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Localization of the hinge region of the Ca^{2+}-ATPase of sarcoplasmic reticulum using resonance energy transfer

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Abstract

The Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum can be labelled at Cys-670 and Cys-674 with 5-[[2-[(iodoacetyl) amino]ethyl]amino]napthalene-1-sulphonic acid (IAEDANS). Resonance energy transfer has been used to measure the distance between Cys-670/Cys-674 and Glu-439 labelled with 5-(bromomethyl)fluorescein as 40 Å. The height of Cys-670/Cys-674 above the phospholipid/water interface has been measured by resonance energy transfer between IAEDANS-labelled ATPase and fluorescein-labelled phosphatidylethanolamine as 54 Å. This locates the hinge region of the ATPase close to the mouth of the pore observed in the cytoplasmic region of the ATPase in electron micrographs. No significant changes in these distances can be detected by resonance energy transfer on binding Ca^{2+} or vanadate. The height of the IAEDANS label above the phospholipid/water interface is the same for bilayers of dimyristoleoylphosphatidylcholine and dioleoylphosphatidylcholine. Conformation changes on the Ca^{2+}-ATPase appear to be localised to small regions of the ATPase.

Key words: ATPase, Ca^{2+}-; Fluorescence; Resonance energy transfer; Sarcoplasmic reticulum; Cysteine; Covalent modification

1. Introduction

The structure proposed for the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SR) consists of two main cytoplasmic regions connected by a narrow stalk to 10 transmembrane α-helices [1]. It has been suggested that the largest of the cytoplasmic regions, between transmembrane helices 4 to 5, contains three domains. On its N-terminal side is the phosphorylation domain, containing the residue (Asp-351) phosphorylated by ATP. In the central region is the nucleotide binding domain, containing the residue (Lys-515) labelled by fluorescein isothiocyanate. On its C-terminal side is a relatively small domain, beginning with Cys-675 and ending at about Asn-739, referred to as the hinge domain since it is thought that it might be involved in the relative movement of the phosphorylation and nucleotide binding domains, as in several kinases. The hinge domain is one of the most highly conserved regions in the P-type ATPase, with three conserved residues between residues 675 and 683, and 12 conserved residues between 701 and 719, found in all known P-type ATPase sequences [2] including the cta3 protein of yeast [3], believed to be an endoplasmic reticulum Ca^{2+}-ATPase.

Chemical labelling studies with analogues of ATP have suggested that the hinge region is associated with the ATP binding site. Adenosine triphosphopyridoxal labels Lys-684 in the presence of Ca^{2+} and both Lys-684 and Lys-492 in the absence of Ca^{2+} [4,5], consistent both with a change in the relative positions of Lys-684 and Lys-492 on binding Ca^{2+} and with a location for these two residues in the ATP binding site [5]. Lys-492 and Arg-678 have been reported to be cross linked by glutaraldehyde [6], each in an ATP-protectable manner, consistent with Arg-678 and Lys-492 being part of the ATP binding site. Ohta et al. [7] have reported that 5′-*p*-fluorosulfonyl benzoyladenosine labels a lysine in the Na^+/K^+-ATPase equivalent to Lys-712 in the Ca^{2+}-ATPase, suggesting this Lys is also in the ATP

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Abbreviations: BrF, 5-(bromomethyl)fluorescein; IAEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulphonic acid; FITC, fluorescein isothiocyanate; PE, dioleoylphosphatidylethanolamine; SR, sarcoplasmic reticulum; di(14:1)PC, dimyristoleoylphosphatidylcholine; di(18:1)PC, dioleoylphosphatidylcholine.

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binding site. Two Asp residues in the Na^+/K^+-ATPase, conserved residues equivalent to Asp-703 and Asp-707 in the Ca^{2+}-ATPase are labelled by adenosine 5'-[*N*-[4-[*N*-(2-chloroethyl)-*N*-methylamino]benzyl]-γ-amidotriphosphate], so that these residues are also presumably part of the ATP binding site [8]. Antipeptide antibodies raised to peptides 659–668 and 731–749 bound to the native ATPase suggesting surface exposure for the two ends of the hinge region [9]. Although antipeptide antibodies raised to peptides 671–682 and 693–704 failed to bind to either native or denatured ATPase, they did bind to their respective peptides, suggesting the possibility of an unusually stable structure in the centre of the hinge region [9]. A number of residues in the hinge region have been mutated and these experiments suggest that the region does not play a critical role in nucleotide binding. Rather, it has been suggested that the hinge domain may constitute an integral part of the phosphorylation domain and be involved in the catalytic events in the phosphorylation site [10].

We have used the technique of resonance energy transfer to locate the positions of defined residues on the ATPase both with respect to other residues and with respect to the lipid/water interface [11–14]. It has been reported that the major sites of labelling of the ATPase with 5-[[2-[(iodoacetyl)amino]ethyl]amino] napthalene-1-sulphonic acid (IAEDANS) are at Cys-670 and Cys-674 [15,16]. Here we use fluorescence methods to locate these residues in the three dimensional structure of the ATPase.

