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ARTICLE *in* BIOCHEMISTRY · MAY 1977

Impact Factor: 3.02 · DOI: 10.1021/bi00626a003 · Source: PubMed

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Fluorescence Energy Transfer between Ca^{2+} Transport ATPase Molecules in Artificial Membranes†

J. M. Vanderkooi,* A. Ierokomas, H. Nakamura, and A. Martonosi

ABSTRACT: The purified ATPase of sarcoplasmic reticulum was covalently labeled with *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) or with iodoacetamidofluorescein (IAF). In reconstituted vesicles containing both types of ATPase molecules fluorescence energy transfer was observed from the IAEDANS (donor) to the IAF (acceptor) fluorophore as determined by the ratio of donor and acceptor fluorescence intensities, and by nanosecond decay measurements of donor fluorescence in the presence or absence of the acceptor. The observed energy transfer may arise by random collisions between ATPase molecules due to Brownian motion or by formation of complexes containing several ATPase molecules. Experimental distinction between these two models of energy transfer is possible based on predictions derived from mathematical models. Up to tenfold dilution of the lipid phase of reconstituted vesicles with egg lecithin had no measurable effect upon the energy transfer, suggesting that random collision between ATPase molecules in the lipid phase is not the principal cause of the observed effect. Addition of unlabeled ATPase in five- to tenfold molar excess over the labeled molecules abolished energy transfer. These observations together with electron microscopic and chemical cross-linking studies support the existence of ATPase oligomers in the membrane with sufficiently long lifetimes for energy transfer to occur. A hypothetical equilibrium between monomeric and tetrameric forms of the ATPase governed by the membrane potential is proposed as the structural basis of the regulation of Ca uptake and release by sarcoplasmic reticulum membranes during muscle contraction and relaxation.

Sarcoplasmic reticulum membranes operate in two distinct functional states during the contraction-relaxation cycle. (1) The relaxation of muscle is initiated by the ATP-mediated accumulation of Ca^{2+} into the sarcoplasmic reticulum tubules which lowers the cytoplasmic Ca^{2+} concentration below 10^{-7} M (MacLennan and Holland, 1976). (2) During excitation the accumulated Ca^{2+} is rapidly released from sarcoplasmic reticulum. The rate of this process is too great to be explained by a simple reversal of the Ca pump (Martonosi, 1972) and presumably involves the formation of Ca^{2+} channels. The Ca^{2+} release is accompanied by depolarization of the sarcoplasmic reticulum membrane as indicated by changes in birefringence (Baylor and Oetliker, 1975) and membrane potential (Bezanilla and Horowicz, 1975). The only hint that a similar process may occur in vitro is the observed Ca^{2+} release from skeletal muscle microsomes under conditions which are assumed to alter membrane potential (Kasai and Miyamoto, 1976).

The Mg^{2+} + Ca^{2+} activated ATPase of sarcoplasmic reticulum plays a major role in the active accumulation of calcium and in the regulation of the passive Ca^{2+} permeability of the membrane.

Cooperativity in the dependence of Ca^{2+} transport and phosphoprotein formation upon the free Ca^{2+} concentration (Coffey et al., 1975), together with indications of "half of the sites" reactivity (Martonosi et al., 1974), provided the first evidence that interaction between elements of the Ca^{2+} transport complex constitutes an important aspect of active Ca^{2+} uptake. Further indication of interaction between Ca^{2+} transport ATPase molecules in the membrane was the demonstration that the number of 85-Å intramembranous particles revealed in native membranes and in reconstituted ATPase vesicles by freeze-etch electron microscopy was 4–5 times less than the number of 40-Å surface particles seen after negative staining (Jilka et al., 1975). As both sets of particles are as-

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sociated with the Ca^{2+} transport ATPase, the possibility arose that the large freeze-etch particles contain clusters of several ATPase molecules which surround a hydrophilic channel (Martonosi, 1975).

