Fluorescence Studies on N-(3-Pyrene)Maleinimide-Labeled Sarcoplasmic Reticulum ATPase in Native and Solubilized Membranes

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Z. Naturforsch. **37 c,** 1170–1179 (1982); received August 16, 1982

Sarcoplasmic Reticulum, ATPase, Protein-Protein-Interactions, Fluorescence Polarization, Excimer Formation

Fluorescence polarization and formation of excimers were studied in N-(3-pyrene)maleinimide-labeled sarcoplasmic reticulum vesicles.

1. The polarization of pyrenemaleinimide labeled vesicles does not change with temperature and shows a pronounced decrease at labeling concentrations larger than 1 mol pyrenemaleinimide per 10 mol ATPase.
2. Solubilization of the membrane with myristoylglycerophosphocholine renders the polarization temperature dependent, but does not affect the concentration dependent depolarization observed in native vesicles.
3. The polarization of labeled vesicles is much smaller than to be expected from the temperature independent polarization indicating that the pyrenemaleinimide polarization did not monitor the rotation of the entire ATPase. Thus segmental motion occurs.
4. Pyrene excimers are observed at label concentrations larger than 1 mol label per 2.5 mol ATPase.
5. The amount of excimers was critically dependent on added detergents. From the fact that non-solubilizing amounts of myristoylglycerophosphocholine strongly reduced the amount of pyrene excimers it is concluded that in the native sarcoplasmic reticulum vesicles at least two ATPase molecules must be in close contact.

Introduction

Various properties of the sarcoplasmic reticulum ATPase from skeletal muscle are affected by the incorporation of small amounts of detergents into native sarcoplasmic membranes [1, 2]. These effects of detergents might be caused by: (i) changes in the phospholipid environment of the enzyme; (ii) an increased mobility of the entire ATPase molecule, or part of it; or (iii) perturbations of inter- or intramolecular protein interactions. To distinguish between these possibilities, we labeled native sarcoplasmic reticulum vesicles with N-(3-pyrene)-maleinimide and measured the following parameters: (i) fluorescence polarization versus temperature, detergent and/or label concentrations and (ii) the formation of pyrene excimers versus label and detergent concentrations. Furthermore, the viscosity of the lipid matrix was monitored by measuring diphenylhexatriene anisotropy to get information concerning the viscosity of the lipid moiety of the membrane which is needed to quantitatively evaluate the measurements (i) and (ii). The fluorescence polarization measurements should give informations about the size of the rotating protein particle. The results indicate that pyrenemaleinimide fluorescence polarization only monitors the motion of the entire ATPase molecule after complete solubilization of the membrane. Within the membrane segmental motion can be observed. Pyrene excimer formation which indicates proximity of two neighbouring labels prove to be very sensitive to the effect of detergents and is discussed in view of an oligomeric form of the ATPase within the membrane.

Abbreviations and symbols used: Pyrenemaleinimide, N-(3-pyrene)maleinimide; diphenylhexatriene, 1,6-diphenyl-1,3,5-hexatriene; Mops: 3-(N-morpholino)propansulfonic acid; p, fluorescence polarization; r, fluorescence anisotropy; τ_Φ, fluorescence lifetime by phase; τ_{mod}, fluorescence lifetime by modulation; *Enzyme:* Ca^{2+}-ATPase (EC 3.6.1.3).

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Materials

The assay components for the experiments were purchased at the following adresses: Mops and N-(3-Pyrene)maleinimide: Serva, Heidelberg (FRG).

ATP: Pharma Waldhof GmbH., Düsseldorf (FRG). Dodecyl octaoxyethylene glycol monoether ($C_{12}E_8$): Nikko Chemicals, Tokyo (Japan). 1,6-di-phenyl-1,3,5-hexatriene: EGA-Chemie, Steinheim/Altbuch (FRG). Heavy liquid paraffin oil (DAB 7): Carl Roth KG., Karlsruhe (FRG). Lasolocid (X-537A) was a generous gift from Hoffmann-La Roche Ltd., Basel (Switzerland).

Methods

Sarcoplasmic reticulum vesicles were prepared according to the procedure described by Hasselbach and Makinose [3] as modified by de Meis and Hasselbach [4]. Protein concentrations were determined by the Biuret method with Kjeldahl calibrated standards.

The calcium-dependent ATPase activity was measured in a solution containing 20 mM histidine, pH 7.0, 5 mM ATP, 5 mM $MgCl_2$, 40 mM NaCl, 0.1 mM $CaCl_2$, 5×10^{-5} M X-537A, 0.4 mg · ml^{-1} protein and the given concentrations of detergent as described by Ronazni *et al.* [5]. ATP splitting was stopped with an equal volume of 2% sodium dodecylsulfate instead of 6% trichloracetic acid to avoid precipitations in the assay mixture for P_i determination.

Diphenylhexatriene-labeled vesicles were prepared as follows: 5 ml vesicle suspension (20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.45 mM EGTA and 0.5 mg · ml^{-1} protein) were incubated with 10 µl diphenylhexatriene (10^{-3} M stock solution in tetrahydrofuran; final concentration 2×10^{-6} M) for 2 h in the dark. Complete incorporation of diphenylhexatriene into the vesicles was checked by measuring time-dependent fluorescence intensity, which reached its maximum after 90 min. Pyrenemaleinimide-labeled vesicles were prepared as follows: 5 ml vesicle suspension (2 mg · ml^{-1} protein in the same buffer as for diphenylhexatriene-labeling) were incubated in the dark with various amounts of pyrenemaleinimide (stock solutions in aceton, final concentrations 1×10^{-7} to 10^{-4} M) for 1 h. Pyrenemaleinimide- and diphenylhexatriene-labeled vesicles were used within 36 h.