2. Materials and methods

Lipids were obtained from Avanti Polar Lipids, fluorescein isothiocyanate (FITC) from Sigma, 5-(bromomethyl)fluorescein (BrF) from Molecular Probes and IAEDANS from Aldrich. Sarcoplasmic reticulum from rabbit skeletal muscle and the purified Ca^{2+}-ATPase were prepared as described in East and Lee [17]. Dioleoylphosphatidylethanolamine (PE) was labelled with FITC and purified on preparative silica gel plates as described [12]. ATPase was labelled with FITC as described in Mata et al. [13] and with BrF as described in Stefanova et al. [14].

SR or purified ATPase was labelled with IAEDANS by incubation in buffer (50 mM Tris-HCl (pH 7.0), 0.2 M sucrose) at 25°C in the dark for up to 3 h. Unbound label was removed by centrifugation through a column of Sephadex G-50. Concentrations of bound label were estimated from the absorbance recorded in 1% SDS (pH 7.2), using an extinction coefficient ϵ_{340} of 6100 $M^{-1}\,cm^{-1}$ [18]. SR was doubly labelled with IAEDANS and BrF by incubating SR in 50 mM Tris-HCl (pH 7.2), 0.2 M sucrose at molar ratios IAEDANS/BrF/ATPase of 3:4:1 for 2 h in the dark, followed by removal of unbound label by passage through two columns of Sephadex G-50. Concentrations of bound BrF were estimated from the absorbance recorded in 1% SDS, 5 mM KOH, using an extinction coefficient ϵ_{495} of 67000 $M^{-1}\,cm^{-1}$ [14]. Concentrations of ATPase were estimated from the absorbance at 280 nm using the extinction coefficient given by Hardwicke and Green [19]. Ammonium vanadate was dissolved in KOH (100 mM) to give a 100 mM stock solution and added to the required final concentration.

Labelled ATPase was reconstituted with exogenous phospholipid (dioleoylphosphatidylcholine (di(18 : 1) PC) plus FITC-PE) as described in Mata et al. [13]. Fluorescence spectra were recorded at 25°C using an SLM-Aminco 8000C fluorimeter, with an excitation wavelength of 337 nm. Spectra were corrected for the inner filter effect using the equation

$$F_\lambda = F_\lambda^o 10^{-A}$$

where F_λ and F_λ^o are the observed and corrected fluorescence intensities, respectively, at wavelength λ, and A is given by $\epsilon_\lambda cl$ where ϵ_λ is the extinction coefficient of FITC-PE at wavelength λ, c is the concentration of FITC-PE and the pathlength l is taken as 0.5 cm. Composite emission spectra were fitted to linear sums of the emission spectra of IAEDANS-labelled ATPase and FITC-PE, according to

$$F_\lambda = w_D D_\lambda + w_A A_\lambda$$

where F_λ, D_λ and A_λ are the fluorescence intensities of the composite system, the donor and the acceptor, respectively, at the wavelength λ and w_D and w_A are the weightings of donor and acceptor, respectively, that give the best fit to the composite spectrum. Spectra were fitted over the wavelength range 400–490 nm covering the IAEDANS emission.

The efficiency of resonance energy transfer E from a donor to an acceptor can be written as:

$$E = 1 - (F/F_o)$$

where F and F_o are fluorescence intensities of the donor in the presence and absence of acceptor, respectively. For the ATPase labelled at single sites by donor and acceptor groups the efficiency of transfer is related to the distance r between the sites by:

$$E = 1/\left(1 + r^6/R_o^6\right)$$

where R_o is the distance at which energy transfer is 50% efficient. R_o (Å) is given by

$$R_o = (9.79 \cdot 10^3)\left(\kappa^2 n^{-4} Q J\right)^{1/6}$$

where κ^2 is the orientation factor, n the refractive index, taken to be 1.33, Q the quantum yield of IAEDANS-ATPase equal to 0.54 [20], and J the spectral overlap integral in units of cm^6/mol. The value of κ^2

has been set to 2/3, the value for rapid isotropic motion. This has been shown to be appropriate for conformationally flexible sites and for probes of mixed polarization [21]. The measured polarization of IAEDANS-ATPase fluorescence emission is low (0.186), indicating a conformationally flexible site [15]. An error in distance measurement of less than 10% has been estimated when the polarization of the donor or acceptor is less than 0.3 [47]. The value of the overlap integral for the IAEDANS-ATPase/BrF-ATPase pair at pH 7.2 has been measured as $1.15 \cdot 10^{-13}$ cm^6 mol^{-1} giving an R_0 value of 47.4 Å. For the IAEDANS-ATPase/FITC-PE pair the overlap integral has been measured as $8.3 \cdot 10^{-14}$ cm^6 mol^{-1} giving an R_0 value of 45 Å; this is significantly less than for the IAEDANS-ATPase/FITC-ATPase pair ($R_0 = 49$ Å [20]) because of the lower extinction coefficient for FITC-PE in lipid bilayers at pH 7.2 than for FITC-ATPase.

