maleimide labeled sarcoplasmic reticulum. The relative fluorescence intensities at emission wavelengths of 375 (*A*), 395 (*B*), and 460 (*C*) nm were calculated from emission spectra similar to those given in Fig. 1 but measured in the presence of different detergents. The fluorescence intensities of the detergent containing solutions were normalized to the corresponding spectra recorded in the absence of detergents and plotted as the function of detergent concentration. For experiments with SDS, solution A was replaced with a solution containing 0.15 M choline chloride, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.45 mM $CaCl_2$, 10 mM histidine, pH 6.8. Symbols: ▲, lysolecithin, □, sodium dodecylsulfate (SDS); ■, K-deoxycholate (DOC); +, Triton X-100; △, octylglucoside; ●, $C_{12}E_8$; ○, Brij-35.

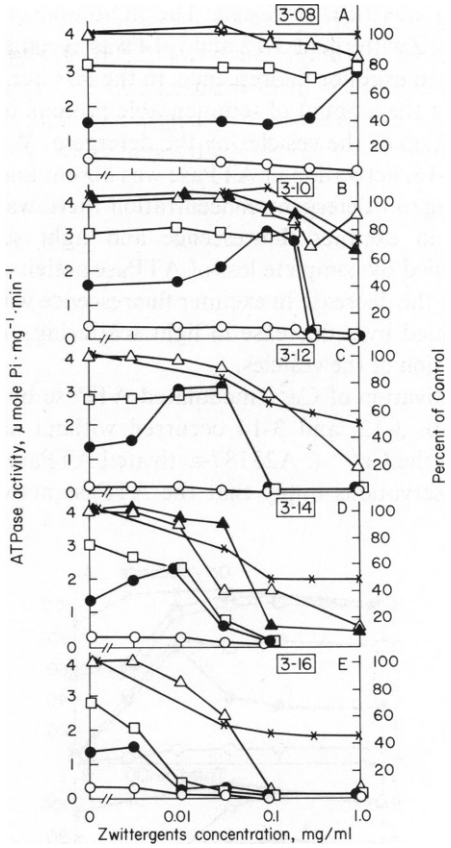

FIGURE 8 Effect of Zwittergents on the ATPase activity, solubility and excimer fluorescence of pyrenemaleimide-labeled sarcoplasmic reticulum (SR). A suspension of SR vesicles (protein concentration of 2 mg/ml) was labeled with 40 μM pyrenemaleimide in a solution containing 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.45 mM EGTA, 20 mM MOPS, pH 7.0, in the dark, on ice, overnight. To 0.5 ml aliquots of the labeled SR (PMI-SR), sufficient amounts of the various detergents were added from stock solutions of either 10 mg/ml or 50 mg/ml to yield protein to detergent ratios in the range of 0.05 to 16.7; the mixture was incubated at 23°C for 10 min. *ATPase determinations.* Aliquots of the detergent treated PMI-SR containing 0.1 mg protein were assayed at 25°C at the final detergent concentrations indicated on the abscissa in 2 ml of the following assay media: *Ca^{2+}-free medium.* (○), 80 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM EGTA, 20 mM MOPS, pH 7.0; *Ca^{2+} medium.* (●), same as the previous solution except that 0.45 mM $CaCl_2$ was included; *Ca^{2+} + A23187 medium.* (□), same as the Ca-free medium except that both 0.45 mM $CaCl_2$ and 1 μM A23187 were included. *Measurement of 90° light scattering,* (△). The light scattering of the PMI-SR was measured under the conditions used in the calcium containing ATPase assay in a Varian SF-330 spectrofluorometer with both excitation and emission wavelengths set at 550 nm. The 90° light scattering is expressed as a percentage of the light-scattering of the detergent-free control sample. *The assay of sedimentable protein,* (▲). After treatment of PMI-SR with detergents as described above, samples containing 0.5 mg of protein were added to 10 ml of a medium of the following composition: 80 mM KCl, 5 mM $MgCl_2$, 0.45 mM EGTA, 20 mM MOPS, pH 7.0. After centrifugation at 70,000 *g* for 30 min, the supernatant was discarded and the pellet was resuspended in 1 ml water for protein assay. The protein content of the pellet is expressed as percent of the protein in the pellet of the detergent-free control sample. *Excimer fluorescence* (x). The fluorescence was measured at 460 nm with excitation at 342 nm, in a medium of 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.45 mM EGTA, 20 mM MOPS, pH 7.0, with detergents added as indicated. (*A*), Zwittergent 3-08. (*B*) Zwittergent 3-10. (*C*) Zwittergent 3-12. (*D*) Zwittergent 3-14. (*E*) Zwittergent 3-16.

intensity of pyrenemaleimide-β-mercaptoethanol adduct was observed at 375 nm in the presence of lysolecithin, lecithin, Triton X-100, and deoxycholate (not shown).