Sodium dodecylsulfate gel electrophoresis was performed in 7% polyacrylamid gels according to Weber and Osborne [6], but using 0.1 M Tris-bicine buffer, pH 8.2 containing 0.1% SDS (R. Leberman, personal communication). If pyrenemaleinimide-labeled vesicles were applied, gels were immediately photographed at 366 nm illumination and afterwards fixed and stained as described by Migala *et al.* [7].

Fluorescence measurements were carried out with an SLM 4800/A spectrofluorometer (SLM instruments Inc., Urbana, Illinois, USA). Fluorescence lifetimes (τ_Φ/lifetime measured by phase shift and τ_{mod}: lifetime measured by modulation) and polarization data were analyzed using SLM interface, programs, and a 97 S I/O Hewlett Packard calculator. Diphenylhexatriene was excited at 375 nm and emission was detected using GG 420 cut off filters (Schott, Mainz, FRG). Pyrenemaleinimide was excited at 340 nm and emission was detected using GG 375 cut off filters (Schott, Mainz, FRG). The standard deviation for the polarization measurements was smaller than 0.02. The standard deviation for diphenylhexatriene-lifetimes was smaller than 0.05 ns and for pyrenemaleinimide-lifetimes smaller than 1 ns. The temperature of the cuvette was measured with a MGU Lauda digital thermometer (R 40) and was constant within 0.1 °C during the measurement. Usually 0.005 mg · ml^{-1} diphenylhexatriene- or pyrenemaleinimide-labeled vesicles were incubated in 2.5 ml of the same buffer solution as was used for the labeling procedure.

The viscosity of the heavy liquid paraffin oil was determined at an angle of 60° in a self-made falling ball viscosimeter which was calibrated with a Brookfield standard viscosity oil (Brookfield Engeneering Laboratories, Inc., Stoughton, MA, USA, 94 cP at 25 °C) and was found to be 133 cP at 25 °C.

Principles of the methods

The fluorescent label N-(3-pyrene)maleinimide was used for the following reasons: (i) the hydrophobic character of the molecule should prevent individual motion of the molecule after its binding to the protein; (ii) the pyrene molecule has a long lifetime of the excitated state (50 – 100 ns) and (iii): the possible formation of excimers allows predictions about the proximity of reactive thiol residues.

A. Polarization studies

The polarization studies were performed according to G. Weber [8, 9] by exciting the preparation in

Table I. Pyrenemaleinimide lifetimes at different degrees of labeling.

Label pyrenemaleinimide : ATPase mol : mol	6 MHz		18 MHz		Calculated	
	τ_Φ [ns]	τ_{mod} [ns]	τ_Φ [ns]	τ_{mod} [ns]	τ_1 [ns]	τ_2 [ns]
1 : 10	7.8	32.9	3.2	14.0	2.2 (70)	42.0 (30)
1 : 5	11.3	38.3	4.3	18.8	2.3 (61)	34.9 (39)
1 : 2.5	13.8	48.2	4.6	23.1	2.5 (54)	49.4 (46)
1 : 1	21.1	52.4	7.3	30.8	3.1 (39)	49.0 (61)
2 : 1	26.6	60.7	8.5	37.6	3.4 (28)	61.6 (72)
5 : 1	24.4	57.9	8.9	35.6	3.8 (36)	52.6 (64)

Lifetimes were determined at 6 MHz and 18 MHz frequencies with a 375 nm cut-off filter in solutions as described for the pyrenemaleinimide polarization experiments (0.005 mg protein · ml^{-1}) τ_1 and τ_2 are the lifetimes obtained after the heterogeneity analysis (SLM program). The numbers in parentheses give the relative amounts of the chromophores present. It should be noted that the small lifetimes calculated using the SLM heterogeneity program correspond to the lifetime of the "intrinsic" chromophore of native vesicles (4.7 ns).

a quarz cell of 1 cm depth with polarized light. The fluorescence anisotropy

$$r = \frac{2p}{3-p} \tag{1}$$

was obtained from the measured polarization

$$p = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}. \tag{2}$$

The relation between the fluorescence anisotropy (r), the viscosity of the surrounding medium (η), the fluorescence lifetime (τ), the size of the rotating particle ($V(r)$) and the relaxation time of the rotation (ϱ) is given by Perrin's equation:

$$\frac{r_0}{r} = 1 + \frac{R \cdot T \cdot \tau}{\eta \cdot V(r)} = 1 + \frac{3 \cdot \tau}{\varrho} \tag{3}$$

where r_0 is the limiting anisotropy, R the gas constant and T the absolute temperature. As shown by G. Weber [8, 9], Perrin's equation derived for spherical molecules can be applied to flat ellipsoids and to prolate ellipsoids of small elongation. The relation can be used even if intramolecular rotations might occur having relaxation times much smaller than the labeled macromolecule and largely independent of the viscosity of the solvent. The relation is especially suitable to monitor qualitative changes of molecular mobility. The range of the relaxation times that can be measured depends besides the lifetime of the pyrenemaleinimide label on the precision of the smallest polarization that can be measured [9]. At a minimal polarization of 0.05 ± 0.01 relaxation times ranging from $5\,\mu s - 0.05\,\mu s$ are calculated with a lifetime of 50 ns for the pyrenemaleinimide label (Table I).