Analysis of resonance energy transfer between IAEDANS-ATPase and FITC-PE needs to take into account the distribution of the fluorescence acceptors in the plane of the membrane. Three models have been used and found to give comparable results [13,14]. The approaches of Koppel et al. [22] and Dewey and Hammes [23] treat the FITC-PE as being distributed randomly on a plane a distance h below the position of the donor on the protein. The method of Gutierrez-Merino et al. [11] considers the ATPase as a cylinder with the position of the label on the ATPase being characterised by its height h above the phospholipid/water interface and the distance d between the site and the circumference of the protein. In the calculations of surface distributions, surface areas of 80 and 1963 Å^2 were assumed for phospholipid and protein molecules, respectively [12].

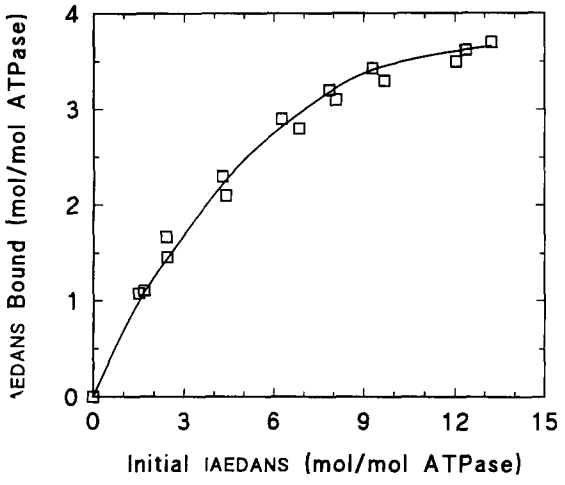

Fig. 1. Stoichiometry of labelling of the Ca^{2+}-ATPase with IAEDANS. The Ca^{2+}-ATPase (3 mg protein/ml) was incubated with IAEDANS at the given molar ratio in 50 mM Tris-HCl (pH 7.2), 0.2 M sucrose, for 3 h at 25°C, and unreacted IAEDANS separated on Sephadex G-50. The molar ratio of bound IAEDANS to ATPase was determined by absorbance measurements.

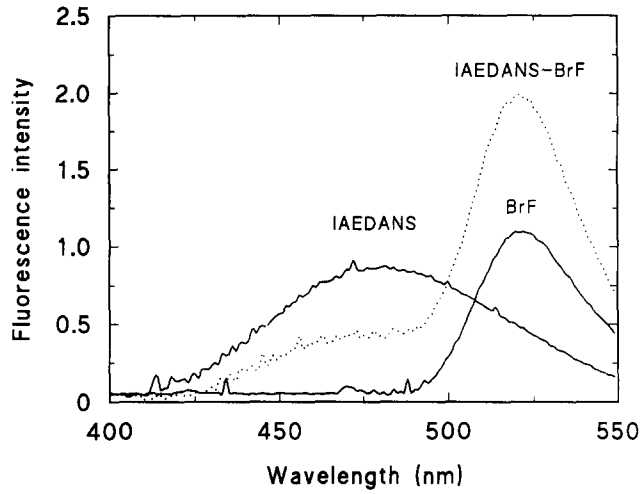

Fig. 2. Fluorescence emission spectra of SR showing energy transfer from IAEDANS to fluorescein on the Ca^{2+}-ATPase. Shown are the emission spectra for the SR labelled with IAEDANS (molar ratio IAEDANS/ATPase 0.9:1) and with BrF (molar ratio BrF/ATPase 0.75:1) and the doubly labelled ATPase (molar ratio IAEDANS/BrF/ATPase 0.75:0.7:1). The spectra for IAEDANS-ATPase and BrF-ATPase are shown at probe concentrations of IAEDANS and BrF, respectively, equal to those in the doubly labelled species.

3. Results

There is disagreement in the literature about the level of labelling obtained when the Ca^{2+}-ATPase is incubated with excess IAEDANS at neutral pH [16,18,20,24]. As shown in Fig. 1 we find that after 3 h incubation at pH 7.2 the labelling ratio increases with molar ratio of added IAEDANS to ATPase, a maximal labelling ratio of approx. 3.7 mol IAEDANS/mol ATPase being obtained with a 15-fold excess of label. Labelling ratios have been expressed in terms of total protein, since experiments were performed with purified Ca^{2+}-ATPase which has been shown by sodium dodecyl-sulphate polyacrylamide gel electrophoresis to be essentially pure [25]. On this basis, labelling ratios of 1:1 were obtained with other labels such as FITC [26] and 4-(bromomethyl)-6,7-dimethoxycoumarin [27] even though the maximal level of phosphorylation of the Ca^{2+}-ATPase was observed to be approx. 0.5 mol [EP]/mol ATPase [28]. At a 2:1 molar ratio of added IAEDANS to ATPase, we obtain a labelling ratio of 1:1 (Fig. 1) in agreement with previous studies [16,18,24].