The Effect of Detergents on the ATPase Activity and Light Scattering of Sarcoplasmic Reticulum

The effect of zwitterionic detergents on the ATPase activity of sarcoplasmic reticulum is precisely defined by the length of the alkyl chain. The Zwittergent 3-08, with an alkyl chain of eight-carbon length produced slight activation of Ca^{2+}-modulated ATPase activity at 1 mg/ml concentration, with some decrease in the light scattering measured at 550 nm (Fig. 8). The excimer fluorescence remained unaffected. With Zwittergents of 10, 12, and 14 carbon alkyl chain length, the ATPase activation occurred at progressively lower detergent concentration, followed by sharp inhibition of ATPase activity as the detergent con-

centration was further raised. The inhibition of ATPase activity by Zwittergent 3-12 and 3-14 was accompanied by decreases in excimer fluorescence, in the 90° light scattering, and in the amount of sedimentable protein, indicating solubilization of the vesicles by the detergent. With Zwittergent 3-16, activation of ATPase was absent and already at 0.03 mg/ml detergent concentration there was a large decrease in excimer fluorescence and light scattering, accompanied by complete loss of ATPase activity (Fig. 8). Note that the decrease in excimer fluorescence was usually accompanied by a decrease in light scattering, indicating solubilization of the vesicles.

The activation of Ca^{2+}-modulated ATPase by Zwittergents 3-10, 3-12, and 3-14 occurred without significant change in the Ca^{2+} + A23187-activated ATPase (Fig. 8). These observations imply that the ATPase activation by these detergents is attributable entirely to an increase in the Ca^{2+} permeability of the membrane, that prevents the increase of intravesicular [Ca^{2+}] to inhibitory levels.

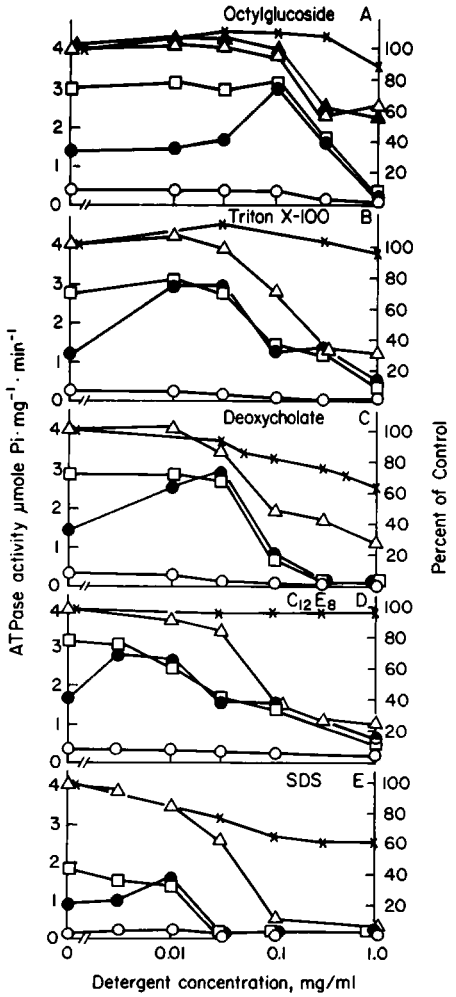

FIGURE 9 The effects of octylglucoside, Triton X-100, deoxycholate, $C_{12}E_8$ and sodium dodecylsulfate on the ATPase activity and solubility of sarcoplasmic reticulum membranes. The experiments were performed as described in Legend to Fig. 8. In the experiment with SDS, the 80 mM KCl was replaced in the labeling buffer and in the assay media by 80 mM choline chloride. (*A*) octylglucoside. (*B*) Triton X-100. (*C*) deoxycholate. (*D*) octaoxyethylene-glycoldodecylether ($C_{12}E_8$). (*E*) sodium dodecylsulfate. For symbols, see Fig. 8.

