Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 176-182

DEPENDENCE OF THE FLUORESCENCE OF FLUORESCEIN LABELLED (Ca^{2+}, Mg^{2+})-ATPase UPON THE LIPID TO PROTEIN RATIO IN SARCOPLASMIC RETICULUM RECONSTITUTED SYSTEMS

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Received October 11, 1985

Reconstituted sarcoplasmic reticulum (SR) vesicles have been prepared mixing fluorescein labelled SR, excess endogenous lipids and deoxycholate by a rapid dilution protocol and several freeze-thaw treatments. We have found that both the steady-state level and the polarization of fluorescein fluorescence of these reconstituted systems monotonically increase as a function of the lipid to protein ratio between 80 and 2000 (on a mole per mole basis). The magnitude of this increase is about 15%. Detergents, such as Triton X-100 and deoxycholate, when added to SR labelled vesicles below their critical micelle concentrations also induce similar changes in fluorescein fluorescence. We suggest that lipid dilution of protein in these reconstituted systems induce a decrease of the level of self-quenching by promoting dissociation of (Ca^{2+}, Mg^{2+})-ATPase.

Sarcoplasmic reticulum (SR) (Ca^{2+}, Mg^{2+})-ATPase undergoes cyclic conformational changes during the hydrolysis of ATP (1, 2). The modulation of this enzyme system by ligands, such as Ca^{2+}, Mg^{2+}, phosphate and nucleotides, has been rationalized in terms of displacements of the equilibrium of this enzyme between two major groups of conformations called E_1 and E_2 (1, 2). The molecular nature of these conformational changes is not clear at present and, thus, is being thoroughly investigated (3-5). In addition, SR (Ca^{2+}, Mg^{2+})-ATPase has been shown to be in equilibrium among different aggregation states (monomers, dimers and tetramers) (5-7). Although the functional relevance of this additional conformational polymorphism is not completely understood yet, it seems that different aggregation states exhibit regulatory differences in some of the steps of the catalytic cycle (7, 8). Therefore, it is of prime importance to characterize the oligomeric state of (Ca^{2+}, Mg^{2+})-ATPase in SR reconstituted vesicles in order to gain a better understanding of the regulatory properties of this enzyme system.

In certain experimental conditions, when mixed with native SR vesicles, fluorescein isothiocyanate has been shown to react solely with the ATP binding site of (Ca^{2+}, Mg^{2+})-ATPase (9,10) and to monitor conformational changes of (Ca^{2+}, Mg^{2+})-ATPase (11). In this paper, we report that the fluorescence

0006-291X/85 $1.50

of fluorescein labelled SR is increased by lipid dilution of protein in reconstituted vesicles. We suggest that this reflects a disaggregation process of (Ca^{2+}, Mg^{2+})-ATPase induced by lipid dilution of protein particles.

MATERIALS AND METHODS

Fluorescein isothiocyanate, ATP, NADH, phosphoenolpyruvate, bovine serum albumin, sucrose, Sephadex G-50, deoxycholate (DOC), Triton X-100, phenyl-methyl-sulfonyl-fluoride, ethyleneglycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 2- [(2-hydroxy-1,1-bis (hydroxymethyl) ethyl-amino] ethanesulphonic acid (Tes) were purchased from SIGMA. Dithiothreitol, pyruvate kinase and lactate dehydrogenase were obtained from Boehringer Mannheim. The rest of the chemicals used were of the highest purity available. The calcium ionophore A23187 was a gift from Elly Lilly SA, USA.

SR vesicles were prepared from New Zealand rabbit skeletal muscle following the procedure of MacLennan (12), except that 0,1 mM phenyl-methyl-sulfonyl-fluoride and 2 mM dithiothreitol were added to all the buffers used in the preparation.

Ca^{2+}-dependent ATPase activity was measured using the coupled enzyme system: pyruvate kinase-lactate dehydrogenase, as in (13). Ca^{2+}-independent ATPase activity was determined in the presence of 3.5 mM EGTA and was usually found to be less than 10% of total ATPase activity. Uncoupled (Ca^{2+}, Mg^{2+})-ATPase activity was measured upon addition to 1 ml of reaction mixture of (5-10) µg A23187 from an ethanolic stock solution. The protein concentration was measured following the method of Lowry (14), using bovine serum albumin as a standard. The total phospholipid content of (Ca^{2+}, Mg^{2+})-ATPase reconstituted vesicles was determined by phosphorous analysis as inorganic phosphate as in (15). Endogenous SR lipids were extracted with chloroform: methanol (2:1 v/v), dried, redisolved in $HCCl_3$ and stored at -20°C under N_2 until use.

