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The position of the ATP binding site on the $(Ca^{2+} + Mg^{2+})$-ATPase

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We present a convenient method to calculate the efficiency of fluorescence energy transfer in two-dimensional membrane systems. We apply it to the analysis of energy transfer between phospholipid molecules labelled with fluorescein and rhodamine groups, and of energy transfer in reconstituted membranes containing $(Ca^{2+} + Mg^{2+})$-ATPase purified from sarcoplasmic reticulum, with the ATPase labelled at the ATP binding site with fluorescein as donor, and rhodamine-labelled lipid as acceptor. The ATP binding site is found to be distant from the plane of the lipid/water interface of the membrane. It is suggested that the ATPase is present in the membrane as a dimer, with the two ATP binding sites in the dimer being close to the protein/protein interface. Addition of vanadate causes no change in quenching, suggesting that the ATP binding site does not move significantly with respect to the lipid/water interface in the E_1-E_2 conformational transition of the ATPase.

Introduction

The $(Ca^{2+} + Mg^{2+})$-ATPase of muscle sarcoplasmic reticulum catalyses the hydrolysis of ATP tightly coupled to active transport of Ca^{2+} [1]. Although it has long been argued that the enzyme exists in two distinct conformations E_1 and E_2 and that it is the transition between these two forms which constitutes the transport event [2–4] little is known about the molecular changes linking phosphorylation to transport. The ATPase is believed to be dimeric or oligomeric within the membrane, but the importance of the state of aggregation is unclear [5–9].

Although the amino-acid sequence of the ATPase is now known [10] little structural information is yet available, and a knowledge of the relative positions of the substrate binding sites on the ATPase is important in developing an explanation for transport at the molecular level. Thus, for example, it has been suggested that the aspartate residue phosphorylated by ATP in the ATPase could be located close to the Ca^{2+}-binding region, and that relatively localised changes in this region of the ATPase result in transport [11]. One approach to the location of binding sites on membrane proteins is the use of fluorescence energy transfer since the efficiency of such transfer

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Abbreviations: FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate; XRITC, 9′-[1*H*,5*H*,9*H*,11*H*,15*H*]-xantheno-[2,3,4-*ij*: 5,6,7-*i′j′*]diquinolizin]-3-one 2′,3′,6′,7′-12′, 13′,16′,17′-octahydro-4(or 5)-isothiocyanato-spiro[isobenzofuran-1(3*H*)]; PE phosphatidylethanolamine.

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is strongly dependent on the distance between the fluorescence donor and acceptor groups [12]. This technique has been used to locate the position of Ca^{2+} and Mg^{2+} binding sites on the ATPase relative to the position of the ATP binding site [13–16]. It has also been used to measure the distances between the lipid headgroup region of the membrane and naturally occurring chromophores found in membrane proteins such as rhodopsin, bacteriorhodopsin and cytochrome b_5 [17,18].

Although the ATPase does not contain any suitable natural chromophore for such measurements, it has been shown that fluorescein isothiocyanate (FITC) specifically labels the ATP binding site [19,20] of the ATPase so that fluorescein-labelled ATPase can be used in fluorescence energy transfer experiments to locate the ATP binding site relative to the lipid headgroup region of the membrane.

Materials and Methods

Lipids were obtained from Lipid Products or Sigma. Fluorescein isothiocyanate (FITC) was obtained from Aldrich, rhodamine isothiocyanate (RITC) from Polysciences Inc. and 9′-[1*H*,5*H*, 9*H*,11*H*,15*H*]xantheno-[2,3,4-*ij*: 5,6,7-*i*′*j*′]diquinolizin]-3-one 2′,3′,6′,7′-12′,13′,16′,17′-octahydro-4(or 5)-isothiocyanato-spiro[isobenzofuran-1(3*H*)] (XRITC) from Research Organics.

Sarcoplasmic reticulum was prepared from female rabbit (New Zealand White) hind leg muscle in the presence of dithiothreitol and PMSF [21]. Purified $(Ca^{2+} + Mg^{2+})$-ATPase was prepared from sarcoplasmic reticulum as described in East and Lee [22]. ATPase was labelled with FITC as described by Highsmith [14] by incubation of FITC and ATPase at a molar ratio of FITC to protein of 0.5 : 1 at pH 7.45 for 30 min followed by separation of unreacted FITC by passage through a short column of Sephadex G-50. Alternatively, the reaction was carried out at a molar ratio of FITC to protein of 0.5 : 1 but at pH 8 for 1 h under which conditions reaction was essentially complete so that separation of bound and free FITC became unnecessary [23].