Based on their effects on ATPase activity, light scattering and excimer fluorescence, the neutral and acidic detergents fall into several groups (Fig. 9). Octylglucoside, Triton X-100, deoxycholate and $C_{12}E_8$ activate Ca^{2+} modulated ATPase activity at detergent concentrations in the range of 0.01–0.1 mg/ml, in agreement with earlier observations from several laboratories (for review, see Møller et al., 1982; Martonosi and Beeler, 1983). This activation is presumably due to an increase in the Ca^{2+} permeability of the membrane, because the maximum activation by detergents did not exceed the ATPase activity observed in the presence of the Ca^{2+} ionophore A23187. The detergent concentration required for maximal activation was ≃0.1 mg/ml for octylglucoside, ≃0.03 mg/ml for Triton X-100 and deoxycholate, and ≃0.01 mg/ml for $C_{12}E_8$ at a protein concentration of 0.05 mg/ml in the ATPase assay medium. At detergent concentrations higher than 0.1 mg/ml octylglucoside, Triton X-100 and $C_{12}E_8$ decreased light scattering, indicating solubilization of the vesicles, with inhibition of ATPase activity, but with little or no change in excimer fluorescence. The presence of excimer fluorescence at $C_{12}E_8$ concentrations of 0.1–1.0 mg/ml, conflicts with the observations of Lüdi and Hasselbach (1982), who found that $C_{12}E_8$ was an effective inhibitor of excimer fluorescence at nonsolubilizing concentrations. The reason for these differences is unknown. Deoxycholate and sodium dodecylsulfate markedly reduce the excimer fluorescence at concentrations of 0.1 mg/ml or greater, with inhibition of ATPase activity and extensive solubilization of microsomes, in agreement with the data of Lüdi and Hasselbach (1982). It is of potential significance that the activation of Ca^{2+}-modulated ATPase activity by several detergents, with the underlying increase in the Ca^{2+} permeability of the membrane, occurred without major change in either light scattering or in excimer fluorescence.

The Properties of the Pyrenemaleimide-Dithiothreitol Adduct

The interpretation of the effect of detergents on the fluorescence of pyrenemaleimide covalently bound to the Ca^{2+}-ATPase requires some information about the fluorescence responses of a simple model system to detergents. We chose the adduct of pyrenemaleimide with dithiothreitol, $(PMI)_2$-DTT, for these studies. As expected from earlier work by Weltman et al. (1973), both SH groups of dithiothreitol react with pyrenemaleimide and in $(PMI)_2$-DTT the two pyrene moieties are located in appropriate positions for efficient excimer fluorescence with an emission maximum near 460 nm measured either in ethanol or in H_2O.

The $(PMI)_2$-DTT adduct is readily incorporated into sarcoplasmic reticulum vesicles or liposomes with retention of the essential aspects of its emission spectrum observed in

ethanol but with dramatic increase in fluorescence intensity compared with the fluorescence of $(PMI)_2$-DTT in 20 mM K-MOPS, pH 7.0 in the absence of sarcoplasmic reticulum or liposomes. The changes in fluorescence intensity together with ultracentrifuge and dialysis data indicate that essentially all $(PMI)_2$-DTT is taken up by the membranes, according to expectations. The fluorescence emission of the $(PMI)_2$-DTT adduct incorporated into egg phosphatidylcholine liposomes is 2.3 times greater using 340 nm, as compared with 280 nm light for excitation; under the same conditions the fluorescence intensity of $(PMI)_2$-DTT adduct in sarcoplasmic reticulum vesicles was nearly equal in excitation at 280 or 340 nm (Fig. 10). It is safe to assume that in liposomes the fluorescence of $(PMI)_2$–DTT arises from direct excitation of pyrene at both wavelengths. Therefore the relative increase in the fluorescence of $(PMI)_2$-DTT in sarcoplasmic reticulum as compared with liposomes at the excitation maximum of tryptophan (≃280 nm) is probably attributable to energy transfer between protein tryptophan and the $(PMI)_2$-DTT adduct dissolved in the lipid phase of sarcoplasmic reticulum (Fig. 10).