The labelling of the enzyme with fluorescein isothiocyanate was done by incubation of both reactants in dark during 30 min at room temperature, at a molar ratio of 0.3 mole of fluorescein isothiocyanate per mole of protein monomer at a pH = 7.45, as in (10). Fluorescein isothiocyanate was added from a freshly made dimethyl formamide solution as in Pick and Karlish (9), and unreacted fluorescein isothiocyanate was removed by passage through a Sephadex G-50 chromatography column (8 cm length x 1 cm diameter). Using an absorption coefficient of 80000 $M^{-1}.cm^{-1}$ for fluorescein bound to SR (9), the extent of labelling determined after passage through the Sephadex G-50 column typically ranged from 0.20 to 0.25 mole fluorescein per mole of (1.1×10^5) dalton protein unit of SR. Fluorescence and polarization measurements were carried out with a spectrofluorimeter Hitachi-Perkin Elmer, mod. 650-40, using excitation and emission wavelengths of 475 and 518 nm, respectively, at room temperature. Polarization of fluorescence was calculated using the following expression:

$$P(\lambda) = \frac{I_{//}(\lambda) - G \cdot I_{\perp}(\lambda)}{I_{//}(\lambda) + G \cdot I_{\perp}(\lambda)}$$

where $I_{//}$ and $I_{\perp}$ are the fluorescence intensities measured with parallel and perpendicularly oriented polarizers, respectively, and G is the correction coefficient for polarization characteristics of the emission monochromator at a fixed emission wavelength. The buffer used in all the experiments was: 0.25 M sucrose, 50 mM Tes, 100 mM KCl and 2 mM β-mercaptoethanol (pH 7.45).

Reconstitution. Labelled SR vesicles, see above, were mixed with deoxycholate and with the appropiate amounts of endogenous lipids. The detergent was added at the following weight ratios: (1 mg detergent per mg of SR protein) plus (0.5 mg detergent per mg excess lipid). The protein concentra-

tion in the solubilized cocktail ranged from 1 to 2 mg/ml in different experimental series. This cocktail was incubated for 30 min at 22°C and then rapidly diluted more than 50-fold with buffer.

RESULTS AND DISCUSSION

The steady-state level of fluorescence of fluorescein labelled SR monotonically increases as a function of time after labelling (results not shown), reaching a steady-state level about 2 hours after labelling. Because the reasons for this change are unclear, we have measured the fluorescence of fluorescein once the steady-state level has been reached.

In addition, when measuring the level of fluorescein fluorescence of the labelled SR in reconstituted systems at different lipid to protein ratios, see the Materials and Methods, we observed that this level changes about 10% in between different samples. However, the reconstituted samples prepared in such a way showed a relatively high variability (about 10%) in their steady-state level of fluorescence. Freezing at -60°C and thawing of these samples makes the intensity and the polarization of the fluorescence of labelled SR to be monotonically dependent on the lipid to protein ratio, see Figs. 1 and 2. We have also found that only two freezing and thawing cycles are needed to completely develop the above mentioned fluorescence changes and that more freeze-thaw cycles (up to five) did not induce any additional fluorescence change. The (Ca^{2+}, Mg^{2+})-ATPase activity of these reconstituted systems was not appreciably changed, however, by these treatments. These results are consistent with the production of a more homogeneous dispersion of the (Ca^{2+},