Protein concentrations were measured either following the method of Lowry et al. [24] with bovine serum albumin as standard or using the extinction coefficient given by Hardwick and Green [25]. Fluorescein was estimated using an extinction coefficient of 80000 at 500 nm [19] after solubilisation of samples in 1% SDS and NaOH (0.1 M). Lipid concentrations were measured as total phosphorus using the method of Bartlett [26].

Egg phosphatidylethanolamine was labelled with FITC, RITC or XRITC using the protocol outlined by Fung and Stryer [27]. Thus, for example, egg phosphatidylethanolamine was labelled with RITC by incubation of RITC (110 mg) and lipid (100 mg) in chloroform (1.5 ml) containing triethylamine (0.1 ml) at room temperature in the dark. Additional RITC (5 mg) was added at 30-min intervals until small samples taken from the incubation mixture no longer contained unreacted phosphatidylethanolamine, as indicated by a lack of staining with ninhydrin. The reaction mixture was then dried in vacuo, redissolved in $CHCl_3$ and purified on preparative silica gel plates using first methanol/chloroform/water (65 : 35 : 4, v/v) and then acetone/water (85 : 15, v/v). R_F values for the labelled lipids developed with methanol/chloroform/water (65 : 35 : 4, v/v) ranged between 0.4 and 0.6.

Labelled ATPase was reconstituted with the desired amount of exogenous lipid (dioleoylphosphatidylcholine plus labelled lipid) by solubilisation in detergent, followed by rapid dilution into buffer [22]. In some experiments, samples were subjected to freeze-thaw treatment [28] after dilution into buffer to ensure mixing of lipid and protein. Fluorescence measurements were made in buffer (40 mM Hepes, 100 mM NaCl, and 1 mM EGTA, pH 7.2) by using either Spex Fluorolog or Perkin-Elmer MPF44A or 650-70 fluorimeters.

Ammonium vanadate was dissolved in KOH (100 mM) to give a 100 mM stock solution and was added to the fluorescence samples to a final concentration of 100 μM.

Analysis of fluorescence energy transfer

A number of theoretical approaches have been presented for the analysis of fluorescence energy transfer in membrane systems [27,29–31] but here we will use the method described by Gutierrez-Merino [32,33].

The extent of quenching of donor fluorescence by Forster energy transfer to an ensemble of acceptors can be expressed as

$$(F_0 - F)/F_0 = \langle E \rangle = (\langle k \rangle / k_0)/\{1 + (\langle k \rangle / k_0)\} \quad (1)$$

where F_0 and F are fluorescence intensities for the donor in the absence and presence of acceptor, respectively, $\langle k \rangle$ is the average rate of Forster energy transfer for the particular distribution of acceptors and k_0 is the rate of energy transfer for a donor-acceptor pair separated by the distance R_0 at which the efficiency of energy transfer $\langle E \rangle$ is 50%.

It has been shown previously [17,32] that the average rate of Forster energy transfer $\langle k \rangle$ in a two-dimensional membrane system can be written as

$$\langle k \rangle = \sum_{i=1}^{n} k_i \quad (2)$$

where the summation extends over all donor-acceptor pairs of the ensemble, and where the rate of transfer for pair i separated by a distance r_i is k_i, provided that the diffusion rate of donor and acceptor is much slower than the lifetime of the excited state of the donor. Elsewhere it has been shown that this expression can be used to analyse the relative positions of lipids and proteins in mixtures [32,33]. The averaging process described by Eqn. 2 for lipid acceptors around a donor covalently attached to a protein can be readily and efficiently computed, because the sixth-power dependence of the efficiency of energy transfer on the distance between donor and acceptor ensures that contributions from acceptor molecules in successive shells of lipid molecules around the protein rapidly become insignificant [32]. Here we extend the analytical approach described by Gutierrez-Merino [32,33] to calculate the average rate of Forster energy transfer as a function of the position of a protein-bound donor with respect to acceptor molecules located in lipid polar headgroups. The approach rests on a number of basic assumptions. First, it is assumed that the number of acceptor molecules per membrane is much greater than the number of donor molecules. Second, it is assumed that the distribution of lipid and protein molecules in the surface of the membrane can be pictured as a continuum of discs of different sizes as in Gutierrez-Merino [33], Lee [34] and East et al. [35]. Third, we assume a random mixing of labelled and unlabelled lipids with no specific interaction with the protein.

