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# Localization of Cys-344 on the ($Ca^{2+}$-$Mg^{2+}$)-ATPase of sarcoplasmic reticulum using resonance energy transfer

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4-Bromomethyl-6,7-dimethoxycoumarin labels the ($Ca^{2+}$-$Mg^{2+}$)-ATPase of skeletal muscle sarcoplasmic reticulum at Cys-344. Resonance energy transfer has been used to measure the distance between this site and Lys-515 labelled with fluorescein isothiocyanate as about 37 Å. The height of Cys-344 above the phospholipid/water interface has been measured by resonance energy transfer for the ATPase reconstituted into bilayers containing fluorescein-labelled phosphatidylethanolamine; the height was found to be about 45 Å. None of these distances was found to alter on changing pH, or on addition of $Mg^{2+}$, $Ca^{2+}$ or vanadate. Quenching of the fluorescence of the coumarin-labelled ATPase with KI suggested that the fluorophore is not fully exposed on the ATPase.

## Introduction

In electron micrographs of the ($Ca^{2+}$-$Mg^{2+}$)-ATPase of skeletal muscle sarcoplasmic reticulum (SR), the bulk of the protein appears on the cytoplasmic side of the membrane as a pear-shaped lobe (65 × 40 × 50 Å) on a narrow stalk, the protein extending some 65–70 Å from the surface of the membrane [1,2]. Further detail about the structure has been obtained using immunological methods to define the trans-membrane topography of the ATPase and to define surface exposed residues [3–7] and using resonance energy transfer to determine distances between defined residues on the ATPase [8–15]. If single sites on the ATPase can be labelled with suitable donor and acceptor fluorophores, then resonance energy transfer can be used to determine the separation of the donor and acceptor labels. Alternatively, if the labelled ATPase is reconstituted into bilayers of fluorescently-labelled phospholipids, then distances can be measured between the label on the protein and the phospholipid bilayer. The problem is to label single sites on the ATPase. Lys-515 in the nucleotide binding domain of the ATPase can be labelled with fluorescein isothiocyanate (FITC) [16] and the distance between FITC and the phospholipid/water interface was measured as about 80 Å, putting Lys-515 on the top surface of the ATPase [8,9]. 5-[[2-[(Iodoacetyl)amino]ethyl]amino] naphthalene-1-sulphonic acid (IAEDANS) labels Cys-670 and Cys-674 on the ATPase, but these two residues are sufficiently close to be treated as a single site for resonance energy transfer measurements; it has been reported that these sites are about 53 Å from FITC at Lys-515 [15]. Fluorescent maleimides have been reported to label two Cys residues on the ATPase, Cys-344 and Cys-364, approx. 36 Å apart; time-dependent resonance energy transfer measurements for the ATPase labelled with both FITC and the fluorescent maleimides could not be fitted to a single donor-acceptor distance but were consistent with two donor-acceptor distances, about 42 Å and about 77 Å [10].

In recently reported work we have shown that the ATPase can be specifically labelled at Cys-344 with 4-bromomethyl-6,7-dimethoxycoumarin (Br-DMC) [17]. Here we report location of this residue with respect to Lys-515 labelled with FITC and with respect to the phospholipid/water interface and a study of the effects of changes in the conformational state of the ATPase on these distances.

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Abbreviations: Br-DMC, 4-bromomethyl-6,7-dimethoxycoumarin; FITC, fluorescein isothiocyanate; IAEDANS, 5-[[2-[(iodoacetyl) amino]ethyl]amino]naphthalene-1-sulphonic acid; NCD-4, *N*-cyclohexyl-*N'*-(4-dimethylamino-1-naphthyl)carbodiimide; SR, sarcoplasmic reticulum.

## Materials and Methods

*Materials*

Lipids were obtained from Lipid Products or Avanti Polar Lipids, FITC from Sigma and Br-DMC from Molecular Probes. SR from rabbit skeletal muscle and the purified ($Ca^{2+}$-$Mg^{2+}$)-ATPase were prepared as described in East and Lee [18]. Dioleoylphosphatidylethanolamine was labelled with FITC using the protocol outlined by Fung and Stryer [19] and was purified on preparative silica gel plates [8]. ATPase was labelled with FITC by incubation of FITC and ATPase at a molar ratio of 2:1 in 50 mM Tris-HCl, 200 mM sucrose (pH 7.0) for 1 h at room temperature, followed by separation of unreacted FITC by centrifugation through a column of Sephadex G-50. ATPase was labelled with Br-DMC by incubation of Br-DMC and ATPase at a molar ratio of 46:1 for 1 h at room temperature in the dark, in the above buffer [17]. Unbound label was again removed by centrifugation through Sephadex G-50. The doubly labelled ATPase was prepared by first incubating the ATPase with Br-DMC for 1 h followed by removal of unreacted Br-DMC, followed by addition of FITC and a further 1 h incubation before removal of unreacted probe. Concentrations of ATPase were estimated from the absorbance at 280 nm using the absorption coefficient given by Hardwicke and Green [20]. Concentrations of bound FITC and DMC were determined following solubilization of samples in 1% SDS and NaOH (0.1 M) using absorption coefficients of $\epsilon_{500} = 80\,000$ $M^{-1}cm^{-1}$ [8] and $\epsilon_{350} = 12\,900$ $M^{-1}cm^{-1}$ [17], respectively.