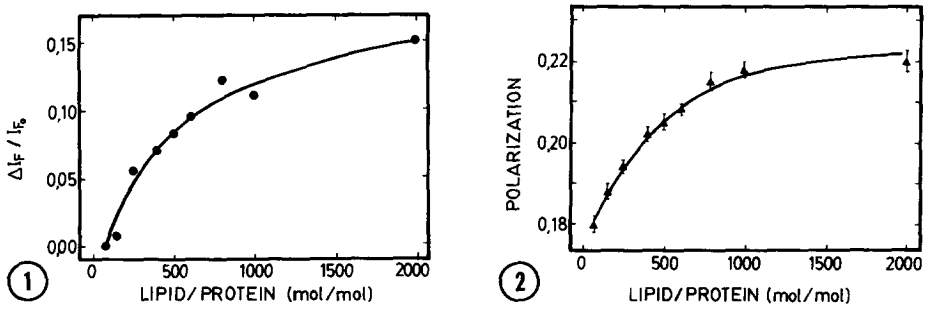


Fig. 1: Dependence of the fluorescence of fluorescein-labelled sarcoplasmic reticulum (Ca^{2+}, Mg^{2+})-ATPase upon the lipid to protein ratio. Emission wavelength = 518 nm; excitation wavelength; 475 nm. Buffer: 0.25 M Sucrose, 50 mM Tes, 100 mM KCl and 2 mM β-mercaptoethanol (pH = 7.45). Protein concentration = 2 - 10 μg protein/ml. The measurements were carried out at room temperature: 20-22°C. See the text for additional experimental details. The data shown are the average of triplicate experiments.

Fig. 2: Dependence of the polarization of the fluorescence of fluorescein-labelled sarcoplasmic reticulum (Ca^{2+}, Mg^{2+})-ATPase upon the lipid to protein ratio. Experimental conditions: same as those stated in the legend for Fig. 1.

Mg^{2+})-ATPase with excess lipids by freeze-thaw treatments. In support to this hypothesis it might be worthy to recall here that Konigsberg (16) has reported that the SR Ca^{2+} pump protein does not disperse homogeneously with excess lipids in SR reconstituted systems prepared by column chromatography, and that freeze-thaw treatments induce packing defects in lipid bilayers which results in an increased probability of fusion of vesicles (17). This makes of freeze-thaw treatments an useful method to reconstitute functionally active membrane vesicles (18).

It is to be noted that the quantum yield of fluorescence attained at high lipid to protein ratios is similar to that of fluorescein-glycine conjugates in the same experimental conditions. Nevertheless, the polarization of fluorescein fluorescence is notably higher in reconstituted labelled SR vesicles than in fluorescein-glycine conjugates in the same buffer (0.23 versus 0.035, respectively). This result shows that the fluorescent dye is largely inmobilized when bound to the protein because the limiting polarization of this dye in glycerol is 0.40 (determined in our experimental conditions).

In order to seek if the change of fluorescence intensity as a function of the lipid to protein ratio can be, at least, partially due to denaturation of the SR (Ca^{2+}, Mg^{2+})-ATPase by DOC at the lower lipid to protein ratios, we have measured the Ca^{2+}-dependent ATPase activity in paralled to the fluorescence intensity of these samples. No significant variation of the Ca^{2+}-dependent ATPase activity in the absence and in the presence of A23187 was found among reconstituted systems of different lipid to protein ratios. Their Ca^{2+} dependent ATPase activity typically ranged between 1.9 and 2.6 μmoles ATP hydrolyzed per mg protein per min at (20-22)°C, and they show a negligible Ca^{2+}-independent ATPase activity. The specific Ca^{2+}-ATPase activity of these reconstituted systems was found to be similar to that obtained for unlabelled reconstituted SR in parallel experiments carried out in our laboratory and to that reported by Fleischer and col. (21). Therefore, the system is not significantly perturbed by this extent of labelling.

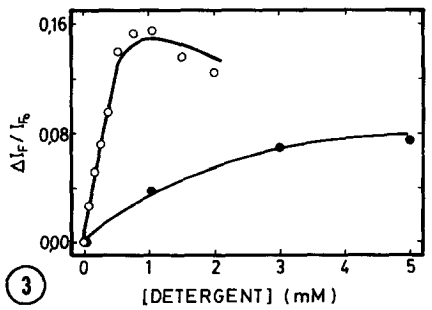
These results suggest that partial self-quenching of fluorescein labelled SR (Ca^{2+} , Mg^{2+})-ATPase does occur in native and in reconstituted SR vesicles at low lipid to protein ratios. Both sets of data, intensity and polarization of fluorescence, are consistent with this hypothesis. Alternatively, the fluorescence changes induced by lipid dilution could be rationalized in terms of fluorescein environmental changes if fluorescein is located nearby the lipid headgroups in labelled reconstituted SR vesicles. However, using fluorescent lipid derivatives capable of being acceptors of the fluorescence of fluorescein in mixed reconstituted systems we have found evidences that the fluorescein is located far away from of the lipid-water interface (C.

Gutiérrez-Merino, A.M. Mata, J.M. East and A.G. Lee, unpublished results). Therefore, it seems unlikely that fluorescein environmental changes, other than self-quenching changes, can be induced in the lipid dilution experiments of Figs. 1 and 2.