The main purpose of this report is to provide additional evidence for the existence of ATPase oligomers in reconstituted membranes by fluorescence energy transfer experiments. The technique proved useful in the analysis of proximity relationships in rhodopsin (Wu and Stryer, 1972), immunoglobulins (Bunting and Cathou, 1973), and ribosomes (Huang et al., 1975) and in demonstrating the formation of gramicidin A dimers as conducting channels in artificial membranes (Veatch and Stryer, 1977). The interaction between ATPase molecules was analyzed in reconstituted ATPase vesicles by measuring the efficiency of energy transfer between two populations of ATPase molecules, one labeled with *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS)[1] serving as energy donor and the other with iodoacetamidofluorescein (IAF) serving as energy acceptor.

Materials and Methods

The Ca^{2+} + Mg^{2+} activated ATPase was prepared from rabbit sarcoplasmic reticulum according to MacLennan (1970). When tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the preparations contained the 100 000-dalton band with only trace amounts of the low molecular weight contaminant usually denoted as proteolipid. The phospholipid composition and other characteristics of the preparation were similar to those reported earlier (MacLennon, 1970; Nakamura et al., 1976). *N*-Iodoacetyl-*N'*-(5-sulfo-1-napthyl)ethylenediamine (1,5-IAEDANS) and its 1,8 isomer were obtained from Aldrich Chemical Co., Milwaukee, Wis. 5-Iodoacetamidofluorescein (IAF) was the product of Molecular Probes, Inc., Roseville, Minn.

Preparation of Fluorescent Derivatives of the Ca^{2+} + Mg^{2+} Activated ATPase. The purified ATPase of sarcoplasmic reticulum was labeled with 1,5-IAEDANS or IAF in a medium of 1 M KCl–0.25 M sucrose–50 mM potassium phosphate buffer (pH 8.0) for 15–18 h at 4 °C using ATPase:dye mole ratios of 1:2 and 1:10. In some experiments 5–10 mM ATP was also present during labeling. The free dye was removed by chromatography on a Sephadex G-50 column (0.9 × 6.0 cm) using 1 M KCl, 0.25 M sucrose, and 50 mM Tris-Cl (pH 8.0) for elution. The ATPase band was collected, concentrated by centrifugation at 80 000*g* for 1 h and dispersed in 1 M KCl–0.25 M sucrose–50 mM Tris-Cl (pH 8.0).

The ATPase activity was measured by incubation at 25 °C for 2, 4, 6, 8, and 12 min in a medium of 0.1 M KCl, 20 mM imidazole, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.45 mM $CaCl_2$, 5 mM ATP, and 0.04 mg of protein per mL; the reaction was stopped with 6% trichloroacetic acid and the inorganic orthophosphate was determined according to Fiske and Subbarow (1925).

The phosphoprotein intermediate was measured essentially as described earlier (Nakamura et al., 1976). A medium of 0.1 M KCl, 20 mM imidazole (pH 7.3), 5 mM $MgCl_2$, 0.5 mM EGTA–0.45 mM $CaCl_2$, 10 μM [^{32}P]ATP, and 0.04 mg of enzyme protein/mL was manually mixed at 4 °C and the reaction stopped after 5 s.

Measurement of the Amount of Bound Dye. The concentration of the bound IAEDANS and IAF was determined by absorption and fluorescence measurements in comparison with standard solutions using the following molar extinction coefficients: for IAEDANS $\epsilon = 6.1 \times 10^3$ at 336 nm and for IAF $\epsilon = 8.5 \times 10^4$ at 492 nm. In the various experiments the concentration of bound dye ranged from 1.5 to 5.9 mol of IAEDANS/mol of ATPase and 0.5–1.1 mol of IAF per mol of ATPase. Addition of 5 mM ATP did not significantly influence the extent of labeling after 15 h of incubation.

Fluorescence Spectra. Steady-state fluorescence spectra were obtained using a Hitachi MPF-2A or an Aminco-Bowman fluorescence spectrophotometer. The sample was maintained at constant temperature with use of circulating water through the cell block. For measurement of fluorescence anisotropy a Polacoat 4B polarizer was used to polarize the emitting beams. Fluorescence polarization, *P*, is defined as:

$$P = \frac{I_{\parallel} - cI_{\perp}}{I_{\parallel} + cI_{\perp}} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed with the analyzer parallel and perpendicular to the excitation polarizer and *c* corrects for instrumental inaccuracies. Temperatures for most fluorescence measurements were at 21 to 23 °C.