Labelled ATPase was reconstituted with the desired amount of exogenous phospholipid (dioleoylphosphatidylcholine plus labelled phospholipid) by solubilization in detergent, followed by dilution into buffer [21]. Typically, phospholipid (300 $\mu$mol) was mixed with buffer (40 $\mu$l; 50 mM potassium phosphate, 1 M KCl, 0.2 M sucrose, pH 8.0) containing $MgSO_4$ (5 mM), ATP (6 mM) and potassium cholate (83 $\mu$g) and sonicated to clarity in a bath sonicator (Megason). ATPase (40 $\mu$g) in a volume of 80 $\mu$l was than added and incubated for 1 h at room temperature. Samples were then diluted with 200 $\mu$l of buffer and stored on ice until use. For fluorescence measurements, samples (130 $\mu$l, equivalent to 15 $\mu$g of ATPase) were diluted into buffer (2.3 ml; 40 mM Hepes-KOH, 100 mM NaCl, 1 mM EGTA, pH 7.2, unless otherwise stated), and fluorescence spectra at a bandwidth of 10 nm were recorded at 25°C using an SLM-Aminco 8000C fluorimeter with an excitation wavelength of 350 nm and a bandwidth of 5 nm. Measurements of fluorescence intensity were corrected for light scatter using samples of the ATPase unlabelled with Br-DMC.

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

*Analysis of resonance energy transfer measurements*

For the ATPase labelled at single sites with donor and acceptor fluorophores, the efficiency of energy transfer ($E$) can be written as:

$$E = 1 - (F/F_0) \tag{1}$$

where $F$ and $F_0$ are fluorescence intensities of the donor in the presence and absence of acceptor, respectively. The efficiency of resonance energy transfer is related to the distance between donor and acceptor ($r$) by:

$$E = r^6/(r^6 + R_0^6) \tag{2}$$

where $R_0$ is the distance at which energy transfer is 50% efficient. $R_0$ (Å) is given by:

$$R_0 = (9.79 \cdot 10^3)(\kappa^2 n^{-4} Q J)^{1/6} \tag{3}$$

where $\kappa^2$ is the orientation factor, $n$ the dielectric constant, $Q$ the quantum yield of the donor in the absence of acceptor and $J$ is the spectral overlap integral in $cm^6/mol$. The quantum yield of DMC-ATPase, measured relative to a value of 0.546 for quinine sulphate [43], was 0.216. The refractive index $n$ was put equal to that for a dilute aqueous solution, 1.33 [8,22]. The value of the orientation factor $\kappa^2$ was taken as 2/3, the value for rapid isotropic motion. There has been much discussion of the $\kappa^2$ factor in the literature, but because the sixth root of $\kappa^2$ is taken in the calculation of $R_0$, small differences between the true and assumed values of $\kappa^2$ will only produce small errors in calculated donor-acceptor distances [23], particularly for conformationally flexible sites and because of the mixed polarizations of the species [24]. As described, any error is likely to be particularly small for transfer from a donor on the protein to a phospholipid acceptor because of the random orientation of acceptor fluorophores in a phospholipid bilayer [9]. The value of the overlap integral $J$ for the coumarin-fluorescein pair has been measured as $7.316 \cdot 10^{-14}$ $cm^6/mol$ giving a value for $R_0$ of 37.5 Å.

Analysis of resonance energy transfer between DMC-ATPase and FITC-PE needs to take into account the distribution of fluorescence acceptors in the plane of the membrane. Three approaches have been adopted. The first takes into account the geometry of the system and has been described in detail elsewhere [9,25]. The ATPase is considered to be a cylinder embedded in a phospholipid bilayer and the position of

the fluorescent donor on the ATPase is then characterised by its height $h$ above the phospholipid/water interface and the distance $d$ between the site and the circumference of the protein. A numerical approach is then used to calculate the efficiency of resonance energy transfer from the donor to the acceptor phospholipids. The two other approaches consider the FITC-PE as being randomly distributed on the surface of a plane a distance $h$ below the position of the donor on the protein. Koppel et al. [26] showed that energy transfer could be represented by the equation:

$$\frac{F}{(F-F_0)} = 1+\sigma^{-1.1}\left[\frac{0.62}{\pi R_0^2}\exp(-0.34r+1.63r^2)^{1.1}\right] \quad (4)$$

where $\sigma$ is the surface density of acceptors and $r$ is defined as:

$$r = h/R_o \quad (5)$$

For the calculation of $\sigma$ surface areas of 80 and 1963 Å$^2$ were assumed for phospholipid and protein molecules, respectively [9]. Alternatively, Dewey and Hammes [27] showed that the extent of quenching of donor fluorescence could be approximated by:

$$F/F_o = (A_2 + A_3)/2 \quad (6)$$

where

$$A_2 = \left[1.0+0.4\left(\frac{R_0}{h}\right)^6\right]\cdot\left[1+0.4\left(\frac{R_0}{h}\right)^6+\left(\pi\sigma\frac{R_0^2}{2}\right)\left(\frac{R_0}{h}\right)^4\right]^{-1} \quad (7)$$

and

$$A_3 = \left[1+\pi\sigma\frac{R_0^2}{2}\left(\frac{R_0}{h}\right)^4+0.0625\left(\frac{R_0}{h}\right)^6\right] \times\left(\left[1+\frac{\pi\sigma R_0^2}{2}\left(\frac{R_0}{h}\right)^4\right]^2+\left[1+\frac{\pi\sigma R_0^2}{2}\left(\frac{R_0}{h}\right)^4\right] \times 0.625\left(\frac{R_0}{h}\right)^6-\frac{\pi\sigma R_0^2}{5}\left(\frac{R_0}{h}\right)^{10}\right) \quad (8)$$

## Results

Fig. 1 shows the fluorescence emission spectra of DMC-ATPase and FITC-ATPase excited at 350 nm. As shown, the spectrum of a 1:1 mixture of DMC-ATPase and FITC-ATPase is, as expected, a simple sum of the two spectra. In contrast, the spectrum of the ATPase doubly labelled with DMC and FITC shows reduced fluorescence emission from the DMC label and increased fluorescence from the FITC. The lack of overlap between the emission spectra of coumarin and fluorescein means that, in the composite spectra, the intensity at 425 nm is due solely to the DMC-ATPase. Table I lists the efficiency of transfer calculated from the decrease in intensity at 425 nm following labelling of DMC-ATPase with FITC and the calculated distance of separation between the coumarin and fluorescein groups on the ATPase. As shown in Table I, changing the pH or addition of $Ca^{2+}$ or vanadate has insignificant effects on the efficiency of transfer.

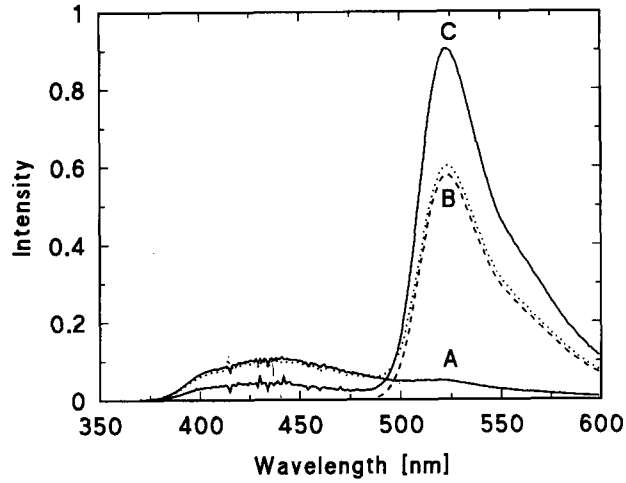


Fig. 1. Fluorescence emission spectra showing energy transfer from coumarin to fluorescein on the labelled ATPase: (A), DMC-ATPase; (B), FITC-ATPase; (C) ATPase doubly labelled with Br-DMC and FITC. The dotted line shows the spectrum of a 1:1 mixture of DMC-ATPase and FITC-ATPase. Fluorescence excitation was at 350 nm.