The effects of different detergents on the emission spectrum of $(PMI)_2$-DTT are summarized in Fig. 11 for sarcoplasmic reticulum vesicles, and in Fig. 12 for egg lecithin liposomes. In both systems the excimer fluorescence of $(PMI)_2$-DTT, measured at 460 nm, was sharply

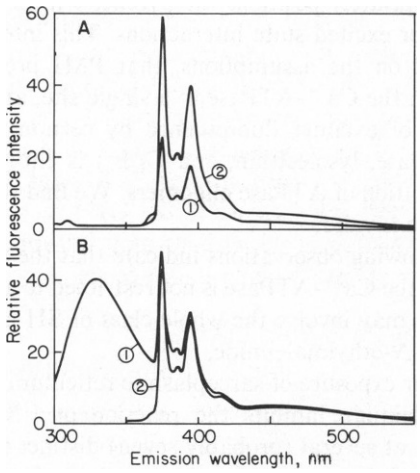

FIGURE 10 Fluorescence spectra of $(PMI)_2$-DTT adduct in liposomes prepared from egg phosphatidylcholine (A) and in SR suspension (B) at 25°C. Stock solutions (10 mM) of PMI and dithiothreitol (DTT) were prepared in acetone and ethanol, respectively. The adduct was formed by incubation of a mixture of 50 μl of PMI and 25 μl of DTT solutions for 90 min at 25°C. The solution was diluted in 0.5 ml ETOH and 40 μl aliquots were added to either 2 ml SR suspension (2 mg/ml protein), or to 2 ml of liposome suspension (2 mg/ml) in 20 mM K-MOPS at pH 7.0. The microsomes were incubated at 2°C overnight. For fluorescence measurements 50 μl of the SR or liposome suspensions were diluted in 2 ml 20 mM K-MOPS, pH 7.0. Liposomes were prepared as described earlier (Jilka et al., 1975); after the addition of $(PMI)_2$-DTT adduct, the liposomes were sonicated two times for 10 s and used for the measurement of fluorescence. (1) Excitation wavelength of 280 nm. (2) Excitation wavelength of 340 nm. Both spectra were recorded under similar conditions.