Fluorescence Decay Measurement. Fluorescence decay profiles were obtained and analyzed as previously described (Vanderkooi et al., 1974). An Ortec photon-counting fluorescence lifetime instrument (Oak Ridge, Tenn.) equipped with an Ortec air spark-gap type flash lamp and an RCA 8850 photon multiplier was used to detect and time resolve the emitted photons. For measurement of the fluorescence decay of IAEDANS-labeled protein, the exciting light was isolated using a Corning 7-60 glass filter (Corning, N.Y.) and the emitted light was isolated using a Kodak Wratten 34A filter (Rochester, N.Y.) plus an interference filter. In the experiments in which the fluorescent acceptor is present, it is necessary to ascertain that fluorescence from the acceptor (IAF) does not contribute to the observed signal. Measured under the above conditions, it took more than 20 times longer to count an integrated signal from the acceptor (IAF) equal to that from the IAEDANS-labeled protein; therefore, it is reasonable to estimate that the contribution from the IAF fluorescence was less than 5% when the donor fluorescence decay was measured in the presence of IAF.

Analysis. The reaction scheme which we are considering is:

$$\begin{array}{ccc} D^* & \xrightarrow{k_T} & A^* \\ {\scriptstyle k_e}\uparrow\downarrow{\scriptstyle k_F} & & \downarrow{\scriptstyle k_{FA}} \\ D & & A \end{array}$$

where k_e is the rate of excitation of the fluorescence donor, D, k_F is the rate of decay of the excited state donor, D*, and k_{FA} is the rate of fluorescence decay of the acceptor molecule, A*. The distance, R_0, at which k_T equals k_F for a given molecular pair is, according to Förster (1965):

$$R_0^6 = 8.785 \times 10^{-25} K^2 \Phi_D n^{-4} \int_0^{\infty} e(\lambda) f(\lambda) \lambda d\lambda$$

where Φ_D is the emission quantum yield of the donor in the absence of acceptor, $e(\lambda)$ is the molar absorbance of the acceptor at wavelength λ, $f(\lambda)$ is the emission intensity of acceptor at wavelength λ, and *n* is the refractive index.

In our calculations, the orientation factor is taken to be $^2/_3$. This orientation factor assumes random orientation between donor and acceptor. The dangers of assuming random orientation have been recently pointed out by Dale and Eisinger (1976). However, in our case it is a reasonable assumption

[1] Abbreviations used are: IAF, iodoacetamidofluorescein; IAEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine.

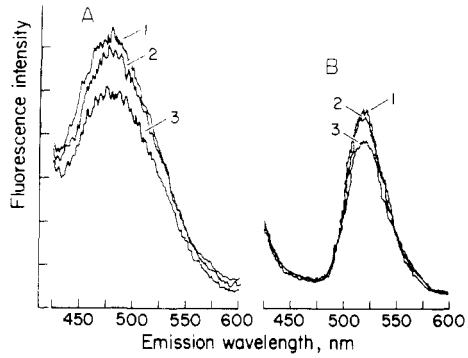

FIGURE 1: The effect of ATP upon the fluorescence of IAEDANS-ATPase (A) and IAF-ATPase (B); medium, 0.1 M KCl-10 mM Tris-maleate (pH 7.0); protein concentration, 0.03 mg/mL; excitation, 320 nm: (1) control; (2) with 1.6 mM $MgCl_2$; (3) with 1.6 mM MgATP.

since the fluorescence polarization of the donor, IAEDANS, is 0.11, indicating considerable rotational mobility. Furthermore, both the IAEDANS and IAF labels are attached to sulfhydryl groups; hence, one might expect a multiplicity of binding sites and a multiplicity of angles of orientation. Finally, the degree of labeling was varied. With the ratio of covalently bound IAEDANS to ATPase varied from 1.5 to 5, the energy transfer did not change significantly.

Using the above assumptions, R_0, the distance between IAEDANS and IAF at which the fluorescence of the donor is 50% quenched (i.e., $k_T = k_F$) is around 48 Å (Wu and Stryer, 1972; Werner et al., 1972). Based upon the molecular weight of the Ca + Mg ATPase and the size of particles revealed by electron microscopy, the radius of the protein can be estimated to be 20–25 Å. Hence, the molecular radius of interaction (the sum of the radii of donor and acceptor molecules) is approximately equal to R_0.

