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Positions of the sites labeled by *N*-cyclohexyl-*N'*-(4-dimethylamino-1-naphthyl)carbodiimide on the (Ca^{2+} + Mg^{2+})-ATPase

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***N*-Cyclohexyl-*N'*-(4-dimethylamino-1-naphthyl)carbodiimide (NCD-4) labels (Ca^{2+} + Mg^{2+})-ATPase at Ca^{2+}-protectable sites, believed to be at or near the two Ca^{2+} binding sites on the ATPase, and at nonspecific sites. The labeled ATPase has been reconstituted into lipid bilayers containing phosphatidylethanolamine labeled with fluorescein isothiocyanate. The distance between NCD-4 and fluorescein groups was measured using Forster energy transfer and the NCD-4 labels were found to be approx. 20 Å from the lipid/water interface suggesting that the Ca^{2+} binding sites on the ATPase are also 20 Å from the lipid/water interface. Addition of vanadate causes no change in the efficiency of energy transfer, suggesting that the Ca^{2+} binding sites on the E1 conformation of the ATPase do not move significantly with respect to the lipid/water interface in the E1–E2 transition.**

Introduction

In the absence of three-dimensional X-ray crystallographic data, indirect methods have be used to determine the main structural features of membrane proteins. (Ca^{2+} + Mg^{2+})-ATPase from skeletal muscle SR is a good candidate for such studies because it can be obtained pure in large amounts, and because its kinetics have been defined in some detail [1,2]. In electron micrographs of negatively stained fragmented SR, the ATPase is seen as particles projecting from the lipid bilayer, consisting of a large headgroup on a narrower stalk [3]. X-ray diffraction techniques suggest that the diameter of the headgroup region is about double that of the stalk region [4]. Low-resolution images of the ATPase have also been obtained from electron micrographs of negatively stained SR, and these show that, in the presence of vanadate, the ATPase is present as a dimer, 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 the diameter of the headgroup being approx. 90 Å [5]. Brandl et al. [6] have related this structural information to their sequence data for the ATPase, and have suggested that the nucleotide binding site is located in the headgroup region of the ATPase and that the two Ca^{2+} binding sites per ATPase molecule are located in the stalk region.

In a previous publication we labeled the ATPase in the ATP binding site with FITC and measured the distance between this site and the surface of the lipid bilayer by fluorescence energy transfer [7]. We found that the ATP binding site was distant from the membrane surface, in agreement with the structure suggested by Brandl et al. [6]. Fluorescence energy-transfer experiments have also shown that the Ca^{2+} binding sites are distant from the ATP site [8,9]. Here, we estimate the distance of the Ca^{2+} binding sites from the membrane surface, thus completing the definition of the relative positions of the substrate binding sites on the ATPase.

The distance between the ATP and Ca^{2+} sites was estimated using Tb^{3+} as a fluorescence donor and either FITC or the ATP analogue 2'(3')-*O*-(2,4,6-trinitrophenyl)adenosine triphosphate (TNP-ATP) as acceptor [9]. Distances to the lipid headgroup region can be estimated using phosphatidylethanolamines

Abbreviations: DOPC, dioleoylphosphatidylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FITC, fluorescein isothiocyanate; FITC-PE, fluorescein isothiocyanate labeled egg phosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NCD-4, *N*-cyclohexyl-*N'*-(4-dimethylamino-1-naphthyl)carbodiimide; SR, sarcoplasmic reticulum.