B. Excimer formation

The occurrence of pyrene excimers at high concentrations of the chromophore in organic solvents was first reported by Förster and Kaspar [10]. In order to form excimers, two pyrene molecules must come into close contact (0.3 nm) after the excitation of one of the pyrene molecules [11]. The effect was recognized by an additional emission peak at 478 nm and was used to determine membrane viscosity [12, 13] and lateral diffusion coefficients in membranes [14, 15]. Excimer formation by pyrenemaleinimide attached to membrane proteins can be expected to occur by (i) a diffusion controlled collision of two proteins, (ii) "double-labeling" of one protein or (iii) a regular arrangement of at least two protein molecules favouring the proximity of two reactive thiol groups to which pyrenemaleinimide is bound.

C. Determination of the membrane viscosity

The interpretation of polarization as well as excimer formation data requires the determination of membrane viscosity before and after the addition of detergents. The membrane viscosity was determined as described by Shinitzky and Barenholz [16], using diphenylhexatriene as a fluorescent probe. For calibration the fluorescence polarization and lifetimes of diphenylhexatriene in heavy liquid paraffin oil and the viscosity of the oil versus

temperature were measured. The limiting polarization was calculated from a plot of $1/p$ *versus* T/η and was found to be 0.452. Therefore the limiting anisotropy is 0.355 (Eqn. (1)), which is in good agreement with previous reports [16]. The calibration curve for determination of membrane viscosity is shown in the inset of Fig. 3 (r_0/r versus $T \cdot \tau/\eta$) and some additional data are given in Table II.

Results

Characterization of pyrenemaleinimide-labeled vesicles

Fig. 1 shows sodium dodecylsulfate gel electrophorograms of pyrenemaleinimide-labeled vesicles (molar ratio 2 : 1; pyrenemaleinimide : ATPase). Whereas gels stained with Coomassie Blue Brilliant contained the usually observed protein bands [7], the fluorescence label was predominantly detected at the position of the ATPase. To determine the amount of pyrenemaleinimide bound to the ATPase protein after the labeling procedure, the vesicles were freeze dried and subsequently extracted with cold acetone and diethylether. This procedure did not markedly affect the ATPase activity [17, 18]. 90% of the label was recovered together with the protein if the vesicles were labeled at a molar ratio of 1 : 1 and 80% was recovered if the ratio was 2 : 1 (pyrenemaleinimide : ATPase). The ATPase activity of pyrenemaleinimide-labeled vesicles (2 mol pyrenemaleinimide : mol ATPase) is 30% lower than that

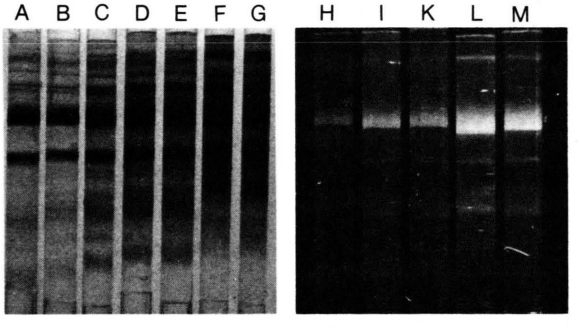

Fig. 1. Sodium dodecylsulfate gel electrophoresis of pyrenemaleinimide-labeled vesicles. Electrophoresis was performed as described under "Materials and Methods". A: Coomassie Blue Brillant staining of a gel loaded with unlabeled vesicles. B–G: Coomassie Blue Brilliant staining of gels loaded with increasing amounts of vesicles labeled with pyrenemaleinimide (2 mol pyrenemaleinimide per mol ATPase). H–M: Same gels as C–G but the gels were photographed after illumination at 366 nm before fixation and staining.

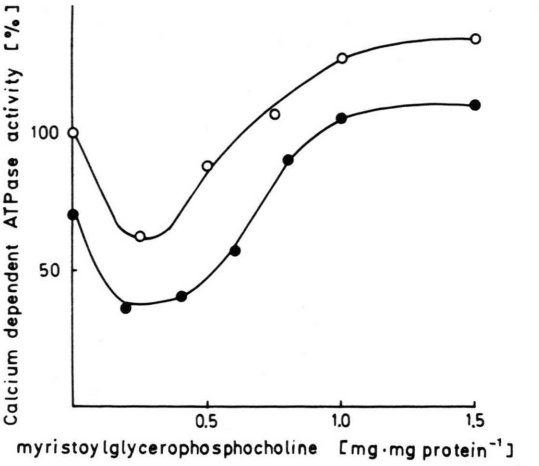

Fig. 2. ATPase activity of pyrenemaleinimide-labeled vesicles at different concentrations of myristoylglycerophosphocholine. ATPase of unlabeled (○) and of pyrenemaleinimide-labeled (●) enzyme (2 mol pyrenemaleinimide per mol ATPase) was determined after the addition of different amounts of myristoylglycerophosphocholine. Normalized activity: 0.65 μmol · mg^{-1} · min^{-1}.

of the control vesicles. This reduction did not affect depth and position of the dip in the activity profile produced by the addition of increasing amounts of myristoylglycerophosphocholine (Fig. 2) [1].