Fig. 2 shows the fluorescence emission spectrum of DMC-ATPase reconstituted into bilayers of dioleoylphosphatidylcholine at a phospholipid-protein molar ratio of 210:1. Fig. 2 also shows that when the labelled ATPase is reconstituted into phospholipid bilayers containing FITC-PE, resonance energy transfer occurs between the coumarin on the ATPase and the fluorescein groups, as shown by a reduction in intensity for

TABLE I

*Resonance energy transfer between coumarin and fluorescein on the doubly labelled ATPase*

The efficiency of resonance energy transfer was measured by the quenching of coumarin fluorescence ($F/F_0$) for the ATPase labelled with Br-DMC and FITC, in various media. Also given are values for the distance of separation $r$ between the probes on the ATPase, calculated as described in the text.

| System | $F/F_0$ | $r$ (Å) |
|---|---|---|
| EGTA, pH 7.0 | 0.46 | 36.5 |
| EGTA, pH 6.0 | 0.46 | 36.5 |
| EGTA, pH 8.0 | 0.44 | 36.0 |
| $Ca^{2+}$, pH 7.0 | 0.46 | 36.5 |
| Vanadate, pH 7.0 | 0.50 | 37.5 |

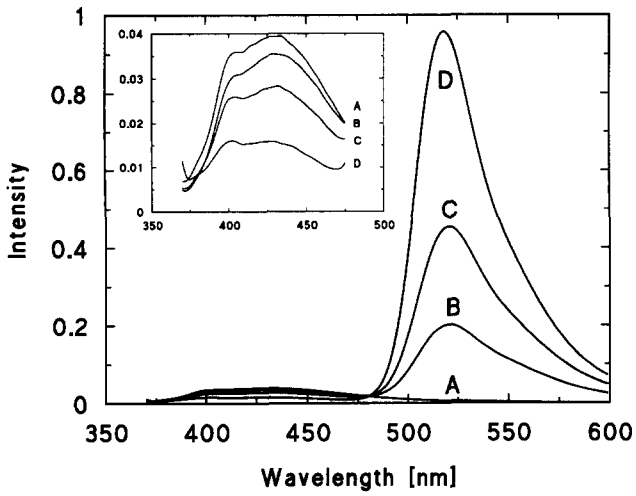


Fig. 2. Fluorescence emission spectra showing energy transfer from DMC-ATPase to FITC-PE in reconstituted systems containing dioleoylphosphatidylcholine and mole fractions of FITC-PE of: (A), 0; (B), 0.02; (C), 0.05; (D), 0.2. The molar ratio of total phospholipid to ATPase was 210:1. The inset shows on an expanded scale the region of the emission spectrum between 350 and 500 nm corresponding to coumarin emission.

coumarin emission. As shown in Figs. 2 and 3, the intensity of DMC-ATPase fluorescence decreases with increasing mole fraction of FITC-PE in the bilayers. Changing the pH from 7.0 to 6.0 or 8.0 has no effect on $F/F_0$ (Fig. 3), and addition of either $Ca^{2+}$ or vanadate also have no effect (data not shown). As shown in Fig. 2, the fluorescence intensity of FITC-PE increases less than linearly with increasing concentration of FITC-PE. This presumably reflects the formation of non-fluorescent dimers or higher aggregates of FITC-PE in the bilayers at relatively high concentrations of FITC-PE.

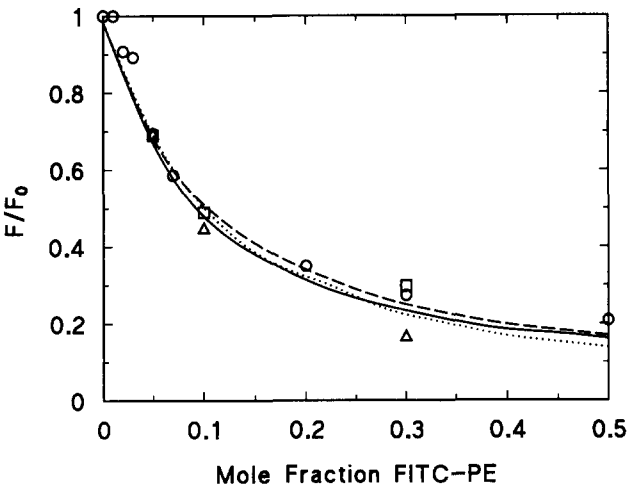


Fig. 3. Fluorescence quenching ($F/F_0$) of DMC-ATPase reconstituted into phospholipid mixtures at a molar ratio of total phospholipid to ATPase of 210:1, as a function of the mole fraction of FITC-PE. Spectra were recorded in buffer containing 1 mM EGTA at: △, pH 6.0; ○, pH 7.0; and □, pH 8.0. The lines are theoretical calculations, using: solid line, the approach of Gutierrez-Merino [9,25] with $d = 5$ Å and $h = 45$ Å; dashed line, the approach of Dewey and Hammes [27] with $h = 45$ Å; and dotted line, the approach of Koppel et al. [26] with $h = 45$ Å.