The Ca^{2+}-ATPase can be labelled to a 1:1 molar ratio with BrF at Glu-439 [14]. Fig. 2 shows the fluorescence emission spectra of SR labelled with IAEDANS and BrF and doubly labelled with IAEDANS and BrF.

Table 1
Resonance energy tranfer between IAEDANS and BrF on the doubly labelled ATPase

Conditions	F/F_0	E	r (Å)
5 mM Mg^{2+}	0.49	0.73	40
5 mM Mg^{2+} + 1 mM vanadate	0.49	0.73	40
0.7 mM Ca^{2+}	0.52	0.69	41
$C_{12}E_8$ [a]	0.57	0.65	43

The efficiency of resonance energy transfer was measured by the quenching of IAEDANS fluorescence for the ATPase doubly labelled with IAEDANS and BrF to molar ratios of IAEDANS/BrF/ATPase of 0.75:0.70:1 (except for the experiment with $C_{12}E_8$ where the ratio was 0.9:0.66:1). The values of E are calculated for a 1:1 molar ratio of BrF/ATPase from the experimental data (F/F_0). Values of r were calculated with R_0 = 47.4 Å. The buffer was 50 mM Hepes-KOH (pH 7.2), 0.3 mM EGTA, 25°C.
[a] 2.0 mg/ml.

The reduced intensity of IAEDANS emission in the doubly labelled species indicates energy transfer from IAEDANS to fluorescein. At the BrF/ATPase labelling ratio of 0.7:1 shown in Fig. 2, F/F_0, the ratio of IAEDANS fluorescence intensity in the doubly labelled species compared to that in the absence of BrF, was 0.49 (Table 1). The efficiency of energy transfer for 1:1 labelling with BrF would then be 0.73 (Table 1), corresponding to a separation of labels of 40 Å (Table 1). As shown in Table 1, the efficiency of energy transfer is unaffected by addition of Ca^{2+} or vanadate or by addition of the detergent $C_{12}E_8$ at concentrations up to 2.0 mg/ml.

Energy transfer was measured for SR doubly labelled to a molar ratio of 0.8:1:1 of IAEDANS/FITC/ATPase. The measured transfer efficiency E of 0.39 corresponded to a distance of separation of 53 Å (data not shown), in good agreement with the published values of 56 Å [20] or 53 Å [29].

Fig. 3 shows fluorescence emission spectra for IAEDANS-ATPase reconstituted into bilayers of mixtures

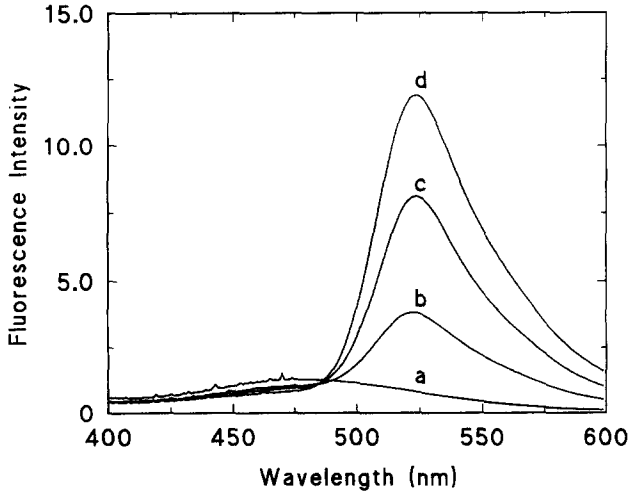

Fig. 3. Fluorescence emission spectra showing energy transfer from IAEDANS-ATPase to FITC-PE in reconstituted systems containing di(18:1)PC and mol fractions of FITC-PE of: (a), 0; (b) 0.01; (c), 0.02; (d), 0.04. The molar ratio of total phospholipid to ATPase was 300:1 and the molar ratio IAEDANS/ATPase was 1:1.