Measurement of Donor and Acceptor Fluorescence Intensities. In control experiments the emission spectra of IAEDANS-ATPase, IAF-ATPase, and a mixture of the two were measured in a medium of 0.1 M KCl-10 mM Tris-maleate (pH 7.0) using an excitation wavelength of 320 nm. Under these conditions the fluorescence intensity of the mixed ATPase preparations was equal to the sum of the donor and acceptor fluorescence intensities measured separately.

In the energy transfer experiments the IAEDANS-ATPase (1 mg/mL), the IAF-ATPase (1 mg/mL), or the mixture of the two was first dissolved in a small volume with 0.5–5.0 mg of deoxycholate per mg of protein, followed 2–20 min later by dilution with 0.1 M KCl-10 mM Tris-maleate (pH 7.0) to a final protein concentration of 0.03–0.06 mg/mL. Upon dilution the vesicles re-form. In mixtures of IAEDANS-ATPase and IAF-ATPase the donor and acceptor molecules will be intermixed within the same vesicle membrane and may interact. Energy transfer is indicated by a decrease in the donor and an increase in the acceptor fluorescence intensities in mixed membranes containing both types of molecules.

Electron Microscopy Studies. Reconstituted ATPase vesicles were negatively stained with 1% potassium phosphotungstate (pH 7.0) on carbon coated parlodion grids and analyzed on a Phillip EM 300 electron microscope. Enlarged negatives of the electron micrographs were used for image enhancement analysis according to Markham et al. (1963).

Results

Characterization of Fluorescent ATPase Derivatives. The labeling of purified ATPase with 1,5-IAEDANS and IAF was usually carried out at dye to protein mole ratios of 2–10 for 15 h at 4 °C in a medium of 1 M KCl-0.25 M sucrose-50 mM potassium phosphate buffer (pH 8.0), and 0.02% sodium azide, with or without 5–10 mM ATP. At dye to protein ratios of 2 and 10, the average amount of IAEDANS covalently bound to the ATPase was about 1.7 and 5.5 mol/mol of ATPase, respectively. The corresponding values for IAF were 0.5 and 0.9 mol/mol of ATPase.

TABLE I: The ATPase Activity of Labeled ATPase Preparations.[a]

Preparation	Dye/Protein Mole Ratio	ATPase Act. (μmol of P (mg of Protein)$^{-1}$ min^{-1})
Purified ATPase	0	2.73 (5)
IAEDANS-ATPase	2:1	3.53 (3)
(−ATP)	10:1	3.56 (1)
IAEDANS-ATPase	2:1	2.67 (2)
(+ATP)	10:1	2.81 (1)
IAF-ATPase	2:1	3.25 (3)
(−ATP)	10:1	2.43 (1)
IAF-ATPase	2:1	1.72 (2)
(+ATP)	10:1	2.25 (1)

[a] For technical details see Materials and Methods. + ATP and − ATP indicate the presence or absence of 5 mM MgATP during labeling. The second column gives the dye:protein mole ratio in the reaction mixture. The numbers in parentheses denote the number of preparations tested.

The emission spectra of IAEDANS- and IAF-labeled ATPase vesicles are shown in Figure 1. The shape of the spectrum did not change markedly upon varying the amount of bound IAEDANS between 2 and 5 mol/mol of ATPase and that of the IAF between 0.45 and 1 mol/mol of ATPase. Both labels were attached to proteins and the extracted lipid had only trace amounts of label.

The intensity of fluorescence of IAEDANS-ATPase decreased upon addition of MgATP in millimolar concentrations; this effect was less pronounced in the presence of 1 mM EDTA (Figure 1). Only slight changes in fluorescence were observed with MgADP or with Mg-β,γ-methylene ATP. As the binding of ATP to the enzyme is not influenced by EDTA, these observations suggest that the effect of ATP upon the fluorescence of IAEDANS-ATPase is connected with one of the elementary steps of the ATPase reaction that follows the binding of substrate.