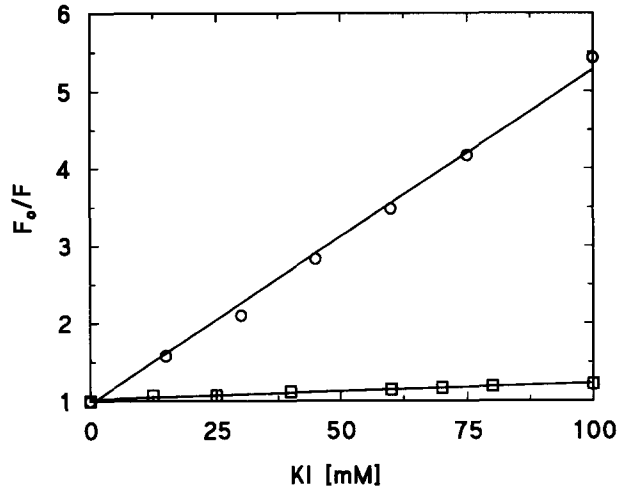


Fig. 4. Stern-Volmer plot of $F_0/F$ against the concentration of added KI for, 6,7-dimethoxycoumarin (○) and for DMC-ATPase (□) at a constant ionic strength. All spectra were recorded at pH 7.0.

To test the accessibility of the DMC label on the ATPase, and to look for effects of conformational changes, we studied quenching of DMC-ATPase fluorescence by iodide. In the simplest cases of dynamic (collisional) or static quenching, fluorescence quenching fits the Stern-Volmer relationship

$$F_0/F = 1 + K_{SV}[Q] \quad (9)$$

where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the quencher concentration and $K_{SV}$ is a constant. As shown in Fig. 4 quenching of free 6,7-dimethoxycoumarin by KI fits well to this equation. For studies of quenching of DMC-ATPase by KI, the ionic strength was maintained constant at 100 mM with KCl. As shown, under these conditions quenching of DMC-ATPase by KI also fits to the Stern-Volmer equation, although with a much smaller quenching constant (Fig. 4).

## Discussion

We have shown that the ($Ca^{2+}$-$Mg^{2+}$)-ATPase reacts with the coumarin derivative Br-DMC to label a single cysteine residue, Cys-344 [17]. Cys-344 is in the phosphorylation domain of the ATPase, close to the residue (Asp-351) phosphorylated by ATP. Although labelling Cys-344 results in only a 25% reduction in the rate of ATP hydrolysis with no effect on the rate of hydrolysis of *p*-nitrophenol phosphate, the fluorescence of DMC-ATPase is sensitive to phosphorylation of the ATPase either by ATP or by $P_i$ [17].

The ATPase can be labelled in the nucleotide binding domain at Lys-515 with FITC [16]. Measurement of

the efficiency of resonance energy transfer between the coumarin and fluorescein labels on the ATPase doubly labelled with Br-DMC and FITC allows an estimation of the separation between Cys-344 and Lys-515. As shown in Fig. 1, energy transfer is observed between these labels, and a separation of about 36.5 Å is calculated (Table I). Bigelow and Inesi [10] found that maleimides labelled Cys-344 and Cys-364 and from time-dependent measurements suggested that one of the maleimide sites (MAL A) was about 42 Å from Lys-515, the other (MAL B) being about 77 Å from Lys-515. The satisfactory agreement between the distance of about 42 Å for MAL A and our determination of the Cys-344 to Lys-515 separation would suggest that MAL A in the experiments of Bigelow and Inesi [10] was Cys-344. This would then give a separation between Cys-364 and Lys-515 of about 77 Å.

We have also used resonance energy transfer between coumarin attached to Cys-344 and FITC-PE to estimate the distance between Cys-344 and the phospholipid/water interface. As shown in Fig. 3, the experimental data on quenching of DMC-ATPase fluorescence by FITC-PE fits well to the three theoretical approaches utilized. The method of Gutierrez-Merino [9,25] characterises the position of the label on the ATPase in terms of its height *h* above the membrane surface and the distance *d* between the site and the circumference of the cylindrical trans-membrane region of the protein. As shown elsewhere [9], when the protein donor is located far from the membrane surface, a wide range of *d* and *h* values give equally good fits to the data when the *d* and *h* values are chosen so that the distance between the protein donor and the annular shells of phospholipids surrounding the ATPase is maintained approximately constant. Thus Fig. 3 shows a good fit to the model with *d* and *h* values of 5 and 45 Å, respectively, but an equally good fit is obtained with, for example, *d* and *h* values of 10 and 41 Å, respectively. The experimental data can also be fitted equally well using the approach of Koppel et al. [26] or Dewey and Hammes [27] both with a distance *h* between the protein donor and the membrane surface of 45 Å (Fig. 3). The observation that three separate methods of calculation give very similar distances suggests that the differences in the approximations and assumptions used in each of the models used are relatively unimportant.