Membrane viscosity

Fig. 3 shows the membrane viscosity as a function of temperature as monitored by diphenylhexatriene anisotropy. Some additional data used for the calculations are given in Table II. In the Arrhenius plot of the data a discontinuity in the temperature dependence of the membrane viscosity was found at 31 °C. It should be noted that the values of the membrane viscosity plotted in Fig. 3 represent the mean values of the two calculations using τ_Φ or τ_{mod}. The difference in τ_Φ and τ_{mod} of diphenylhexatriene incorporated into sarcoplasmic reticulum vesicles were also observed if diphenylhexatriene was incorporated into sonicated egg lecithin vesicles and therefore could not be a consequence of diphenylhexatriene bound to the protein. In addition, the application of a lifetime heterogeneity program (SLM instruments) did not reveal two different lifetimes.

The results shown in Fig. 4 demonstrate that the incorporation of myristoylglycerophosphocholine into the sarcoplasmic reticulum vesicles only weakly

Table II. Fluorescence lifetimes and anisotropy of diphenylhexatriene in heavy liquid paraffin oil and sarcoplasmic reticulum vesicles at different temperatures and viscosities [η].

Heavy liquid paraffin oil					Sarcoplasmic reticulum vesicles				
Temp. [°C]	η [cP]	r	τ_{mod} [ns]	τ_Φ [ns]	Temp. [°C]	η [P]	r	τ_{mod} [ns]	τ_Φ [ns]
2.9	763	0.276	10.13	9.72	3.5	3.3	0.217	10.58	9.44
10.5	387	0.227	10.13	10.00	10.8	2.4	0.178	10.36	9.24
19.8	168	0.162	10.20	10.29	20.8	1.6	0.153	10.09	9.19
30.0	95	0.104	10.09	10.35	30.2	1.2	0.126	9.69	8.88
39.5	57	0.068	9.92	10.05	39.3	0.9	0.108	9.38	8.53

affects the membrane viscosity at concentrations smaller than 2.5 mg · mg protein^{-1}. Significant reduction of the pyrenemaleinimide polarization requires myristoylglycerophosphocholine concentrations higher than 1 mg·mg protein^{-1}. These concentrations nearly effect complete solubilization of all membrane components. Hence, the concentrations of myristoylglycerophosphocholine which are needed to reduce pyrenemaleinimide and diphenylhexatriene polarization are much larger than those affecting ATPase activity (comp. Fig. 2).

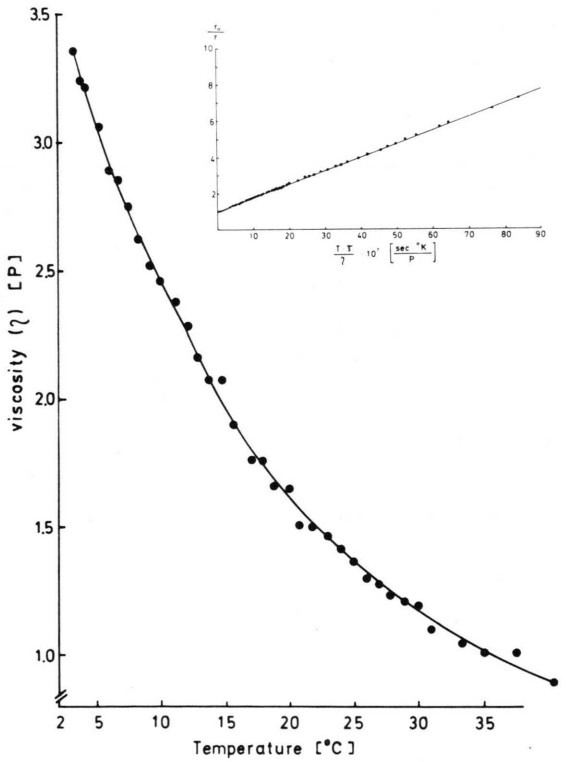

Fig. 3. Temperature dependence of the viscosity (η) of sarcoplasmic reticulum vesicles. The temperature dependence of the fluorescence anisotropy and lifetime of diphenylhexatriene incorporated into sarcoplasmic reticulum vesicles was determined as it is described under "Materials and Methods". Using the calibration curve obtained in heavy liquid paraffin oil (inset), the viscosity of the membrane (η) was calculated. The data shown represent the mean values obtained using τ_Φ or τ_{mod} for the calculations. (Compare to Table I.)