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labeled with FITC or rhodamine isothiocyanate as markers [7,10]. Potentially then, distances from the Ca^{2+} binding sites on the ATPase to the lipid headgroup region could be estimated using Tb^{3+} or other lanthanides as fluorescent donors and labeled lipid as acceptors, but it is known that lanthanides and other polyvalent ions will bind directly to lipid bilayers [8,11,12] and such binding would considerably complicate the interpretation. However, it has been suggested that carbodiimides can be used to label the ATPase at or near the Ca^{2+} binding sites. García de Anios and Inesi [35] have shown that DCCD labels the ATPase on both the A_2 and A_1 tryptic fragments. Labeling of the A_2 tryptic fragment only occurred in the absence of Ca^{2+} and, since the A_2 fragment contains several negatively charged residues likely to be involved in complexing Ca^{2+} [6], it was suggested that the groups labeled by DCCD in the absence of Ca^{2+} were normally involved in the binding of Ca^{2+} [35]. The A_1 fragment was found to be labeled in both the presence and absence of Ca^{2+}, but undergoes DCCD-induced cross-linking only when derivatization was carried out in the absence of Ca^{2+}. It was therefore suggested that fragment A_1 participates with A_2 in formation of the Ca^{2+}-binding domain [35] (see also 25). Chadwick and Thomas [13,14] introduced a fluorescent carbodiimide NCD-4 and showed that this labeled the ATPase only on the A_2 tryptic fragment. They also showed that about half the labeling was at calcium-protected sites and half was calcium-independent [13,14]. These experiments therefore suggest very strongly that the sites labeled by NCD-4 will be at, or close to, the Ca^{2+} binding sites on the ATPase, as also suggested by Pick and Weiss [15]. Here we measure the distance between the sites labeled by NCD-4 and the lipid/water interface.

Materials and Methods

Lipids were obtained from Lipid Products. FITC and spin-labeled fatty acids were obtained from Aldrich and NCD-4 was from Molecular Probes.

SR was prepared from female rabbit (New Zealand White) hind leg muscle and (Ca^{2+} + Mg^{2+})-ATPase was purified as described by East and Lee [16]. ATPase was labeled with NCD-4 by incubation of ATPase (10 μM) with NCD-4 (150 μM) in 100 mM KCl/50 mM Hepes-KOH/1 mM EGTA/0.2 M sucrose (pH 6.2) for 3 h at 25°C. Unbound NCD-4 was separated from the labeled ATPase by centrifugation through Sephadex G-50 columns preequilibrated with 25 mM Hepes-KOH/100 mM KCl/1 mM EGTA/0.2 M sucrose (pH 7.0) [17].

Egg phosphatidylethanolamine was labeled with FITC using the protocol outlined by Fung and Stryer [18] and was purified on preparative silica gel plates [7]. Labeled ATPase was reconstituted with the desired amount of exogenous lipid (DOPC plus labeled lipid) by solubilisation in detergent, followed by rapid dilution into buffer [7]. Typically, lipid (300 μmol) was mixed with buffer (40 μl; 50 mM potassium phosphate/*i* M KCl/0.2 mM sucrose (pH 8.0) containing $MgSO_4$ (5 mM), ATP (6 mM) and potassium cholate (0.3 mg) and sonicated to clarity in a bath sonicator (Megason). ATPase (0.125 mg) in a volume of 30 μl was then added and incubated at 25°C for 20 min followed by 40 min at 5°C. Samples were then diluted with 200 μl of buffer and stored on ice until use. For fluorescence measurements, samples (100 μl, equivalent to 46 μg of ATPase) were diluted into buffer (2.45 ml; 40 mM Hepes/100 mM NaCl/5 mM $MgSO_4$/1 mM EGTA (pH 7.0)) and fluorescence spectra were recorded on a Perkin-Elmer MPF44A fluorimeter. Spectra were excited at 338 nm and fluorescence emission of NCD-4 was detected at 430 nm. Emission spectra were corrected using the spectrum of quinine sulphate [19] as standard.

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.