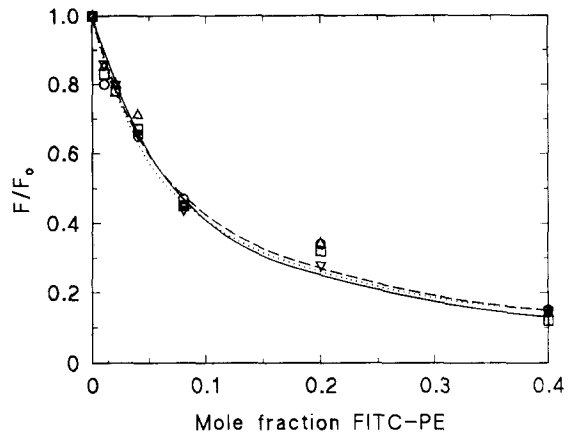

Fig. 4. Fluorescence quenching (F/F_0) of IAEDANS-ATPase reconstituted into phospholipid mixtures at a molar ratio of total phospholipid to ATPase of 300:1, as a function of the mol fraction of FITC-PE. The molar ratio of IAEDANS/ATPase was 1:1. Spectra were recorded at pH 7.2 in buffer containing 0.3 mM EGTA plus 5 mM Mg^{2+} (□), 0.7 mM Ca^{2+} (○); 1 mM vanadate and 5 mM Mg^{2} (△); or thapsivillosin A at a 1:1 molar ratio to ATPase (▽). The lines are theoretical calculations using: dotted line, the approach of Gutierrez-Merino [11] with d = 5 Å and h = 55 Å; dashed line, the approach of Dewey and Hammes [23] with h = 55 Å; and solid line, the approach of Koppel et al. [22] with h = 54 Å.

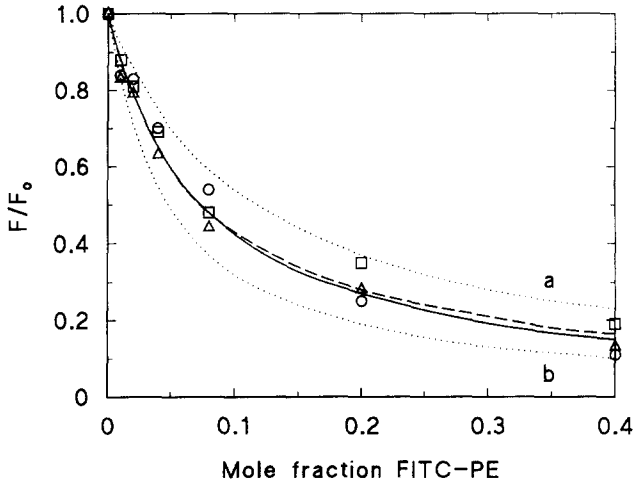

Fig. 5. Fluorescence quenching (F/F_0) of IAEDANS-ATPase reconstituted into phospholipid mixtures as a function of the mol fraction of FITC-PE, for the ATPase labelled to molar ratios of IAEDANS/ATPase of: 0.5:1, (△); 1:1, (○); and 1.7:1 (□). The solid line shows the theoretical curve calculated using the approach of Dewey and Hammes [23] with h = 54 Å, assuming a single site of labelling on the ATPase. The dotted lines show the theoretical curves for (a), h = 61 Å and (b), h = 47 Å and the broken line shows the theoretical curve for labelling of two sites with equal fluorescence intensities with h values of 61 and 47 Å.

of di(18:1)PC and FITC-PE at a molar ratio of total lipid/ATPase of 300:1 and recorded at pH 7.2 in the presence of 0.3 mM EGTA. As shown, the intensity of fluorescence at approx. 460 nm characteristic of the IAEDANS probe decreases with increasing mol fractions of FITC-PE in the mixture. At high molar ratios of FITC-PE, fluorescein shows significant absorbtion at the wavelengths of IAEDANS fluorescence emission; this was corrected for as described in Materials and methods. Relative intensities of IAEDANS-ATPase emission were obtained by least squares fitting of the composite spectra to the sum of the spectra of IAEDANS-ATPase reconstituted with di(18:1)PC and of unlabelled ATPase reconstituted with the appropriate mixture of di(18:1)PC and FITC-PE. Fig. 4 shows the relative intensity of IAEDANS fluorescence as a function of the mol fraction of FITC-PE in the lipid mixture.

Addition of a final free concentration of 0. 7 mM Ca^{2+} to IAEDANS-labelled ATPase reconstituted in di(18:1)PC led to a 6% increase in intensity of IAEDANS fluorescence recorded at 465 nm, but, as shown in Fig. 4, to no significant change in the fluorescence quenching curve in mixtures with FITC-PE. Similarly, addition of 5 mM Mg^{2+} led to a 3.6% decrease in IAEDANS fluorescence intensity and subsequent addition of 1 mM vanadate led to a further 40% decrease in fluorescence intensity but, as shown in Fig. 4, again no significant change in the fluorescence quenching curve. Finally, addition of thapsivillosin A to IAEDANS-ATPase resulted in no change in fluorescence intensity and no significant change in the fluorescence quenching curve.