An alternative way of increasing the average protein to protein particle distance is by the addition of detergents to native SR membranes. We have used DOC and Triton X-100 for this purpose. Figs 3 and 4 show that both detergents, at concentrations below their critical micelle concentration, enhance the fluorescence intensity and of fluorescence polarization of fluorescein in labelled reconstituted SR vesicles. The magnitude of the observed increase closely agree with that found when diluting protein particles with excess SR endogenous lipids.

Because self-quenching might be associated to an oligomerization process, we have attempted to fit the experimental lipid dilution data to the simplest case of a dimerization process (ie. 2 · monomer $\rightleftharpoons$ dimer). The solid line of Fig. 1 is the best polynomial fit to the equation A-3, see the Appendix. It can be seen that the data fit reasonably well to this equation and, thus, it appears that a dissociation process like: dimer $\rightarrow$ 2.monomer or tetramer $\rightarrow$ 2.dimer, with an apparent association constant, K, of (0.05-0.1) could explain these data. Because fluorescein has been shown to selectively label the sustrate (ATP) binding site of SR (Ca^{2+}, Mg^{2+})-ATPase (9,10), this suggests a close spatial proximity of the ATP binding sites in ATPase oligomers.

Finally, we wish to note the relevance of these findings to properly understand the changes of the fluorescence of fluorescein labelled SR vesicles induced by different ligands of this enzyme (11). Work is actually in progress in this laboratory in this latter direction.



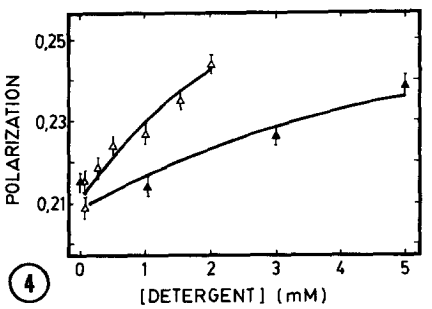


Fig. 3: Effect of Triton X-100 (O) and of deoxycholate (●) on the intensity of fluorescence of fluorescein-labelled SR Other experimental conditions as in Fig. 1, except that the protein concentration used in these measurements ranged from 50 to 60 μg protein/ml. The data shown are the average of triplicate experiments.

Fig. 4: Effect of Triton X-100 (Δ) and of deoxycholate (▲) on the polarization of fluorescence of fluorescein-labelled SR. Other experimental conditions as in Fig. 3.

Appendix. Dependence of the monomer molar fraction in monomer ⇋ dimer equilibrium upon the lipid to protein ratio.

As stated in eq. 19 of Ref. 19, for this case the concentration of monomers per vesicles (C_M) can be written:

$$C_M = [-1 + (1 + 8.\ K.\ n_p)^{1/2}] / 4\ .\ K \qquad (A\text{-}1)$$

where: n_p is the average number of protein particles per vesicle and K is the equilibrium constant: $K = C_D/C_M^2$, being C_D and C_M the concentration of dimers and of monomers per vesicle, respectively. The average value of n_p can be related to the lipid to protein ratio, assuming ideal mixing, as follows. The total surface of the vesicle (S_T) must be coupled by lipid (S_L) and protein (S_P). Therefore, we can write:

$$S_T = S_L + S_P = \pi\ .\ n_p\ [r.\ (\phi_L/2)^2 + (\phi_P/2)^2] \qquad (A\text{-}2)$$

where ϕ_L and ϕ_P are the cross-sectional diameters of lipid and protein monomers, respectively, and r is the lipid to protein ratio.

Combining eqs. A-1 and A-2, we can finally obtain:

$$\frac{C_M}{C_T} = [-1 + (1 + 8\ .\ K\ .\ n_p)^{1/2}]/\ 4\ .\ K\ .\ n_p \qquad (A\text{-}3)$$

and

$$n_p = \frac{S_T}{\pi\ .\ [r.\ (\phi_L/2)^2 + (\phi_P/2)^2]}$$

Taking ϕ_L = 10 Å (20), ϕ_P = 50 Å (5) and an average vesicle diameter of 1000 Å (21, 22), the value of K can be estimated from the polynomial fit to eq. A-3 of any set of experimental data reflecting the dissaggregation process as a function of the lipid to protein ratio.

ACKNOWLEDGEMENTS

This work has been supported by Grant number 2813/83 of the Spanish CAICYT. Part of this work was presented at the Special FEBS Meeting: "Metal Ions, Proteins and Membranes", held at the Algarve (Portugal) April, 1985.

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