Fig. 5 shows the distances between Lys-515 and Cys-344 superimposed on the structure of the ATPase deduced by Stokes and Green [28] from studies of negatively stained crystals of the ATPase. The extramembranous domain of the ATPase is believed to consist of a larger and a smaller lobe, connected to the membrane by a relatively narrow stalk (Fig. 5) [2]. The ATPase extends about 65 Å above the surface of the membrane [1,2]. The definition of the surface of the

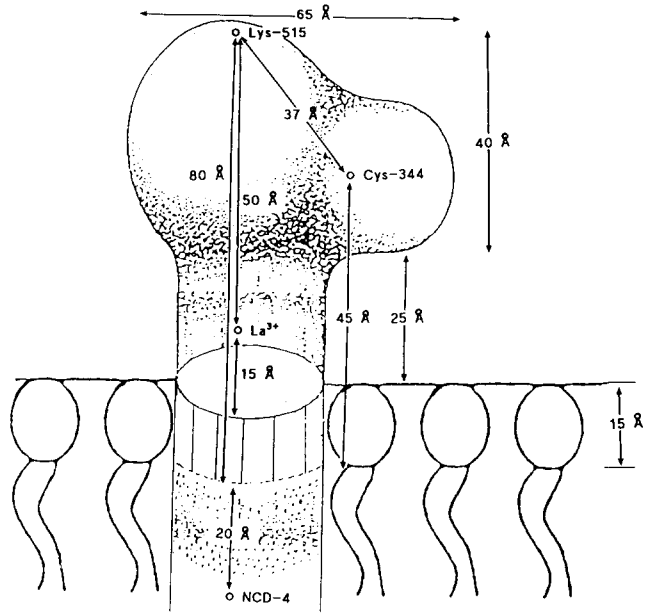


Fig. 5. Location of Lys-515 and Cys-344 on the structure of the ATPase deduced by Stokes and Green [28] from studies of negatively stained crystals of the ATPase. Also shown is the location of the sites labelled by NCD-4, believed to be associated with the binding of $Ca^{2+}$ [9] and the possible location of site(s) binding lanthanides.

membrane is, however, not straightforward. 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 [29]. As argued previously [9] it seems likely that FITC-PE will adopt a similar conformation with the non-polar region of the fluorophore located at the glycerol backbone region and the carboxyl group located in the headgroup region so that the phospholipid/water interface as defined with this probe will correspond to the level of the glycerol backbone of the phospholipid. The surface of the membrane, as defined by electron microscopy of negatively stained material, corresponds to the plane of minimum penetration of negative stain and is likely to correspond to the phospholipid headgroup region. Since a phosphatidylcholine headgroup extends approx. 15 Å from the glycerol backbone region in crystals of the phospholipid [30], definitions of the membrane surface by fluorescence and electron microscopy will differ by about 15 Å (Fig. 5). Location of Lys-515 80 Å above the glycerol backbone region would put Lys-515 on the top surface of the larger lobe of the ATPase, defining this lobe as the nucleotide binding domain. Cys-344 could then be located in the smaller lobe, representing the phosphorylation domain (Fig. 5).

Sites at or near the $Ca^{2+}$ binding sites on the ATPase have been labelled with the fluorescent carbodiimide *N*-cyclohexyl-*N'*-(4-dimethylamino-1-naphthyl)carbodiimide (NCD-4) and the labelled sites were

located about 20 Å from the phospholipid/water interface [31]. Originally it was suggested that the sites were in the cytoplasmic region of the ATPase, but the energy transfer measurements would be equally consistent with a location within the trans-membranous region of the ATPase; such a location would be consistent with the observed quenching of fluorescence by spin-labelled fatty acids which will partition into the phospholipid bilayer [9]. Experiments using site-directed mutagenesis have suggested that $Ca^{2+}$ binding involves residues in postulated trans-membranous $\alpha$-helices [32]. If the $Ca^{2+}$ binding sites are located within trans-membrane regions of the ATPase with Lys-515 at the top surface of the ATPase, separation distances are greater than those estimated using lanthanides as probes for the $Ca^{2+}$ binding site (Fig. 5). Scott [11] using $Tb^{3+}$ as probe has suggested a separation of 47 Å, which, with a location for Lys-515 80 Å above the surface [8] would put the $Tb^{3+}$ binding site about 30 Å above the surface. X-ray diffraction studies have located binding sites for lanthanides about 12 Å above the phospholipid polar headgroup region of the bilayer [33]; with a thickness of the headgroup region of 15 Å, the site locations estimated by X-ray diffraction and fluorescence would be in very close agreement. It is unclear whether the lanthanides bind at the 'true' $Ca^{2+}$ binding sites or at some other site(s) [34–36]. Squier et al. [14] have measured a separation between bound $Pr^{3+}$ and IAEDANS at Cys-670 and Cys-674 as 18 Å. If it can be assumed that all the lanthanides bind at the same site on the ATPase then this would serve to locate Cys-670 and Cys-674 within the structure. It has been estimated that the distance between $Eu^{3+}$ and Cr-ATP on the ATPase is less than 10 Å [13] but it seems likely that in these experiments, $Eu^{3+}$ binds to a metal-ion subsite of the nucleotide binding site, normally occupied by $Mg^{2+}$.