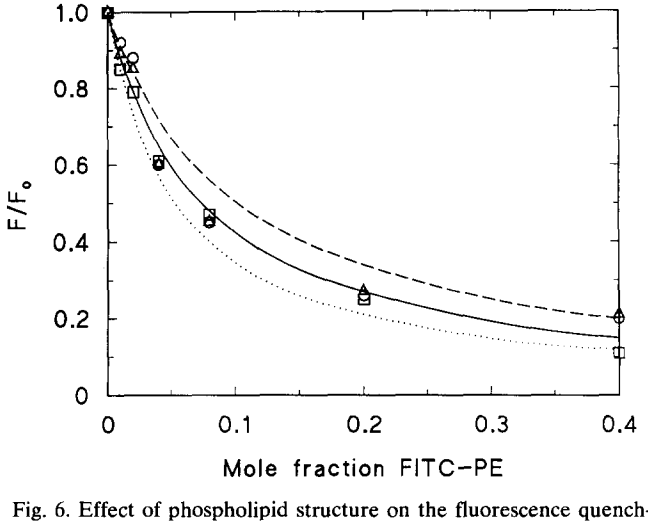

Fig. 6. Effect of phospholipid structure on the fluorescence quenching (F/F_0) of IAEDANS-ATPase reconstituted into phospholipid mixtures containing FITC-PE and: di(14:1)PC, (○); di(14:1)PC+cholesterol at a molar ratio of sterol/total phospholipid of 1:1, (△); and di(14:1)PC+androstenol at a molar ratio of sterol/total phospholipid of 1:1 (□). The solid, broken and dotted lines show the theoretical curves calculated using the approach of Dewey and Hammes [23] with h = 54, 60 and 50 Å, respectively.

Fig. 5 shows plots of F/F_0 for IAEDANS-ATPase in mixtures with FITC-PE for the ATPase labelled with IAEDANS to molar ratios of IAEDANS/ATPase of 0.5:1, 1:1 and 1.7:1. As shown, identical plots are obtained at all three labelling ratios.

The effect of phospholipid structure is shown in Fig. 6 where the labelled ATPase was reconstituted with mixtures of FITC-PE and either dimyristoleoylphosphatidylcholine (di(14:1)PC) or di(14:1)PC plus cholesterol or androstenol at a molar ratio of sterol/total phospholipid of 1:1.

4. Discussion

The Ca^{2+}-ATPase of sarcoplasmic reticulum has been predicted to contain 10 transmembrane α-helices [1,9,30]. Most of the extramembranous residues are in two cytoplasmic regions separated by transmembrane helices M3 and M4. The first (the β-strand or transduction domain) has a predicted anti-parallel β-structure, and is linked to the membrane by α-helices S2 and S3 [31]. The second region, between transmembrane helices M4 and M5, is larger and has been subdivided into three domains. The phosphorylation domain and nucleotide binding domain are both predicted to consist of alternating β-strands and α-helices. The pattern is similar to that found in kinases where ATP, bound to the second of two parallel β-sheet domains, phosphorylates a substrate bound to the other domain. The phosphorylation domain contains the residue (Asp-351) phosphorylated by ATP. The nucleotide binding domain contains a conserved sequence around Lys-515 which can be labelled in an ATP-protectable manner by fluorescein isothiocyanate (FITC) and is thus believed to contain the binding site for ATP. Finally at the C-terminal side of this second region is a central or hinge domain which, since it is labelled by γ-phosphate affinity labels [4,5], must be close to the phosphorylation site in the three dimensional structure of the ATPase.

Resonance energy transfer can be used to measure distances between labelled sites in membranes, and to study changes in these distances on ligand binding. We have used this technique to measure distances between Cys-344 and Glu-439 in the phosphorylation domain, and Lys-515 in the nucleotide-binding domain of the ATPase and the distances between these sites and the lipid/water interface [11,13,14]

The Ca^{2+}-ATPase contains 24 cysteine residues [1] of which about 14 can be modified chemically and so are believed to be exposed to the surface of the ATPase [24,32,33]. *N*-Ethylmaleimide has been shown to label both Cys-344 and Cys-364 [34,35]. 4-(Bromometh-

yl)-6,7-dimethoxycoumarin has been found to label only Cys-344 [27]. As shown in Fig. 1, reaction of the Ca^{2+}-ATPase with excess IAEDANS at pH 7.2 leads to the incorporation of up to 3.7 mol IAEDANS/mol ATPase at a molar ratio of total added IAEDANS/ATPase of 15:1, in agreement with Birmachu et al. [18] who obtained a labelling ratio of 3 mol IAEDANS/mol ATPase at an added ratio of 10:1 although Squier et al. [20] reported a labelling ratio of approx. 1.5 under these conditions. When the ATPase was reacted with a 2:1 molar ratio of IAEDANS for 3 h we obtained a bound molar ratio of IAEDANS/ATPase of 1:1 (Fig. 1) in agreement with previous studies [16,18,24]. The major site of labelling, accounting for approx. 75% of the label, has been shown to be Cys-674 [16,24]. However, the related 6-(iodoacetamido)fluorescein has been shown to label both Cys-674 and Cys-670, and, on the basis of the competition between IAEDANS and 6-(iodoacetamido)fluorescein for labelling the ATPase, it has been suggested that IAEDANS can also label Cys-670 [15,36].