The fluorescence of IAF-ATPase was only marginally affected by ATP.

The ATPase activity of labeled preparations was slightly activated (Table I) without significant change in the steady-state concentration of phosphoprotein measured with Mg and Ca as activators.

Electron microscope analysis of the IAEDANS-ATPase and IAF-ATPase preparations after negative staining with 1% potassium phosphotungstate (pH 7.0) revealed vesicles of greatly different sizes ranging in diameter from 700 to 2000 Å; the surface of the vesicles was covered with particles of about 40-Å diameter which were similar to those observed on unlabeled ATPase or sarcoplasmic reticulum vesicles (Figure 2).

Energy Transfer. Upon mixing IAEDANS-labeled ATPase vesicles with IAF-ATPase vesicles in the absence of detergents, the fluorescence spectrum is usually the sum of the spectra of the two components measured separately, indicating that no significant energy transfer occurs. Under these conditions the donor and acceptor fluorophores are segregated in different

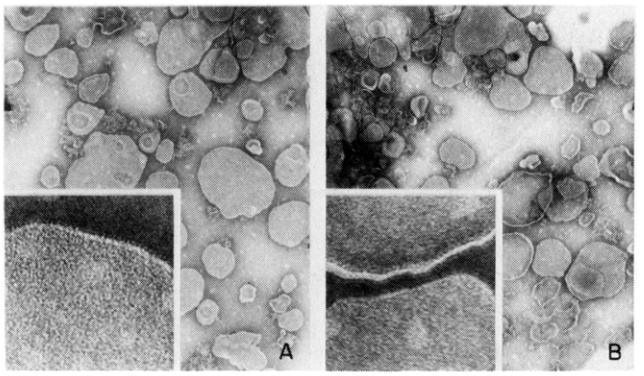

FIGURE 2: Electron microscopy of negatively stained IAEDANS-ATPase and IAF-ATPase vesicles. For conditions see Materials and Methods: (A) IAEDANS-ATPase, ×21 580; insert, ×125 000; (B) IAF-ATPase, ×21 580; insert, ×125 000.

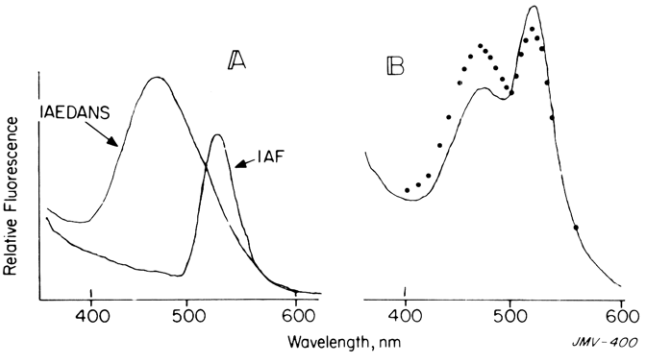

FIGURE 3: Emission spectra of IAEDANS- and IAF-labeled ATPase proteins. (A) Sample contained 0.1 mg of IAEDANS- or IAF-labeled protein/1 mL, 0.1 mg of deoxycholate/mL, 0.1 M KCl, and 10 mM Tris-maleate (pH 7.0). (B) (●) Numerical sum of spectra presented in Figure 1A; (—) spectra of 0.1 mg of IAEDANS protein, 0.1 mg of IAF, and 0.2 mg of deoxycholate/mL in 0.1 M KCl and 10 mM Tris-maleate (pH 7.0). The IAEDANS- and IAF-labeled proteins were incubated with deoxycholate for 20 min before dilution with the buffer; excitation wavelength, 320 nm; slits, 7 nm; temperature, 22 °C.

vesicles which are too distant for efficient energy transfer.

When the concentrated suspensions of donor and acceptor labeled vesicles are dissolved in the presence of 0.5–5.0 mg of deoxycholate per mg of protein, followed a few minutes later by dilution with 0.1 M KCl–10 mM Tris-maleate buffer (pH 7.0), vesicles containing both donor and acceptor labeled ATPase molecules form spontaneously. Upon the mixing of equal molar concentrations of IAEDANS- and IAF-labeled ATPase proteins, there is a 15–20% quenching of IAEDANS fluorescence and a 10–15% increase in IAF fluorescence (Figure 3). The magnitude of these differences was similar at deoxycholate concentrations ranging from 0.5 to 5.0 mg of deoxycholate per mg of protein, although the intensity of scattered exciting light diminished with increasing deoxycholate concentration. These data are consistent with the occurrence of long-range energy transfer (Förster, 1965).