Location of Lys-515 on the top surface of the ATPase would be consistent with the considerable surface exposure of the protein in this region as defined by the binding of antibodies to the native ATPase [3,5]. Studies of the binding of anti-peptide antibodies raised to the phosphorylation domain of the ATPase also suggest that much of this domain is also surface exposed, with, for example, anti-peptide antibodies raised to sequences 324–339 and 366–377 binding to the native ATPase [5]. Ball and Loftice [37] have reported that an antipeptide antibody raised to the region of the $Na^+/K^+$-ATPase including the Asp residue phosphorylated by ATP (corresponding to Asp-351 in the $(Ca^{2+}$-$Mg^{2+})$-ATPase) failed to bind to the native ATPase, suggesting that this region of the ATPase is buried. We studied the possible surface exposure of Cys-344 by studying the quenching of the fluorescence of DMC-ATPase by iodide (Fig. 4). As shown, quenching of free 6,7-dimethoxycoumarin by KI fits the Stern-Volmer equation, with a quenching constant $K_{SV}$ of $45 \pm 0.7$ $M^{-1}$. Quenching of DMC-ATPase is much less efficient, but at constant ionic strength, still fits to the Stern-Volmer equation with a quenching constant of $2 \pm 0.2$ $M^{-1}$ (Fig. 4). Johnson and Yguerabide [39] have argued that quenching constants for a bound fluorophore will always be less than for the fluorophore free in solution, because the translational and rotational diffusion coefficients of the bound fluorophore will be smaller than those of the free fluorophore. They estimated that this would result in a reduction in $K_{SV}$ by a factor of 2. The observed reduction in value of $K_{SV}$ by a factor of about 20 would therefore indicate shielding of the bound coumarin on the ATPase. The efficiency of quenching differs little in the presence or absence of $Ca^{2+}$ or on variation of pH between 6 and 8, or on addition of $Mg^{2+}$ (data not shown). If the ionic strength of the solution is not maintained constant, then a curved Stern-Volmer plot is obtained (data not shown) as observed in a number of other systems [38].

Stahl and Jencks [40] have suggested a conformational change on the ATPase following binding of ATP to the ATPase in the presence of $Ca^{2+}$, serving to relocate the nucleotide binding and phosphorylation domains on the ATPase, bringing the $\gamma$-phosphate of ATP close to Asp-351. The experiments reported here give no evidence for any major relocation of Cys-344 in the phosphorylation domain, either with respect to Lys-515 in the nucleotide binding domain or with respect to the phospholipid/water interface. Thus changing pH, which alters the balance between the two major conformational states of the ATPase, E1 and E2 [41,42], or addition of $Ca^{2+}$ or vanadate, an analogue of $P_i$, have no effect on the efficiency of transfer between coumarin and fluorescein on the ATPase (Table I). Similarly, these changes have no effect on energy transfer from DMC-ATPase to FITC-PE (Fig. 3 and data not shown). We have also shown that addition of $Ca^{2+}$ or vanadate has no effect on the distance between Lys-515 and the phospholipid/water interface [8] or on the separation between the sites labelled by NCD-4 and the phospholipid/water interface [9]. We conclude that the conformational differences between the E1 and E2 states of the ATPase and between $Ca^{2+}$ bound and free forms must be localised in small regions of the structure. This is also consistent with the observation that most monoclonal antibodies binding to the native ATPase have no significant effect on activity [3].