Structure predictions using the method of Garnier et al. [37] suggest that the region around Cys-674 will be α-helical. Molecular modelling with an α-helical structure predicts a Cys-670 to Cys-674 distance of 6.1 Å. It has therefore been suggested that these two Cys residues can be treated as a single site in energy transfer experiments [15,20,36]. Using time-resolved energy transfer measurements, a single distance between Cys-670/Cys-674 and FITC at Lys-515 has been determined as 56 Å [20] or 53 Å [29]. However, in their time-resolved energy transfer measurements on this same system, Birmachu et al. [18] detected two distances, 52 Å and 31–40 Å which they suggested could corresponded to the Cys-670 to Lys-515 and Cys-674 to Lys-515 distances, respectively. The minimum distance between Cys-670 and Cys-674 would then be between 12 and 21 Å, considerably greater than the expected separation if this region were α-helical. Modelling shows that the separation between Cys-670 and Cys-674 in a fully extended conformation is 14.5 Å. The distances reported by Birmachu et al. [18] would thus equal the maximum possible difference in Cys-670 and Cys-674 to Lys-515 distances, corresponding to an extended conformation in which the vectors between Cys-670 and Cys-674 and between the two Cys residues and Lys-515 were parallel, which seems unlikely. Our estimate of 53 Å for the IAEDANS-FITC distance based on fluorescence intensities agrees with that of Bigelow and Inesi [29]. In the analysis below, we will assume that Cys-670 and Cys-674 can be treated as a single site in distance measurements.

As shown in Table 1, we estimate an IAEDANS-BrF separation of 40 Å. To distinguish between intermolecular and intramolecular effects, we studied the effect of the detergent $C_{12}E_8$ on the efficiency of energy transfer. $C_{12}E_8$ at 2.0 mg/ml has been shown to solubilize the ATPase in monomeric form [38] but had no significant effect on the efficiency of transfer, demonstrating that energy transfer was intramolecular (Table 1). The measured distance is included with other determined distances in Fig. 7. Bigelow and Inesi [29] have determined the separation between IAEDANS and two Cys residues designated MAL A and MAL B labelled with maleimide derivatives as 37 and 28 Å. The measured separation between MAL A and FITC at Lys-515 is the same as that measured between Cys-344 and Lys-515 [13] suggesting that MAL A is Cys-344 and thus giving the Cys-344 to IAEDANS distance as 28 Å (Fig. 7). These measurements suggest a location for Cys-344 (and thus of the residue phosphorylated by ATP, Asp-351) towards the centre of the ATPase molecule.

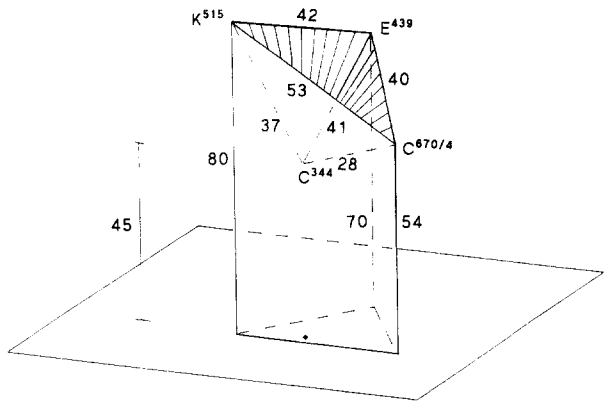

Fig. 7. Distances on the Ca^{2+}-ATPase determined using resonance energy transfer.

The height of the IAEDANS label above the lipid/water interface has been determined by measuring energy transfer between IAEDANS-ATPase and FITC-PE (Fig. 4). As shown in Fig. 4 the data fit well to the three theoretical approaches utilized. The methods of Koppel et al. [22] and of Dewey and Hammes [23] consider the donor and acceptor fluorophores as being located in two planes a distance h apart; the two approaches give distances of 54 and 55 Å, respectively. The method of Gutierrez-Merino et al. [11] characterizes the position of the label by a distance d between the site and the circumference of the protein and its height h above the lipid/water interface. It has been shown that when the protein donor is located far from the membrane surface, a wide range of values of d and h give equally good fits to the data, as long as the distances d and h are chosen so as to keep the distance between the protein donor and the annular shells of lipid around the ATPase a constant. As shown in Fig. 4, a good fit to the data is obtained with $d = 5$ Å and $h = 55$ Å. Equally good fits are obtained with, for example, $d = 1$ Å and $h = 57$ Å or $d = 10$ Å and

$h = 50$ Å (data not shown). Measurements of energy transfer between the tryptophan residues of the ATPase and IAEDANS have given a very approximate location for IAEDANS 36–40 Å above the membrane surface; the analysis assumes that all Trp residues are located within the trans-membrane region of the ATPase [39].