In a few preparations, change in the donor–acceptor ratio was observed even in the absence of deoxycholate; this usually required long incubation times and may have been caused by spontaneous fusion of the vesicles and intermixing of their contents.

Light scattering and absorption artifacts were minimized by keeping the concentration of the proteins low, but it was of interest to examine the fluorescence decay of the donor, since the decay rate is less affected by absorption and turbidity artifacts. The excited state lifetime of IAEDANS-ATPase was determined in the presence and absence of IAF-ATPase at a deoxycholate:protein ratio of 1 (Figure 4). The decay curve was fitted by computer to two exponentials. The lifetimes in the absence of IAF-ATPase were 2.7 and 18.8 ns and in the presence of IAF-ATPase 0.4 and 15.1 ns. An average of 3 measurements gave 1.3 and 18.6 ns in the absence of acceptor and 0.8 and 15.4 ns in the presence of acceptor. These observations are clearly consistent with energy transfer. The fluorescence lifetime of IAEDANS-ATPase was unchanged when the donor and acceptor preparations were mixed in the absence of deoxycholate.

Two models were considered to explain these observations. In one case energy transfer was assumed to occur when the donor and acceptor molecules collide (or come within the distance R_0) due to Brownian motion. The other model assumes the existence of ATPase oligomers in which the donor and acceptor are in close proximity permitting energy transfer to occur within the complex.

Dilution of the lipid phase with added lipids should increase the average distance between ATPase molecules in the membrane. If the observed energy transfer was due to random collision between ATPase molecules it should diminish with increasing lipid:protein ratio. In fact, the donor–acceptor ratio of fluorescence intensity remained essentially unaffected by the addition of 5 mg of egg lecithin per mg of protein. This amount of egg lecithin should increase the lipid phase of the membrane about tenfold and the average distance between ATPase molecules about threefold.

Similar observations were made using fluorescent decay measurements. Under conditions given in the legend of Figure 4, the fluorescence lifetimes were 1.2 and 16.1 in the presence of 5 mg of egg lecithin per mg of protein and 15.9 and 0.9 in the absence of egg lecithin. The small difference in fluorescence lifetimes between the two systems is within experimental error and is consistent with energy transfer within ATPase oligomers.

It is also expected that the collision frequency between ATPase molecules would decrease with decreasing temperature. Yet the magnitude of the energy transfer was similar at 6, 23, 33, and 37 °C. The lipid dilution and the temperature dependence studies are clearly consistent with energy transfer within ATPase oligomers. If this interpretation is correct, addition of a large amount of unlabeled ATPase should result in fewer donor and acceptor pairs and a decrease in the energy transfer under conditions of Figure 4. A tenfold excess of unlabeled ATPase restored the fluorescence lifetime of IAEDANS-ATPase in mixed vesicles containing both donor and acceptor to the unquenched levels.

Effect of ATP and Ca on the Donor–Acceptor Ratios. In dispersions of IAEDANS-ATPase and IAF-ATPase reconstituted with deoxycholate, addition of 1.6 mM ATP decreases the ratio of donor and acceptor fluorescence intensities. A major part of this change may be explained by the effect of ATP upon the fluorescence intensities of individual components without postulating an effect upon energy transfer. In accordance with this conclusion 5 mM MgATP had no significant effect upon the excited state lifetime of reconstituted IAEDANS + IAF-ATPase vesicles measured by nanosecond decay.