Pyrenemaleinimide-polarization studies

Fig. 5 shows that pyrenemaleinimide polarization of intact sarcoplasmic reticulum vesicles is practically temperature-independent and the value for the limiting polarization is quite small. The temperature independence indicates that the relaxation time of the ATPase molecule in the membrane does not fall in the range which is monitored with the pyrenemaleinimide label. At the given lifetime of the pyrenemaleinimide label (Table I) the limiting factor is the relatively high viscosity of the lipid matrix. Yet, after complete solubilization of the

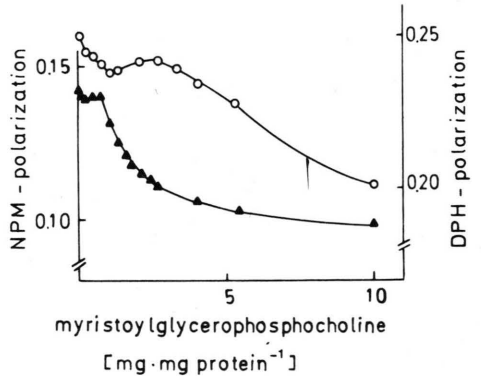

Fig. 4. Polarization of pyrenemaleinimide-labeled and diphenylhexatriene-labeled vesicles at increasing amounts of myristoylglycerophosphocholine. Pyrenemaleinimide-labeled vesicles (molar ratio 1 : 1) 0.05 mg protein · ml^{-1} were incubated at 20 °C and the polarization was determined after the addition of myristoylglycerophosphocholine (○–○). The same experiment was repeated using diphenylhexatriene-labeled vesicles (▲–▲). NPM: pyrenemaleinimide; DPH: diphenylhexatriene.

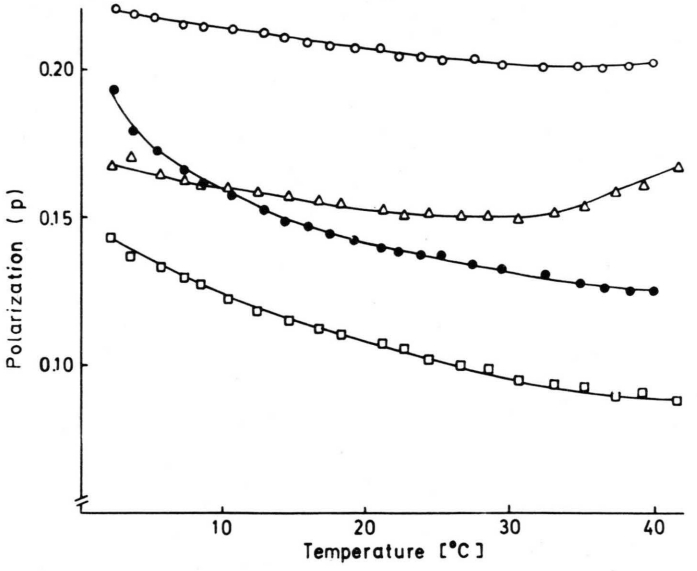

Fig. 5. Temperature dependence of pyrenemaleinimide polarization using intact or solubilized vesicles. Vesicles (0.005 mg · ml^{-1}) labeled at a molar ratio of 1:10 (pyrenemaleinimide: ATPase) (○, ●) and 1:1 (△, □) were incubated at different temperatures and the polarization was determined. (○, △) before solubilization, (●, □) after the solubilization with myristoylglycerophosphocholine (10 mg · mg protein^{-1}).

membrane with myristoylglycerophosphocholine the pyrenemaleinimide polarization decreases with increasing temperature. That is because in the complete solubilized preparation the viscosity determining the rotation of the ATPase molecule embedded in a detergent micelle is the low viscosity of the buffer solution. As described by Weber [8, 9] the relaxation time and the molecular weight can be obtained from anisotropy values at two temperatures. The procedure yields molecular weights for the ATPase-detergent micelle of 70 kD – 180 kD depending on the temperature range taken for the calculation. These values result from the anisotropy values obtained with the labeling concentration of 1 mol pyrenemaleinimide/10 mol ATPase. This indicates that solubilization resulted in the formation of small rotating units, possibly ATPase monomeres. The temperature dependence of the pyrenemaleinimide polarization is reversible. Usually native or solubilized vesicles were incubated at about 3 °C

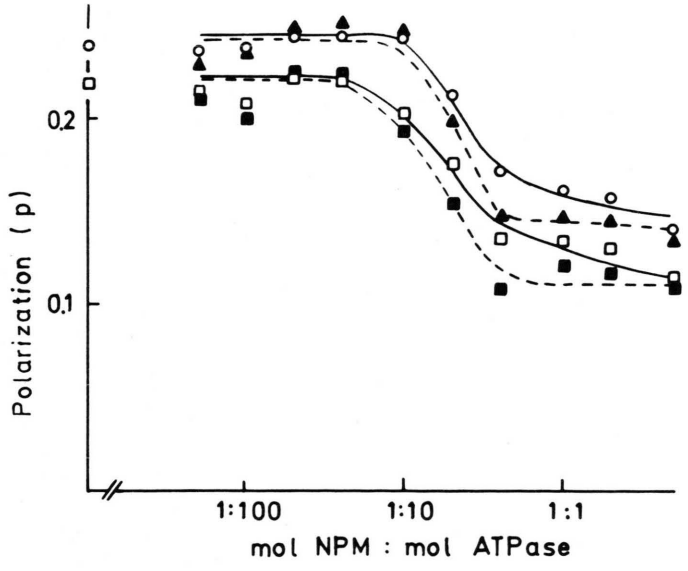

Fig. 6. Concentration depolarization of pyrenemaleinimide-labeled vesicles. The polarization of the vesicles (0.005 mg · ml^{-1}) was determined as described under "Materials and Methods" at different labeling concentrations before (○) and after (□) solubilization with myristoylglycerophosphocholine (50 mg · mg^{-1}). (....) values obtained after the correction using Eqn. (4), see text). (▲..▲) native vesicles; (■..■) solubilized vesicles. NPM: pyrenemaleinimide.

and the temperature was stepwise increased up to 40 °C. The polarization measured after cooling the sample back to 3 °C did not differ from the polarization determined at the beginning of the experiment.