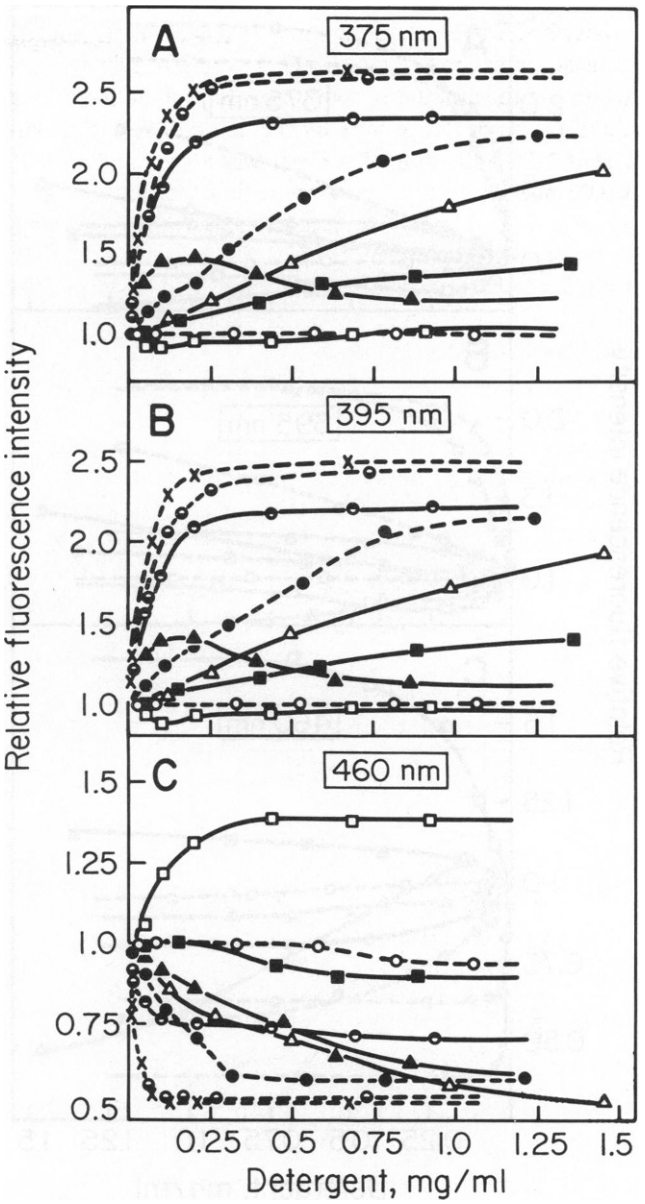

FIGURE 11 The effect of detergents on the fluorescence of pyrene-maleimide-dithiothreitol adduct in sarcoplasmic reticulum vesicles. The $(PMI)_2$-DTT adduct was prepared and incorporated into sarcoplasmic reticulum vesicles as described in the legend to Fig. 10. The detergents were added from concentrated stock solutions to the final concentrations indicated on the abscissa, and the spectra were recorded on an SLM 4800 fluorescence spectrometer using light of 340 nm for excitation; slit width, 4 nm (excitation) and 2 nm (emission). The following Zwittergents were used (broken lines): ○, 3-08; ●, 3-12; ⊖, 3-14; +, 3-16. Other detergents were (solid lines): □, $C_{12}E_8$; ■, Triton X-100; △, deoxycholate; ⊖, lysolecithin; ▲, sodium dodecylsulfate. All data were normalized with respect to the fluorescence emissions measured in the absence of detergents. Panel *A*, emission at 375 nm; panel *B*, emission at 395 nm; panel *C*, emission at 460 nm.

reduced by Zwittergent 3-12, 3-14, 3-16, and deoxycholate, whereas $C_{12}E_8$ significantly increased the excimer fluorescence; other detergents had intermediate effects. The increased excimer fluorescence caused by $C_{12}E_8$ is also decreased by Zwittergent 3-16 at concentrations of 0.5–1.0