At very high molar ratios of lipid to protein, protein molecules can be considered to be effectively isolated within the membrane and surrounded by a series of shells of phospholipid molecules. For R_0 values of 40–60 Å, significant energy transfer will occur from a central protein donor to a lipid acceptor in the first 8–10 shells of lipid, beyond which energy transfer becomes insignificant. At lower molar ratios of lipid to protein, however, where protein-protein distances will be shorter than the distance to the 10th shell of lipid some of the space within the 8–10 significant shells of lipid around a central protein molecule will be occupied by other protein molecules (Fig. 1). To estimate the possible importance of this factor, we calculate the average protein-protein separation d_{av} at the given molar ratio of lipid to protein, assuming lipid, r_l, and protein, r_p, radii of 5 and 25 Å, respectively. We then place all protein molecules at this average separation, which is equivalent to assuming an hexagonal lattice for the protein molecules. For the first j shells where no space in the lipid shells is occupied by adjacent protein molecules we can write Eqn. 2 as

$$\langle k \rangle = \sum_j n_j k_j \text{ for } j < (d_{av} - 2r_p)/2r_l \quad (3)$$

where the summation is carried out over the j shells of lipid around the protein and the number of lipid molecules in the jth shell is n_j. For the following shells where space will be occupied by adjacent protein molecules, the equation is modified to

$$\langle k \rangle = \sum_j n_j (1 - \alpha_j) k_j \text{ for } (d_{av} - 2r_p)/2r_l < j < d_{av}/2r_l \quad (4)$$

where the parameter α_j accounts for occupation of space by the adjacent protein molecules. For subsequent shells, $\langle k \rangle$ is calculated using Eqn. 3.

The number of lipid molecules in the jth shell is calculated as:

$$n_j = \frac{\pi\{r_p + (2j-1)r_l\}}{r_l} \quad (5)$$

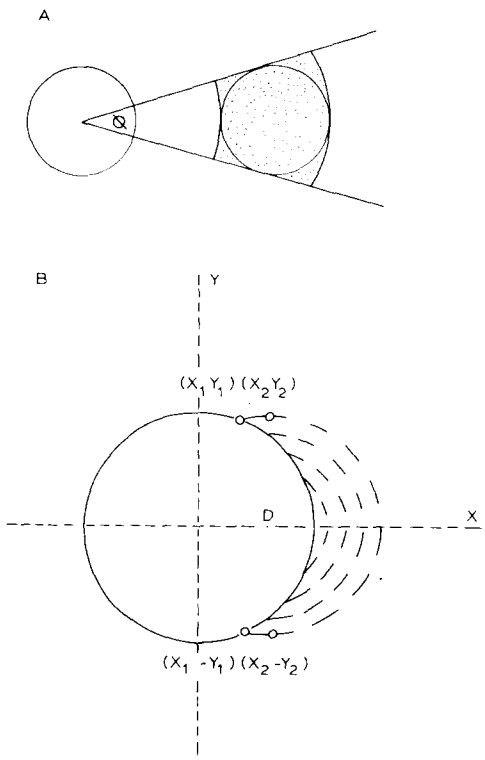

Fig. 1. (a) Illustration of the method used to calculate the space (shaded) considered to be occupied by a neighbouring protein in the lipid shells surrounding a central protein A. (b) Illustration of the method used to calculate the crossing points for lipid shells centred on the donor D. The dotted circles pass through the centres of the lipid molecular cross sections in a given j shell. The solid line represents the protein molecular cross-section.

where r_p and r_l are the cross-sectional radii of protein and lipid molecules, respectively.

The factor α_j is calculated geometrically as (Fig. 1)

$$\alpha_j = 6\{\arcsin[r_p/(d_{av})]/\pi\} \tag{6}$$

where

$$\alpha_j = 6\phi_j/2\pi \tag{7}$$

where ϕ_j is the solid angle illustrated in Fig. 1. We stress that this approach was used only to obtain an estimate of the lipid/protein ratio beyond which protein molecules can be considered to be effectively isolated within the membrane. These calculations were performed assuming that the ATPase was monomeric, but similar results would be obtained for a dimer. As described below, the experimental measurements were made at lipid/protein molar ratios very considerably greater than the lower limits estimated here for effectively isolated molecules.