The polarization of membrane bound pyrenemaleinimide-labeled ATPase is unexpectedly small (Figs. 4 and 5). These low polarization values are neither caused by depolarization due to light scattering, nor by concentration-depolarization. Light scattering-depolarization is unlikely due to the low optical density of the sample (0.015 at 340 nm) and due to the fact that sarcoplasmic reticulum vesicles labeled with N-(7-dimethylamine-4-methyl-3-cumaryl)-maleinimide under the same conditions exhibit a much higher polarization value (0.36 at 20 °C). Concentration-depolarization was analyzed as shown in Fig. 6. An increase of pyrenemaleinimide polarization was observed if less than every 3rd ATPase molecule was labeled. This increase was independent on the solubilization of the membrane, but in general the polarization of the solubilized vesicles was reduced by about 20%. The maximum polarization reached a value of 0.24 if only every 10th ATPase molecule was labeled. But this value is still much smaller than the limiting polarization. Yet, even without labeling the vesicles the same degree of polarization was obtained which is due to an "intrinsic" polarization at an excitation wavelength of 340 nm. Complete solubilization of the membrane did not abolish the "intrinsic polarization", excluding that it is produced by light scattering of the suspension. Furthermore, a small absorption band at 340 nm was observed with native and solubilized vesicles as well as with purified ATPase preparations and an emission maximum could be detected at 385 nm if the vesicles were excited at 340 nm. This indicated that there exists an unknown chromophore in native sarcoplasmic reticulum vesicles (see also Table I). We, therefore, treated the polarization data according to the equation

$$\bar{p} = \frac{\sum F_i p_i}{\sum F_i} \tag{4}$$

where $\bar{p}$ is the measured polarization, F_i is the intensity and p_i the polarization of the i-th chromophore [8]. After the determination of the relative fluorescence intensities of unlabeled and labeled vesicles and of the "intrinsic polarization" the effective polarization of pyrenemaleinimide could be determined. At low labeling concentrations the measured polarization was not much different from the calculated pyrenemaleinimide polarization. At higher labeling concentrations the calculated pyrenemaleinimide polarization was smaller than the measured polarization (Fig. 6, dotted lines). The low pyrenemaleinimide polarization of native sarcoplasmic reticulum vesicles on the one hand and the temperature independence of the polarization on the other hand indicates that the pyrenemaleinimide label monitors segmental motion which is not determined by the viscosity of the buffer solution.

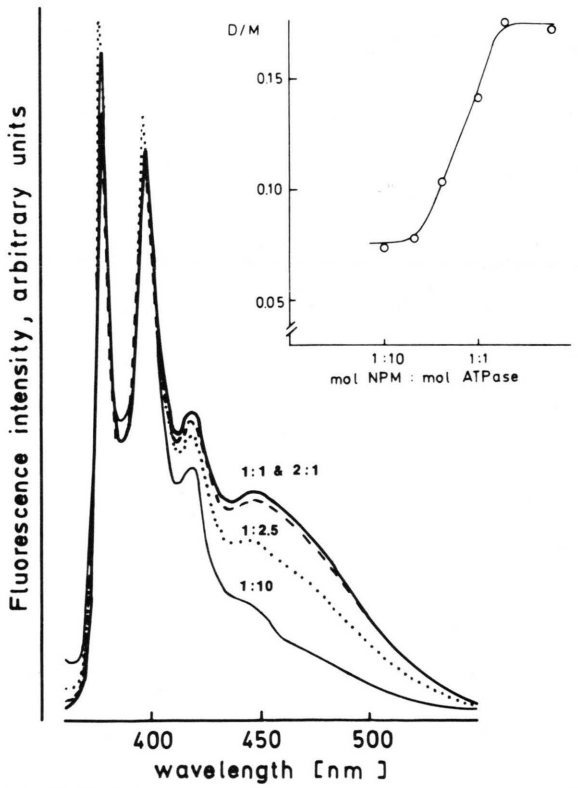

Fig. 7. Emission spectra of vesicles labeled with different amounts of pyrenemaleinimide. Emission spectra of pyrenemaleinimide-labeled vesicles (0.05 mg protein · ml^{-1}) were obtained at an excitation wavelength of 340 nm. The vesicles were labeled with different amounts of pyrenemaleinimide. The molar ratio of pyrenemaleinimide : ATPase is given in the curves. Inset: Relative peak intensity of the pyrenemaleinimide-excimer (450 nm) to the peak intensities of the pyrenemaleinimide "monomer" (376 nm, 397 nm, 416 nm) (D/M) as a function of labeling concentration. Note: the D/M-value for pyrenemaleinimide (10^{-8} M) in benzol is 0.85. NPM: pyrenemaleinimide.