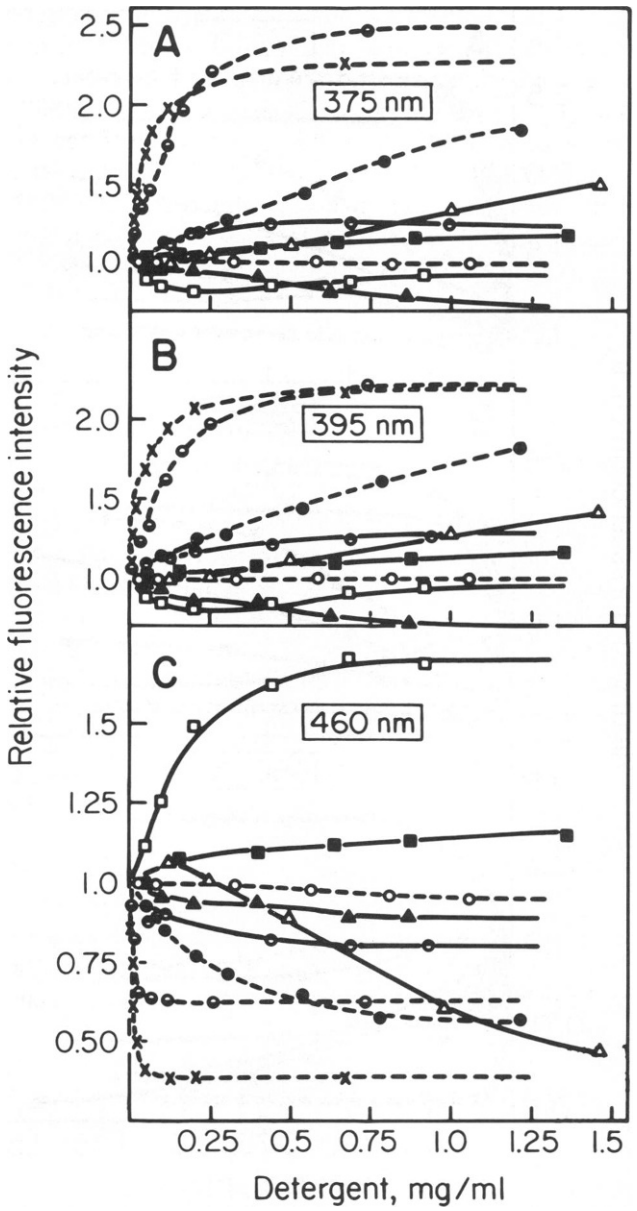

FIGURE 12 The effect of detergents on the fluorescence of pyrenemaleimide-dithiothreitol adduct in liposomes prepared from egg lecithin. The experiments were performed essentially as described in legend to Fig. 11, except that the $(PMI)_2$-DTT adduct was incorporated into liposomes as described in Fig. 10.

mg/ml, that are one order of magnitude higher than the effective Zwittergent concentration required for inhibition of excimer fluorescence in the absence of $C_{12}E_8$. The effects of Zwittergent 3-16 and deoxycholate indicate that detergents of appropriate structure can interfere with the formation of excited state dimers between the two pyrene moieties in $(PMI)_2$-DTT, whereas $C_{12}E_8$ may facilitate such interactions.

The decrease in excimer fluorescence caused by detergents is usually accompanied by an increase in monomer fluorescence at 375 and 395 nm (Figs. 11 and 12).

The effects of detergents on the $(PMI)_2$-DTT adduct incorporated into sarcoplasmic reticulum or phospholipid vesicles (Figs. 11 and 12) are qualitatively similar to the effects of the same detergents on the fluorescence of the PMI covalently bound to the Ca^{2+}-ATPase of sarcoplasmic reticulum (Figs. 5 through 7).

In light of these observations, together with other evidence outlined earlier in this report, the effects of deoxycholate and lysolecithin on excimer fluorescence described by Lüdi and Hasselbach (1982) cannot be used as evidence for the dissociation of preexisting ATPase oligomers by detergents.

DISCUSSION

Pyrenemaleimide reacts with a class of SH groups in sarcoplasmic reticulum giving rise to enhanced fluorescence at 375, 395, and 415 nm, corresponding to the emission of pyrene monomer, and to a broad band of fluorescence at 460 nm that is due to pyrene excimers. We confirmed the observation of Lüdi and Hasselbach (1982, 1983) that much of this reaction involves the Ca^{2+} transport ATPase and there was little incorporation of pyrenemaleimide in other proteins of sarcoplasmic reticulum. The ATPase activity was not affected significantly by the incorporation of 1–2 mol of PMI per mol of ATPase.

According to Lüdi and Hasselbach (1982, 1983) the two pyrene moieties forming the excimer are located on two distinct ATPase molecules within an oligomeric structure that appropriately positions the pyrenes within the 3–4 Å distance for excited state interactions. This interpretation was based on the assumptions, that PMI preferentially reacts with the Ca^{2+}-ATPase at a single site, and that the inhibition of excimer fluorescence by certain detergents (deoxycholate, lysolecithin, and $C_{12}E_8$) is entirely due to the dissociation of ATPase oligomers. We find that neither assumption is valid.

The following observations indicate that the reaction of PMI with the Ca^{2+}-ATPase is not restricted to a single SH group, but may involve the whole class of SH groups that react with *N*-ethylmaleimide.

(*a*) Prior exposure of sarcoplasmic reticulum vesicles to pyrenemaleimide inhibits the reaction of [^{14}C]*N*-ethylmaleimide at several (probably seven) distinct SH groups as indicated by the decreased labeling of several SH peptides produced by peptic hydrolysis. There were also indications of multiple bands of fluorescence after separation of peptic peptides of pyrenemaleimide-labeled sarcoplasmic reticulum on urea-gel, or by thin layer chromatography.

(*b*) Pyrenemaleimide reacts with 50–60 nmol of SH equivalents per mg sarcoplasmic reticulum protein, if it is supplied in sufficient concentration; this represents conservatively, 5–6 SH groups per mole of ATPase. It is not plausible to assume that at lower labeling ratios the reaction would be limited to a single SH group, since the rapid decline in the rate of pyrenemaleimide reaction at

low PMI concentration is satisfactorily explained by the decrease in PMI concentration.