We characterise the position of the probe within the protein by a height h of the probe above the plane of the lipid/water interface and a distance d between the probe and the circumference of the protein (Fig. 2). Energy transfer is then calculated using Eqn. 2 in terms of transfer to shells of lipid centred at the donor group (Fig. 1b). To calculate the number of lipids in each of the donor-centred shells, the points of intersection of each lipid shell with the circumference of the protein (points X_1, Y_1 and X_1, $-Y_1$ in Fig. 1b) were calculated numerically. Since we are dealing with lipids of discrete size, we have introduced cut-off points in the calculations (points X_2, Y_2 and $X_2, -Y_2$ in Fig. 1b) where

$$|X_1 - X_2| \geq 2.5(2j-1)$$

for the jth lipid shell. The factor $(2j-1)$ was introduced to account approximately for the different abscissa projection of lipids in different j shells at the intersection points. Finally, for protein and lipid diameters, as used here, the factor α_j

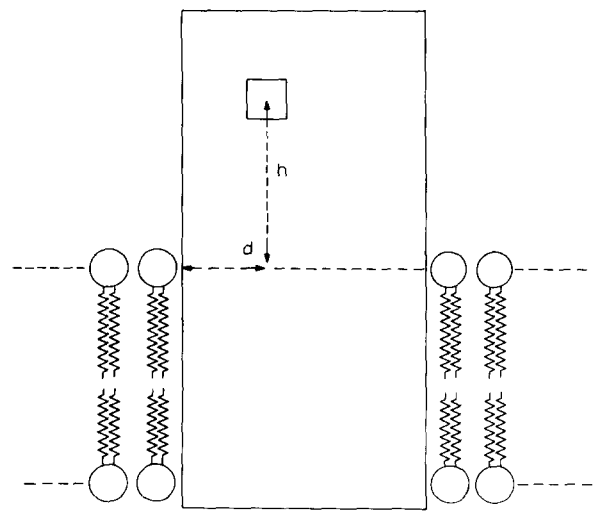

Fig. 2. Cross-sectional view of the ATPase, showing the parameters d and h used in the calculations.

(Eqn. 6) can be put equal to the values calculated for protein-centred cells.

The distance R_0 is calculated as [45,46]

$$R_0 = 9.765 \cdot 10^3 (K^2 \cdot J \cdot Q_D \cdot n^{-4})^{1/6} \quad (8)$$

where J is the overlap integral (cm^6/mol), Q_D is the donor quantum yield in the absence of acceptor and n is the refractive index of the medium. K^2 is an orientation factor and accounts for the relative orientation of the donor emission and acceptor absorption transition dipoles. For isotropic motions of the dipoles, $K^2 = 2/3$.

An estimate of the deviation of K^2 from 2/3 can be made from polarisation measurements. If just motion of the donor is considered, then it can be shown [12,36] that the actual separation of donor and acceptor r can be related to the apparent value r' calculated assuming $K^2 = 2/3$ by the equation

$$r = \alpha r' \quad (9)$$

where α has values in the limits

$$[0.75(1 - \cos^2\theta)]^{1/6} \leqslant \alpha < (6 \cos^2\theta)^{1/6} \quad (10)$$

and θ is the semiangle of a cone in which the donor is supposed to be rapidly rotating. The angle θ can be calculated from the emission anisotropies A_0 and A_1 of the donor in the absence and presence of rotational motion

$$A_1/A_0 = \cos^2\theta(1 + \cos^2\theta)/4 \quad (11)$$

Motion of the acceptor as well as the donor will, of course, reduce the deviation of K^2 from 2/3 even further. Thus, in general, it has been found that $K^2 = 2/3$ fits the data well for membrane systems [23,32,33].

As discussed by Moog et al. [37] the refractive index n is put equal to that of a dilute aqueous solution (1.33).

Results

The efficiency of fluorescence energy transfer will depend on the surface density of acceptors, on the lipid/protein ratio and on the position of the protein-bound donor with respect to the lipid headgroup region. Assuming a cylindrical shape for the protein molecule we define the position of the donor by an effective distance from the circumference of the protein (d) and its height (h) above the lipid/water interface (Fig. 2). Calculations of energy transfer efficiency as a function of lipid/protein ratio for fixed positions of the donor and a fixed molar ratio of donor to acceptor, illustrate the effect of occupation of space around one protein molecule by other protein molecules (Fig. 3). At molar ratios of lipid/protein below approx. 100 : 1 such effects become very important, so that in this region the exact details of the distribution of the protein molecules become important. For lipid/protein molar ratios greater than 200 : 1, effects of neighbouring protein molecules are very small. We have, therefore, chosen to work at a molar ratio of lipid/protein of 300 : 1.

The effect of the position of the donor molecule within the protein is illustrated in Fig. 4. Not surprisingly, the effects of varying h and d (Fig.