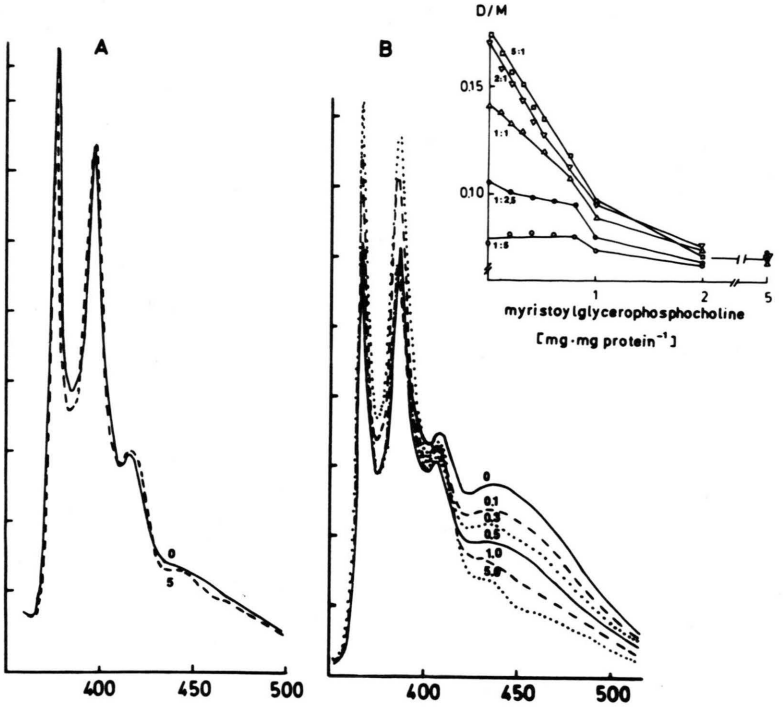

Fig. 8. Emission spectra of pyrenemaleinimide-labeled vesicles after the addition of myristoylclycerophosphocholine. Pyrenemaleinimide-labeled vesicles were excited at 340 nm. A: Emission spectra of vesicles (0.05 mg/ml) labeled with 0.2 mol pyrenemaleinimide per mol ATPase before and after addition of myristoylglycerophosphocholine. B: Same as in A, but the vesicles were labeled at a molar ratio of 2:1 (pyrenemaleinimide:ATPase). The numbers given in the curves give the amount of myristoylglycerophosphocholine added (mg · mg^{-1} protein). Inset: D/M-values of vesicles labeled with different amounts of pyrenemaleinimide after the addition of myristoylglycerophosphocholine. The numbers indicate the molar ratio of pyrenemaleinimide : ATPase used for the labeling procedure.

The same applies to the intrinsic chromophore. Furthermore, the fact that small amounts of detergents do not affect polarization of pyrenemaleinimide-labeled vesicles (Fig. 4) indicates that neither the mobility of the entire ATPase molecule nor that of the pyrene label and the intrinsic chromophore are increased.

Pyrene-excimer formation

The occurrence of pyrene-excimers was monitored at varying label and detergent concentrations. At a labeling concentration exceeding 1 mol pyrenemaleinimide per 2.5 mol ATPase, excimer formation becomes significant (Fig. 7). The excimers did not result from a "double-labeling" of the ATPase, since after the complete solubilization of vesicles previously labeled with 2 mol pyrenemaleinimide per mol ATPase gave the same spectra as obtained with labeling concentrations not leading to excimer formation (Fig. 8). Excimers disappeared when vesicles labeled with 2 mol pyrenemaleinimide per mol ATPase were solubilized with sodiumdesoxycholate (1.5 mg · mg^{-1} protein). If the detergent was subsequently removed by gel filtration and dialysis [19], excimer formation was considerably restored. A D/M value of 0.12 was obtained (comp. Fig. 7, inset). This largely excludes the participation of unbound pyrenemaleinimide in excimer formation since free pyrenemaleinimide should be removed by the procedure. This conclusion is further supported by the fact that the addition of pyrenemaleinimide (2×10^{-5} M) reacted with cystein in excess (0.1 M, pH 7.0) to pyrenemaleinimide-labeled vesicles did not increase the amount of excimers. On the contrary, the amount of excimers strongly decreases if more than 10 – 20% of the pyrenemaleinimide present in the cuvette was cystein-pyrenemaleinimide. This effect was observed at all labeling degrees.

In contrast to the polarization of pyrenemaleinimide-labeled vesicles, the formation of excimers proved to be sensitive to low concentrations of myristoylglycerophosphocholine (0.1 mg · mg protein^{-1}) or dodecyl octaoxyethylene glycol monoether ($C_{12}E_8$, not shown).

Discussion

Diphenylhexatriene-fluorescene polarization was originally applied by Shinitzky and Barenholz to

determine the "microviscosity" of phospholipid bilayers [16]. The values shown in Fig. 3 are in good agreement with the values reported for egg lecithin liposomes [16, 20] and sarcoplasmic reticulum [21]. The disadvantages of the method arise from the fact that the calibration curve is determined in a medium where diphenylhexatriene undergoes isotropic motion, whereas in the lipid membranes the rotation of diphenylhexatriene is anisotropic. Therefore, the absolute values for the membrane viscosity obtained with this method were doubted by different groups determining the formation of pyrene excimers [12–14] or the time-resolved polarization of diphenylhexatriene [22] to evaluate the membrane viscosity. Absolute values, one order of magnitude smaller than these shown in Fig. 1, were reported. But even if these values are used in equation [3], the polarization of pyrenemaleinimide-labeled vesicles remained temperature-independent, even if the monomeric form of the ATPase (MW 100 kD) was taken as the rotating entity. As it is shown in Fig. 5 and can be calculated from Eqn. (3), the viscosity has to be reduced by a factor of 100 to get temperature-dependent polarization. This reduction of the viscosity was obtained after the solubilization of the vesicles with myristoylglycerophosphocholine when the viscosity determining the rotation of the ATPase molecule is not much different from the viscosity of water.