Electron Microscope Studies. Previous observations established that the number of intramembranous particles revealed by freeze-etch electron microscopy on reconstituted membranes containing only the purified ATPase is 4–6 times less than the number of surface particles observed after neg-

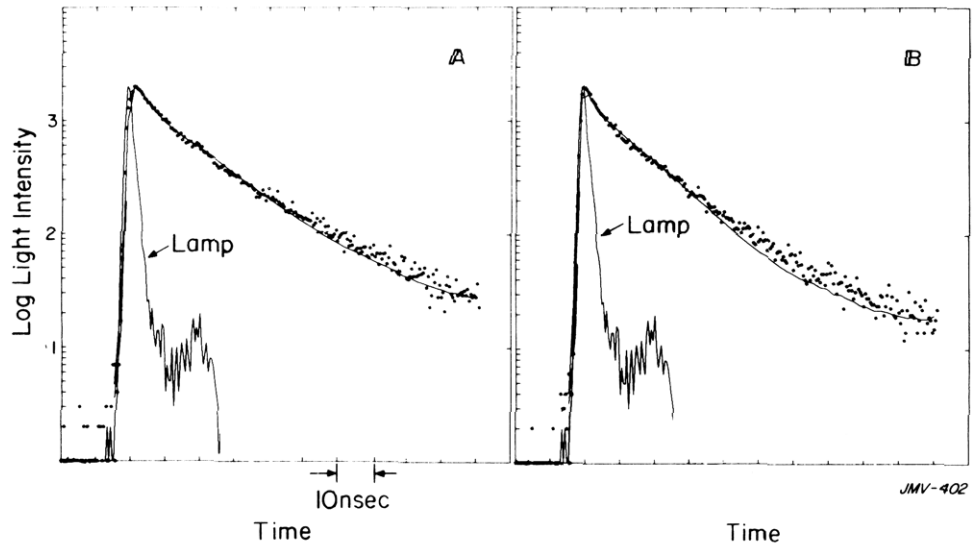

FIGURE 4: Fluorescence decay of IAEDANS-labeled ATPase protein. (A) IAEDANS-ATPase sample is identical with that in Figure 3A. Solid line is the computer best fit to the data points using 18.8 ns and 2.7 as fluorescent lifetimes. (B) IAEDANS-ATPase + IAF-ATPase sample is identical with that in Figure 1B. Solid line is the computer best fit to the data using 15.1 and 0.4 ns as fluorescent lifetimes.

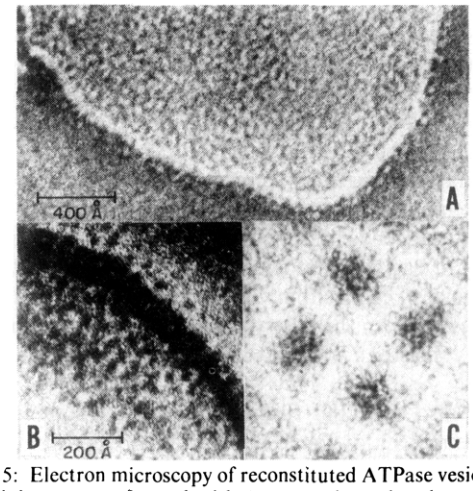

FIGURE 5: Electron microscopy of reconstituted ATPase vesicles. Negative staining was performed with 1% potassium phosphotungstate as described under Materials and Methods: (A) ×255 000; (B) inverted ×455 000; (C) enlarged image of a tetramer obtained by the rotation technique. For technical details see Markham et al. (1963).

ative staining (Jilka et al., 1975). The possibility was considered that the intramembranous particles contain clusters of several ATPase molecules (Martonosi, 1975).

Further analysis of the electron micrographs of negatively stained, reconstituted ATPase vesicles by image enhancement techniques revealed a pattern of four subunits which occurred in favorably oriented regions of the surface and required some selection (Figure 5).

The clusters of four subunits may represent tetramers of ATPase molecules in equilibrium with a less orderly arrangement of monomers and dimers. Alternatively the polypeptide chain of the ATPase may be folded into several helical segments with their axis oriented perpendicularly to the plane of the membrane. In this interpretation the 40-Å subunits seen by negative staining may correspond to the nonhelical bends in the polypeptide chains which project into the water phase.