We can explore the consequences of two different heights for Cys-674 and Cys-670 above the lipid/water interface using the theoretical approaches described above. Thus if Cys-674 and Cys-670 are labelled by IAEDANS to give equal intensities of fluorescence emission, then the fluorescence quenching profiles expected from the approach of Dewey and Hammes [23] for one site located a distance of 47 Å from the surface and the other a distance of 61 Å from the surface (corresponding to the maximum possible differences in height of the two Cys residues in a fully extended conformation) would be indistinguishable experimentally from two sites both located a distance of 54 Å from the surface (Fig. 5). However, if at low IAEDANS labelling ratios, labelling favoured one of the two sites (as is suggested by sequencing experiments [16,24]) then quenching profiles would vary significantly with labelling ratio (Fig. 5). Since identical quenching plots are obtained at molar ratios of IAEDANS/ATPase of 0.5:1, 1:1, and 1.7:1, Cys-674 and Cys-670 are probably located at very similar heights above the lipid/water interface, as expected if this region of the ATPase adopts an α-helical conformation. The height of Cys-670/Cys-674 above the lipid/water interface is comparable to that determined for Cys-344 [13] (Fig. 7). It has been argued that the lipid/water interface as defined with FITC-PE will correspond to the level of the glycerol backbone of the phospholipid whereas that defined by electron microscopy is more likely to be defined by the lipid headgroup region; these definitions will differ by up to 15 Å [14]. The total height of the ATPase molecule as defined by electron microscopy is 120 Å, extending some 75 Å above the membrane surface [40]. The cytoplasmic region has been likened to the head and neck of a bird, the neck corresponding to the stalk region, some 25 Å long. Toyoshima et al. [40] have located a groove in the structure of the ATPase about 40 Å above the surface of the membrane as defined by electron microscopy which they have suggested could represent the ATP binding site. The resonance energy transfer measurements would therefore be consistent with the hinge domain making up the mouth of this ATP binding cleft on the ATPase. It has been suggested that Lys-684 is part of the ATP binding domain [4,5] and that Lys-492 can be cross-linked to Arg-678 in an ATP-protectable manner so that Arg-678 is also likely to be part of, or close to, the ATP binding site [6]. The long distance (53 Å) between IAEDANS at Cys-670/Cys-674 and FITC at Lys-515 suggests that these regions would represent opposite ends of the ATP binding domain. Further, it appears that Lys-515 may not be one of the residues that actually line the ATP binding site since it has been shown using site directed mutagenesis that Lys-515 is not an essential residue in ATP binding [41]. The inhibition of ATP binding that follows from labelling this residue with FITC may follow from steric effects attributable to the fluorescein group locating Lys-515 further away from the 'true' binding site for ATP.

It has been shown that IAEDANS fluorescence intensity is sensitive to conformational changes on the ATPase, with Ca^{2+} binding leading to an increase in fluorescence intensity and phosphorylation with ATP to a decrease [16,42]. We find identical plots of F/F_o against mol fraction of FITC-PE in the absence or presence of Ca^{2+} and in the presence of vanadate (Fig. 4). Similarly, the separation between IAEDANS and BrF does not change significantly on binding Ca^{2+} or vanadate (Table 1). Thus any motion in the hinge region must be fairly subtle with distances between labelled residues changing by less than approx. 5 Å. It has been suggested from X-ray diffraction studies that substantial net movement of the ATPase mass into the core of the membrane occurs on phosphorylation [43]. The resolution of the diffraction studies is not, however, sufficient to identify whether or not significant changes occur in the height of the ATPase above the surface of the lipid bilayer. The lack of any measurable change in measured distance for Cys-670/Cys-674 (Fig. 4) together with the lack of effect on the measured heights of Lys-515 [11], Cys-344 [13] or Glu-439 [14] argue against any major change in conformation.

Inhibitors of the Ca^{2+}-ATPase of the sesquiterpene lactone class such as thapsivillosin A, have been shown to bind to the ATPase locking it in a conformation unable to bind Ca^{2+} [44,45]. As shown in Fig. 4, binding of thapsivillosin A to IAEDANS-ATPase results in no change in energy transfer to FITC-PE in reconstituted systems. It has been shown that on reconstitution of the Ca^{2+}-ATPase with the short-chain phospholipid di(14:1)PC the stoichiometry of Ca^{2+} binding changes from the usual two Ca^{2+} ions bound per ATPase molecule to one Ca^{2+} ion bound per ATPase molecule, and that this effect can be reversed by addition of sterols such as cholesterol or androstenol [46]. As shown in Fig. 6, plots of F/F_o against mol fraction of FITC-PE are identical in mixtures with di(18:1)PC, di(14:1)PC or 1:1 mixtures of di(14:1)PC and cholesterol. It has been shown that the Ca^{2+} binding stoichiometry remains at 1:1 in mixtures of di(14:1)PC and di(18:1)PC down to 50% di(14:1)PC so that the similarity between the quenching curves for mixtures of FITC-PE and di(14:1)PC or di(18:1)PC at high mol fractions of phosphatidylcholine argues for very similar locations of

the IAEDANS label above the lipid/water interface in bilayers of di(18:1)PC and di(14:1)PC. These experiments therefore suggest that conformational changes on the Ca^{2+}-ATPase are localised to small regions of the structure.

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