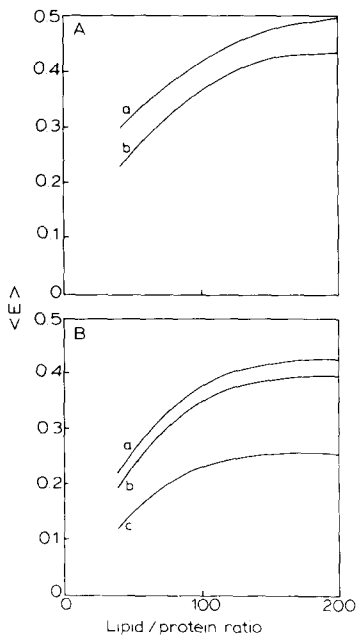

Fig. 3. Calculated energy transfer efficiency $\langle E \rangle$ as a function of molar ratio of lipid to protein, at an acceptor surface density (molar ratio) of 0.01 for: (A), $h = 0$ Å and d values (Å) of: A, 10; B, 20; and (B), $d = 20$ Å and h values (Å) of: A, 0; B, 20; C, 40; all with $R_0 = 50$ Å.

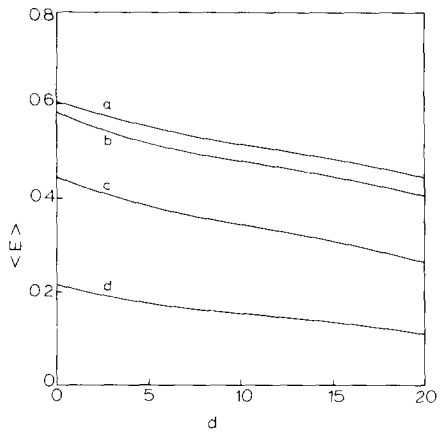

Fig. 4. Calculated energy transfer efficiency $\langle E \rangle$ as a function of d (Å) for a molar ratio of lipid to protein of 300:1, at an acceptor surface density (molar ratio) of 0.01 with $R_0 = 50$ Å, and h values (Å) of: A, 0; B, 20; C, 40; D, 60.

2) are different. Fig. 5 illustrates the effect of changing the surface density of acceptors, keeping the total molar ratio of lipid to protein constant.

Values of R_0 were calculated from absorption and corrected emission spectra, and the spectroscopic constants are summarised in Table I. The quantum yield of FITC-ATPase was determined as 0.65, based on a value of 0.85 for FITC [38].

The values of R_0 and the method of calculation of energy transfer were checked by measuring energy transfer between phosphatidylethanola-

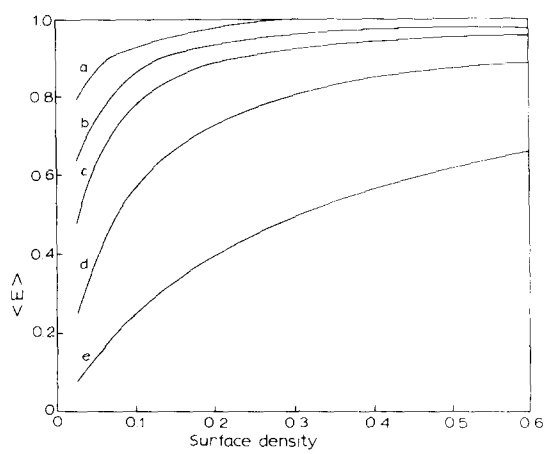

Fig. 5. Calculated energy transfer efficiency $\langle E \rangle$ as a function of the acceptor surface density (molar ratio) at a molar ratio of lipid to protein of 300:1 with $R_0 = 50$ Å, with d and h values (Å) of: A, 0,0; B, 20,20; C, 30,30; D, 40,40; E, 100,0.

TABLE I

FLUORESCENCE PROPERTIES OF ACCEPTOR MOLECULES

Acceptor	Extinction coefficient [a] ($M^{-1} \cdot cm^{-1}$)	Overlap integral [b] J (cm^6/mol)	R_0 [b] (Å)
RITC – PE	$6.83 \cdot 10^4$	$2.68 \cdot 10^{-13}$	55.2
XRITC-PE	$5.29 \cdot 10^4$	$1.86 \cdot 10^{-13}$	52.0

[a] Measured at wavelength of maximum absorption.
[b] From FITC-labelled ATPase, using $Q_D = 0.65$, $n = 1.33$ and $K^2 = 0.67$.

mine labelled with FITC (FITC-PE) and with RITC (RITC-PE). As shown in Fig. 6, there is efficient energy transfer between the fluorescein and rhodamine groups in this system. Since the fluorescence intensity measured at 517 nm derives only from the fluorescein fluorophore when fluorescence is excited at 475 nm, fluorescence intensities measured at this wavelength in the presence of the rhodamine derivative give directly the extent