Discussion

Fluorescence energy transfer was observed between ATPase molecules in artificial membranes. The energy transfer was not altered noticeably by dilution of membrane lipids with egg lecithin which is expected to increase the average distance between ATPase molecules about threefold and by varying the temperature between 6 and 37 °C. Addition of a five- to tenfold excess of unlabeled ATPase abolished the energy transfer. These observations together with electron microscopic findings led to the tentative conclusion that the energy transfer takes place in oligomers of the ATPase molecules.

The exact magnitude of the contribution of energy transfer during random collision between ATPase molecules due to Brownian motion was not determined. Further work is needed to settle this point, although the temperature dependence and lipid dilution studies suggest that this contribution may be minor. Some predictions regarding the contribution of diffusion to the energy transfer may be derived based upon reasonable assumptions about the distribution and concentration of Ca^{2+} transport ATPase in the membranes and distance requirements for energy transfer. The emission spectrum of IAEDANS-labeled ATPase overlaps with the absorption spectrum of IAF-labeled ATPase molecules permitting efficient energy transfer, provided the distance between donor and acceptor fluorophores is less than 50 Å. The average distance between the ATPase molecules is in excess of 100 Å, if they are non-aggregated and randomly distributed. Since under conditions of our experiments we have approximately equimolar amounts of donor and acceptor molecules, the average distance between donor and acceptor pairs becomes greater than $(2^{1/2})100$ or 150 Å. The diffusion coefficient of the ATPase molecule is unknown; however, based upon the size of the molecule and the diffusion coefficient of other proteins in membranes, a diffusion coefficient of 10^{-9} cm^2/s can be taken. Since the lifetime of the donor is about 18 ns, a rough estimate based upon average distance between donor and acceptor pairs and diffusion coefficient suggests that the contribution of diffusion to the observed energy transfer is likely to be very small.

It is apparent that considering the uncertainties of our calculations which include the shape and size of the ATPase, the exact effective concentration of donor and acceptor in the membrane and the position and orientation of donor and acceptor molecules, no clear decision can be made about the role of aggregation or diffusion in the observed energy transfer. In fact, an intermediate form of the two extremes is possible, with

an equilibrium between aggregated and nonaggregated forms. Systematic studies at varying donor:acceptor ratios and at widely varying concentrations of acceptor present obvious future possibilities for a more precise quantitative assessment of the contribution of collisional transfer.

Although the observation of energy transfer occurs between labeled ATPase monomers suggests that aggregation between the monomers occurs, the stoichiometry of aggregation cannot be ascertained from the data. Analysis of the electron micrographs of the negatively stained membranes by image enhancement techniques reveals a pattern of four subunits within the clusters. The array of four subunits could be observed only on portions of the surface. These clusters may represent tetramers of four ATPase molecules in equilibrium with monomers and dimers. This interpretation accounts for the observation that the number of intramembranous particles in reconstituted membranes of the ATPase determined by freeze-etch electron microscopy is 4–6 times less than can be observed through negative staining (Jilka et al., 1975; Martonosi, 1975). The existence of ATPase tetramers is further supported by the observation of 400 000-dalton oligomers in detergent-solubilized sarcoplasmic reticulum preparations (Le Maire et al., 1976). Although perhaps less likely, the possibility also exists that the polypeptide chain of the ATPase molecule is folded into several helical segments with the axis oriented perpendicularly to the plane of the membrane. If so, the 40-Å subunits seen by negative staining may correspond to nonhelical bends in the polypeptide chains which project into the water phase. An arrangement similar to this has been described for the structure of bacteriorhodopsin in the purple membrane (Henderson and Unwin, 1975).

It is tempting to speculate that the association between ATPase molecules constitutes the structural basis of the rapid Ca^{2+} release during excitation (Ashley and Caldwell, 1974) and contributes to the unique permeability characteristics of the sarcoplasmic reticulum membrane (Duggan and Martonosi, 1970; Jilka et al., 1975). Application of the energy transfer method to more physiological muscle preparations may allow an experimental test of the proposition (Martonosi et al., 1976) that the Ca^{2+} permeability of sarcoplasmic reticulum is regulated by an equilibrium between monomeric and tetrameric forms of the Ca^{2+} transport ATPase under the influence of the membrane potential.

Acknowledgments

The authors would like to thank Dr. Carlos Gitler for interesting discussions and Dr. B. Chance for use of equipment.

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