The fact that in pyrenemaleinimide-labeled vesicles the measured polarization did not correspond to the limiting polarization even at very low labeling concentrations, where concentration depolarization did not occur (*e.g.* 1 pyrenemaleinimide molecule per 100 ATPase molecules), suggests an additional motion of pyrenemaleinimide which does not monitor the rotation of the entire ATPase molecule within the membrane. This kind of segmental motion was originally described by Bürkli and Cherry [23] and Restall *et al.* [24], using flash-induced dichroism measurements of the triplet probe 5-iodoacetamideosin bound to sarcoplasmic sarcoplasmic reticulum ATPase. The occurrence of segmental motion was also supported by the fact that nonsolubilized vesicles showed a small decrease of the pyrenemaleinimide polarization at increasing temperature. But above 35 °C the polarization again increased. These changes in polarization were very small, but repeatedly observed with 5 different preparations and at all degrees of labeling (Fig. 5). Furthermore, the discrepancies in the size of the calculated rotating entity after the complete solubilization which were obtained using different temperature ranges for the calculations, suggest the existence of a motion which is independent on the rotation of the entire ATPase molecule. Obviously, a segmental motion could not explain the concentration-dependent decrease of polarization shown in Fig. 6 which occurs at very small labeling concentrations. In addition myristoylglycerophosphocholine did not shift the profile obtained with native sarcoplasmic reticulum vesicles towards higher label concentrations which is expected from the fact that the mean distance of labeled protein should increase. The reason for these quite striking effects might be the observed "intrinsic polarization" the origin of which is unknown. It may be speculated to be the consequence of "tryptophan-excimer formation". It should be noted that vesicles labeled with another fluorescent maleinimide, namely N-(7-dimethylamino-4-methyl-3-cumaryl)-maleinimide did not show a significant concentration-dependent depolarization at labeling concentrations between 1:10 and 1:1 (mol maleinimide per mol ATPase). In addition, the polarization of the cumarylmaleinimide was 0.36 at 20 °C, which was much higher than the polarization of pyrenemaleinimide. This indicates (i) that the results shown in Figs. 4, 5, and 6 were specific for pyrenemaleinimide and (ii) that light scattering does not significantly decrease pyrenemaleinimide polarization and therefore was not the reason for the low values obtained. Excimer formation did not lead to a depolarization of pyrenemaleinimide fluorescence and does not contradict the interpretation of the low pyrenemaleinimide polarization observed (Figs. 4, 5, and 6). As shown in Figs. 7 and 8, excimer formation and pyrenemaleinimide depolarization occurred at two different but very narrow ranges of pyrenemaleinimide concentrations. As demonstrated in Fig. 8 the formation of excimers was not due to the fact that pyrenemaleinimide molecules were bound to the same ATPase molecule, since it seems to be very unlikely that the addition of detergent would completely abolish the interaction between two pyrenemaleinimide molecules linked to the same ATPase. In addition, the lateral diffusion coefficient for membrane proteins was reported to be in the range of $1-6 \cdot 10^{-9}\,cm^2 \cdot s^{-1}$ [25] and the rotational correlation time of the ATPase was shown to be in the

order of 50–200 μs [26–28]. If these values are compared with the fluorescence lifetime of pyrenemaleinimide (50 ns), it results that the two pyrenemaleinimide molecules involved in excimer formation have to be in close contact before excitation. This implies an arrangement of at least two ATPase molecules, which favours the proximity of two of their reactive thiol groups. The reduction of excimers by low concentrations of detergent (0.2 mg · mg protein^{-1}) must therefore be the consequence of the disruption of protein-protein-interactions, *i.e.* a random distribution of the ATPase molecules replaces the ordered arrangement after the addition of non-solubilizing amounts of detergents. This is in agreement with previous reports, where it has been shown that these low concentrations of detergent affect several properties of the ATPase (*e.g.* phosphorylation of the protein from inorganic phosphate, ATPase activity, calcium affinity, calcium-dependent tryptophanfluorescence and reduction of the immobilized component in the electron spin resonance spectrum of spin-labeled fatty acids [1, 29].

The reduction of excimers after the vesicles were solubilized and reformed, using the reconstitution method of Brunner *et al.* [19] also supports the existence of a regular arrangement of the ATPase in native membranes. The reduction of excimers is best explained by the fact that the ATPase molecules were symmetrically incorporated in the reconstituted vesicles [30–33]. The reconstitution experiment excluded that the small amounts of pyrenemaleinimide bound to other proteins (Fig. 1) are responsible for excimer formation, since these proteins are removed from the vesicles during the reconstitution procedure. Furthermore, unbound freely diffusing pyrenemaleinimide reacted with cystein did not increase the amount of excimers. Thus excimers are not due to the interaction between unbound and protein-bound pyrenemaleinimide.

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