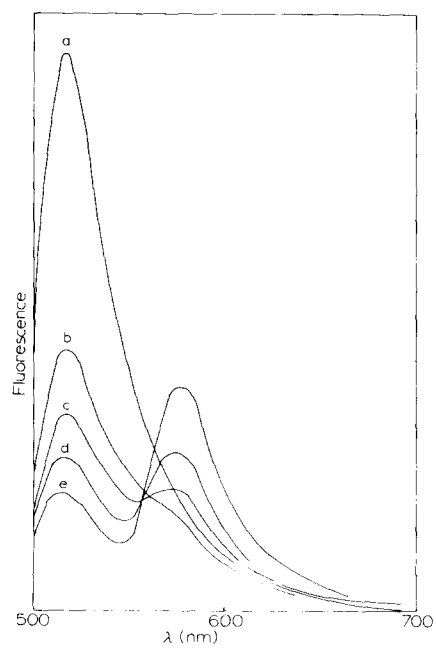

Fig. 6. Fluorescence emission spectra showing energy transfer from FITC-PE to RITC-PE in vesicles of dioleoylphosphatidylcholine containing a mole fraction of RITC-PE of (a) 0, (b) 0.004, (c) 0.012, (d) 0.024, (e) 0.08. The total lipid concentration was 200 μM and the concentration of FITC-PE 0.2 μM. Fluorescence excited at 475 nm.

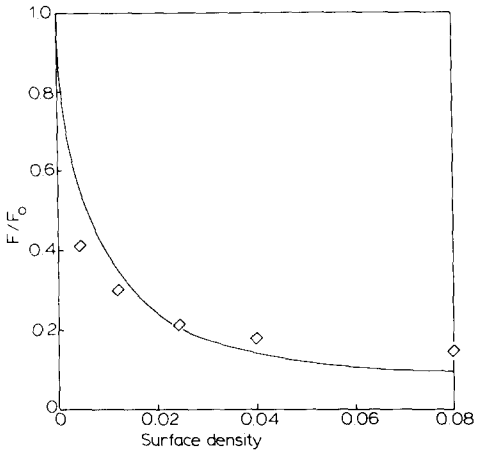

Fig. 7. Fluorescence quenching (F/F_0) of FITC-PE as a function of the mole fraction of RITC-PE in vesicles of dioleoylphosphatidylcholine. Points, experimental data taken from Fig. 6. Solid line, theoretical calculation for $R_0 = 55.2$ Å.

of fluorescein fluorescence quenching. Fig. 7 shows a plot of fluorescence quenching as a function of the molar ratio of acceptor in vesicles of dioleoylphosphatidylcholine. The good fit between experiment and theory shows that the assumption that $K^2 = 2/3$ is valid for this system.

Fig. 8 shows that quenching of fluorescein fluorescence by RITC-PE is considerably less efficient when the fluorescein fluorophor is located on the ATPase than when it is on a phospholipid. This immediately shows that the fluorescein fluorophore on the ATPase must be located a considerable distance from the membrane surface (Fig. 5). When the protein donor is located far from the membrane surface a wide range of pairs of d and h values will give equally good fits to the data (see Fig. 4). Fig. 9 illustrates a fit of the quenching of

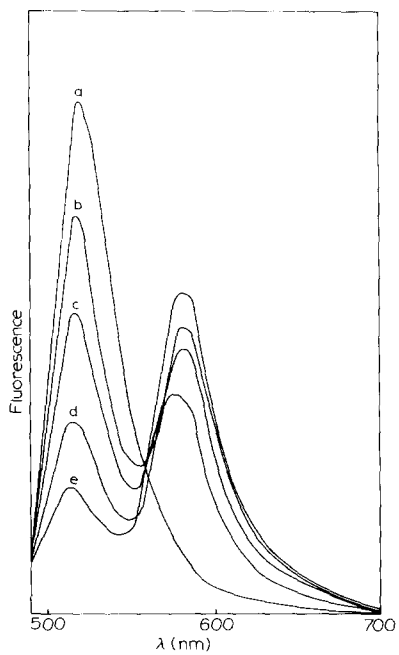

Fig. 8. Fluorescence emission spectra showing energy transfer from FITC-ATPase to RITC-PE in reconstituted systems containing dioleoylphosphatidylcholine and a mole fraction of RITC-PE of (a) 0, (b) 0.05, (c) 0.1, (d) 0.3, (e) 0.5. The molar ratio of total lipid to ATPase was 300:1 and the FITC:ATPase ratio 0.5:1. Fluorescence excited at 475 nm.