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## References


1 Dux, L., Taylor, K.A., Ting-Beall, H.P. and Martonosi, A. (1985) J. Biol. Chem. 260, 11730–11743.
2 Stokes, D.L. and Green, N.M. (1990) Biophys. J. 57, 1–14.
3 Colyer, J., Mata, A.M., Lee, A.G. and East, J.M. (1989) Biochem. J. 262, 439–447.
4 Matthews, I., Colyer, J., Mata, A.M., Green, N.M., Sharma, R.P., Lee, A.G. and East, J.M. (1989) Biochem. Biophys. Res. Commun. 161, 683–688.
5 Mata, A.M., Matthews, I., Tunwell, R.E.A., Sharma, R.P., Lee, A.G. and East, J.M. (1992) Biochem. J. 286, 567–580.
6 Matthews, I., Sharma, R.P., Lee, A.G. and East, J.M. (1990) J. Biol. Chem. 265, 18737–18740.
7 Clarke, D.M., Loo, T.W. and MacLennan, D.H. (1990) J. Biol. Chem. 265, 17405–17408.
8 Gutierrez-Merino, C., Munkonge, F., Mata, A.M., East, J.M., Levinson, B.L., Napier, R.M. and Lee, A.G. (1987) Biochim. Biophys. Acta 897, 207–216.
9 Munkonge, F., East, J.M. and Lee, A.G. (1989) Biochim. Biophys. Acta 979, 113–120.
10 Bigelow, D.J. and Inesi, G. (1991) Biochemistry 30, 2113–2125.
11 Scott, T.L. (1985) J. Biol. Chem. 260, 14421–14423.
12 Teruel, J.A. and Gomez-Fernandez, J.C. (1986) Biochim. Biophys. Acta 863, 178–184.
13 Herrmann, T.R., Gangola, P. and Shamoo, A.E. (1986) Eur. J. Biochem. 158, 555–560.
14 Squier, T.C., Bigelow, D.J., De Ancos, J.G. and Inesi, G. (1987) J. Biol. Chem. 262, 4748–4754.
15 Birmachu, W., Nisswandt, F.L. and Thomas, D.D. (1989) Biochemistry 28, 3940–3947.
16 Mitchinson, C., Wilderspin, A.F., Trinnaman, B.J. and Green, N.M. (1982) FEBS Lett. 146, 87–92.
17 Stefanova, H.I., East, J.M., Gore, M.G. and Lee, A.G. (1992) Biochemistry 31, 6023–6031.
18 Lee, A.G., East, J.M., Jones, O.T., McWhirter, J., Rooney, E.K. and Simmonds, A.C. (1982) Biochemistry 21, 6441–6446.
19 Fung, B.K.K. and Stryer, L. (1978) Biochemistry 17, 5241–5248.
20 Hardwicke, P.M. and Green, N.M. (1974) Eur. J. Biochem. 42, 183–193.
21 East, J.M. and Lee, A.G. (1982) Biochemistry 21, 4144–4151.
22 Moog, R.S., Kuki, A., Fayer, M.D. and Boxer, S.G. (1984) Biochemistry 23, 1564–1571.
23 Dale, R.E., Eisinger, J. and Blumberg, W.E. (1979) Biophys. J. 26, 161–194.
24 Haas, E., Katchalski-Katzir, E. and Steinberg, I.Z. (1978) Biochemistry 17, 5064–5070.
25 Gutierrez-Merino, C. (1981) Biophys. Chem. 14, 259–266.
26 Koppel, D.E., Fleming, P.J. and Strittmatter, P. (1979) Biochemistry 18, 5450–5457.
27 Dewey, T.G. and Hammes, G.G. (1980) Biophys. J. 32, 1023–1036.
28 Stokes, D.L. and Green, N.M. (1990) Biochem. Soc. Trans. 18, 841–843.
29 Ghiggino, K.P., Lee, A.G., Meech, S.R., O'Connor, D.V. and Phillips, D. (1981) Biochemistry 20, 5381–5389.
30 Pearson, R.H. and Pascher, I. (1979) Nature 281, 499–501.
31 Chadwick, C.C. and Thomas, E.W. (1984) Biochim. Biophys. Acta 769, 291–296.
32 Clarke, D.M., Loo, T.W., Inesi, G. and MacLennan, D.H. (1989) Nature 339, 476–478.
33 Asturias, F.J. and Blasie, J.K. (1991) Biophys. J. 59, 488–502.
34 Imamura, Y. and Kawakita, M. (1991) J. Biochem. Tokyo 110, 214–219.
35 Fujimori, T. and Jencks, W.P. (1990) J. Biol. Chem. 265, 16262–16270.
36 Ogurusu, T., Wakabayashi, S. and Shigekawa, M. (1991) Biochemistry 30, 9966–9973.
37 Ball, W.J. and Loftice, C.D. (1987) Biochim. Biophys. Acta 916, 100–111.
38 Eftink, M.R. (1991) in Biophysical and Biochemical Aspects of Fluorescence Spectroscopy (Dewey, T.G., ed.), pp. 1–41, Plenum Press, New York.
39 Johnson, D.A. and Yguerabide, J. (1985) Biophys. J. 48, 949–955.
40 Stahl, N. and Jencks, W.P. (1987) Biochemistry 26, 7654–7667.
41 De Meis, L. and Vianna, A.L. (1979) Annu. Rev. Biochem. 48, 275–292.
42 Froud, R.J. and Lee, A.G. (1986) Biochem. J. 237, 197–206.
43 Miller, J.N.(1981) Standards in Fluorescence Spectroscopy, Chapman and Hall, London.