(*c*) The polarization of pyrene fluorescence decreases during the course of progressive labeling of sarcoplasmic reticulum. A decrease in polarization was also observed when the labeling of PMI was carried out in the presence of EGTA, that promotes the incorporation of PMI into the sarcoplasmic reticulum. These observations suggest that the class of rapidly reacting SH groups provides an environment conducive to higher polarization than the slower reacting SH groups.

These observations clearly establish that the labeling of Ca^{2+}-ATPase by PMI occurs at several distinct sites. Therefore, in the absence of evidence to the contrary, the excimer emission observed at 460 nm in PMI-labeled sarcoplasmic reticulum may be attributed partly or entirely to multiple labeling of ATPase molecules, giving rise to intramolecular excimers between pyrene groups covalently attached to adjacent thiols within a single ATPase molecule.

The inhibition of excimer fluorescence by deoxycholate, lysolecithin, and $C_{12}E_8$ (Lüdi and Hasselbach, 1982, 1983) cannot be attributed without independent evidence exclusively to the dissociation of hypothetical ATPase oligomers for the following reasons:

(*a*) According to our observations, the loss of excimer fluorescence in the presence of detergents is generally accompanied by a decrease in light scattering and a change in the sedimentation pattern of membrane proteins, indicating concomitant solubilization of microsomes. Solubilization of microsomes occurred at lower concentrations of octylglucoside, Triton X-100, and deoxycholate than those required to decrease excimer fluorescence. In fact, in our hands $C_{12}E_8$ did not decrease the excimer fluorescence, even at concentrations that caused large decrease in light scattering and an inhibition of ATPase activity, although under similar conditions $C_{12}E_8$ causes the dissociation of Ca^{2+}-ATPase oligomers into monomers (Møller et al., 1982; Martin and Tanford, 1984; Martin et al., 1984). These findings conflict with those of Lüdi and Hasselbach (1982, 1983), and raise the possibility that the observed loss of excimer fluorescence with some detergents is due to conformational changes in the Ca^{2+}-ATPase or to a direct effect of detergents on excited state interactions.

(*b*) Several detergents influenced the excimer fluorescence of the $(PMI)_2$-DTT adduct incorporated either into sarcoplasmic reticulum or into egg lecithin liposomes in a qualitatively similar manner to their effects on PMI-labeled sarcoplasmic reticulum. Therefore we must consider the possibility that detergents can directly influence the excited state interactions of pyrene in a manner that seems to reveal specific structural requirements. For example, deoxycholate and Zwittergent 3-16 effectively inhibited the excimer fluorescence of $(PMI)_2$-DTT adduct, while $C_{12}E_8$ enhanced the excimer fluorescence. The effects of detergents on the fluorescence of $(PMI)_2$-DTT described in this report extend earlier observations indicating a relationship between the physical properties of the membrane environment and the excimer fluorescence of 1,3-di-(1-pyrenyl)propane (Zachariasse et al., 1980, 1982; Almeida et al., 1982, 1984) and 1,3-α-dinaphthylpropane (Turro and Okubo, 1981; Turro et al., 1979). Therefore the effects of detergents on the excimer fluorescence of PMI covalently bound to the Ca^{2+}-ATPase may also reflect such a direct effect of detergents on excited state interactions, independently of their possible effects on ATPase conformation or on the interactions between ATPase molecules.

The conclusive evidence indicating multiple labeling of ATPase molecules by pyrenemaleimide, together with the similar effects of detergents on the excimer fluorescence of the pyrenemaleimide adducts of dithiothreitol and sarcoplasmic reticulum lead us to the conclusion that intramolecular excimer formation between pyrenemaleimide adducts on adjacent thiol groups within a single ATPase molecule, must be considered as a contributing, possibly dominant, and perhaps exclusive, mechanism of the observed excimer fluorescence. While the observations of this report do not exclude contributions by ATPase-ATPase interactions to the excimer fluorescence, they suggest that such contributions may not be sufficiently exclusive or dominant to consider the excimer fluorescence a reliable and established indicator of ATPase-ATPase interactions. If intramolecular mechanisms account largely or exclusively for the excimer fluorescence, then the changes observed in the presence of detergents may be related to conformational changes in the Ca^{2+}-ATPase or to a direct effect of detergents on excited state interactions between pyrene molecules.

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