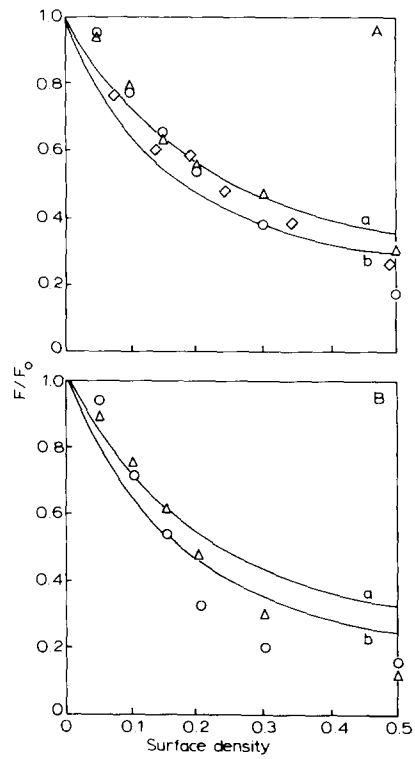

Fig. 9. Fluorescence quenching (F/F_0) of FITC-ATPase reconstituted into lipid mixtures at a molar ratio of total lipid:protein of 300:1, as a function of the mole fraction of (A) RITC-PE or (B) XRITC-PE in the lipid mixture. Points, experimental: (○) and (◇) ATPase samples labelled at a molar ratio of FITC:ATPase of 0.5:1 at pH 8 and (△) ATPase labelled at a molar ratio of FITC:ATPase of 0.5:1 at pH 7.45, excess FITC being removed by column centrifugation (see Methods). Solid lines, theoretical simulations with the R_0 values given in Table I and d and h values (Å) of (A): (a) (0,107), (25,93) or (45,82); (b) (0,100), (25,87) or (45,75) and (B): (a) (0,100), (25,87) or (45,73); (b) (0,92), (25,77) or (45,67).

the fluorescein fluorescence of FITC-ATPase by RITC-PE and XRITC-PE to the theoretical model developed above assuming pairs of values of d and h (Å) of (0,100), (25,87) or (45,75) and (0,107), (25,93) or (45,82) for the RITC-PE system and (0,100), (25,87) or (45,73) and (0,92), (25,77) or (45,67) for the XRITC-PE system.

It is noticeable in Fig. 8 that the increase in intensity of rhodamine fluorescence with increasing concentrations of RITC-PE is less than expected presumably due to extensive concentration quenching at the high molar ratios of RITC-PE employed.

Addition of vanadate ion (100 μM) to FITC-ATPase reconstituted with lipid mixtures containing RITC-PE causes no significant change in the fluorescence quenching profile from that shown in Fig. 9.

The fluorescence polarisation of FITC-ATPase is 0.22, compared to a value of 0.45 for FITC labelled glycine in glycerol at room temperature. These values define the limits of α (Eqn. 10) as being between 0.7 and 1.3 with an 80% probability of being between 0.75 and 1.15, in the absence of rotation of the acceptor molecule: the range of α values will be narrower than this in the presence of acceptor motion.

Discussion

It has been shown that fluorescein isothiocyanate selectively labels the ATP binding site of the purified $(Ca^{2+} + Mg^{2+})$-ATPase presumably in the adenine binding region since labelling is totally inhibited by ATP [19,20,44]. The modified lysine is close to the middle of the protein sequence, being at position 515 [10]. Since it is possible to place a fluorescence donor group specifically in this region of the protein, it is possible to use fluorescence energy transfer measurements to locate the position of this region of the protein in space. We choose to use phospholipid molecules labelled in the head group region as fluorescence acceptors, and so measure the distance between the ATP binding site and the lipid/water interface.

A comparison of the mole fractions of lipid acceptors needed to quench the fluorescence of FITC-labelled lipid (Fig. 6) and FITC-labelled ATPase (Fig. 8) immediately indicates that the ATP binding site on the ATPase must be located far away from the lipid/water interface region of the bilayer. In order to estimate what this distance is, it is necessary to set up a geometrical description of the membrane. Here we introduce a very efficient algorithm that allows the calculation of energy transfer in two-dimensional systems [32,33]. As shown in Fig. 7, the algorithm can be used to calculate the energy transfer between lipid donors and acceptors and can also be used to reproduce the energy transfer measurements of Fung and Stryer [27] (not shown). The calculations show that for energy transfer measurements in the ATPase system at molar ratios of lipid to protein greater than approx. 150 : 1, the ATPase molecules can be pictured as effectively isolated within the membrane (Fig. 3): to be on the safe side we performed our experiments at a lipid : protein molar ratio of 300 : 1. Assuming a cylindrical shape for the ATPase (monomer or dimer), the position of the ATP binding site with respect to the lipid/water interface can be defined by a height h of the site above the plane of the lipid-water interface and a distance d between the site and the circumference of the ATPase (Fig. 2). The experimental data for the quenching of FITC-ATPase by RITC-PE and XRITC-PE can be fitted to such a model, although agreement between the experimental data and the simulations is rather poor at high mole fractions of the labelled lipid, particularly in the case of the XRITC-PE system (Fig. 9B). We believe that this can be attributed to perturbation of the membranes at high mole fractions of these labelled lipids. It has been shown that the fluorescence properties of phosphatidylethanolamine labelled with the dansyl group in the headgroup region are consistent with a conformation in which the dansyl group is folded back and penetrates into the bilayer [39]. It is likely that RITC-PE and XRITC-PE will also adopt such a conformation, particularly with the more hydrophobic XRITC group.

Because the donor group is located far from the lipid/protein interface, a range of possible pairs of d and h values will fit the data (Fig. 9). A choice between these possible pairs of values can be made on the basis of structural information about the ATPase obtained from 3D image recon-

struction of electron micrographs of negatively stained preparations [40]. In the presence of vanadate ions, the ATPase is dimeric, with the hydrophilic portion of the ATPase dimer extending approx. 60 Å above the surface of the membrane (as defined by a minimum penetration of negative stain) with a diameter of approx. 90 Å [40]. The ATPase is also believed to be dimeric in the absence of vanadate [41]. The phosphatidylcholine headgroup extends approx. 15 Å from the glycerol backbone region in crystals of the lipid [42] so that if it is assumed that the negative stain is excluded from the lipid headgroup region, then the hydrophilic portion of the ATPase extends approx. 75 Å above the glycerol backbone region of the bilayer. If the ATPase were monomeric, with a radius of approx. 25 Å then the minimum height of the ATP site above the glycerol back bone region to fit our data would have to be approx. 77–97 Å, with the ATP site located along the central axis of the ATPase. If the ATPase were dimeric with a radius of 45 Å, then the minimum height of the ATP site above the glycerol back bone region to fit our data would have to be approx. 60–80 Å, now with the ATP site located along the central axis of the ATPase dimer. Formation of larger aggregates (e.g. tetramers) would have relatively little further effect on the calculated distances. Our data is, therefore, consistent with a dimeric model for the ATPase, with the ATP site being located at close to the maximum possible height above the plane of the lipid bilayer and close to the protein/protein interface of the dimer. This model assumes that the ATPase dimer has two-fold rotational symmetry, as indicated by the image reconstruction techniques [40]. A location for the ATP binding site close to the protein/protein interface in dimers or aggregates has been suggested in Ref. 43.

In our distance calculations, we have used a value of 0.67 for the orientational parameter K^2. From Eqn. 10, the fluorescence polarisation data for FITC-ATPase shows that this assumption will introduce little error.

The kinetics of the ATPase have been interpreted in terms of a two-conformation model, with one conformation E_1, having Ca^{2+} binding sites of high affinity exposed to the outer surface of the sarcoplasmic reticulum and the other conformation, E_2, having Ca^{2+} binding sites of low affinity exposed to the inner surface: the conformation change between E_1 and E_2 is the transport event (see Ref. 23). Vanadate ions bind to the ATPase and to the ATPase labelled with FITC and have been proposed to stabilise the ATPase in the E_2 conformation, presumably acting as an analogue of orthophosphate [44]. The fluorescence quenching profile of FITC-ATPase reconstituted with lipid mixtures containing RITC-PE is identical in the presence and absence of vanadate (100 μM), suggesting that the ATP binding site does not move significantly with respect to the lipid/water interface in the E_1-E_2 conformational transition.

Scott [16] has used fluorescence energy transfer between the fluorescein group and bound lanthanides to estimate the distance between the ATP and Ca^{2+} binding sites. The efficiency of transfer was low and the estimated distance between the sites was 47 Å [16]. We have also observed a low efficiency of transfer between fluorescein and bound lanthanides for both vesicles of sarcoplasmic reticulum and for purified ATPase (Levinson and Lee, unpublished observations). The Ca^{2+}-ATP site distance is comparable to half the estimated diameter of the hydrophilic portion of the ATPase dimer. With the two ATP binding sites located close to the protein/protein interface of the dimer, the Ca^{2+} binding sites will then most probably be located at the other extremes of the protein. The two Ca^{2+} binding sites in each monomer have been estimated to be relatively close together, with a separation of approx. 11 Å [16].

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