Fluorescence energy transfer was analysed in one of three ways. The first takes into account the geometry of the system and has been described in detail elsewhere [20,21]. Briefly, the position of the fluorescent label on the ATPase is characterised by its height, *h*, above the plane of the lipid/water interface and the distance, *d*, between the site and the circumference of the protein (Fig. 1). A numerical approach is then used to calculate the rate of Forster energy transfer from the donor group on the protein to the acceptor groups located in the lipid polar headgroups. The second approach is that of Koppel et al. [22], in which the lipid-bound acceptors

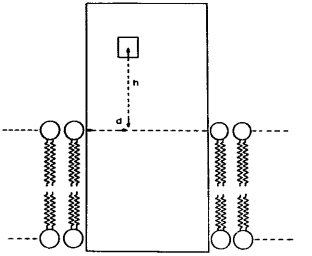

Fig. 1. Cross-sectional view of the ATPase, showing the parameters *d* and *h* used in the calculations.

are considered to be randomly distributed on the surface of a plane a distance, h, below the position of the acceptor on the protein. Koppel et al. [22] showed that energy transfer could be represented by the equation

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

where F_0 and F are the acceptor fluorescence intensities in the absence and presence of acceptors, respectively, R_0 is the distance between the donor and acceptor for which the efficiency of energy transfer is 50%, σ is the surface density of acceptors and r is defined as

$$r = h/R_0 \quad (2)$$

For the calculation of σ, surface areas of 80 and 1963 Å^2 were assumed for lipid and protein molecules, respectively [7]. The third approach is that of Dewey and Hammes [23], which again considers donor and acceptor fluorophores to be located in planes separated by a distance h. Dewey and Hammes [23] showed that the extent of quenching of donor fluorescence was given approximately by

$$F/F_0 = (A_2 + A_3)/2.0 \quad (3)$$

where

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

and

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

The value of the overlap integral between the emission of NCD-4-labeled ATPase and FITC-labeled egg phosphatidylethanolamine was found to be $8.5 \cdot 10^{-14}$ cm^6/mol and with a value of dielectric constant n of 1.33 [24], a quantum yield of NCD-4 fluorescence of 0.06 [13] and a value for the orientation factor K^2 describing the relative orientation of the donor emission and acceptor absorption transition dipoles equal to the value appropriate for isotropic motion of the dipoles, $K^2 = 2/3$, the value of R_0 is calculated to be 31.2 Å. There has been much discussion of the K^2 factor in the literature [36]. Depending on the relative orientation of donor and acceptor, K^2 can take values between 0 and 4. In the calculation of R_0, the sixth root of K^2 is taken so that small differences between the true and assumed values of K^2 will only produce small errors in calculated donor-acceptor distances. It has generally been found that a value of K^2 of 2/3 fits the data well for membrane systems [7,10,20,21]. In particular, we have determined the fluorescence polarisation of FITC-PE in lipid bilayers at high dilution in the bilayer (where concentration depolarisation is negligible) to be approx. 0.1, consistent with considerable mobility for the fluorescein group in the headgroup region of the bilayer. It has been estimated that, when the polarization of the donor or the acceptor is less than approx. 0.2, errors in distance caused by assuming a K^2 value of 2/3 are less than 10% [36]. In the case of energy transfer from a membrane protein to a lipid bilayer containing a high concentration of labeled lipid as acceptor, the error is likely to be even less than this for the following reasons. We have shown that the fluorescence polarisation of FITC-PE in bilayers of phosphatidylcholine drops rapidly with increasing concentration of FITC-PE in the bilayer, and by a mole fraction of FITC-PE of 0.1 is zero (data not shown). This loss of polarisation results from energy transfer between FITC-PE molecules and indicates that the orientation of fluorescein groups in the plane of the bilayer is random. Therefore, for the labeled ATPase reconstituted into bilayers containing FITC-PE, energy transfer between donor groups on the protein and acceptor groups in the lipid can be expected to occur between donor and acceptor groups whose orientations are random. It has been shown that for a random distribution of donor and acceptors whose orientations are fixed, the appropriate value of K^2 is 0.476 [37]. In the presence of motion, this value will move toward that appropriate for motional averaging, 0.66. Thus, even in the absence of any motion, the error in distance estimates resulting from assuming $K^2 = 2/3$ is likely to be less than 5%.

Results

Fig. 2 shows the fluorescence emission spectrum of NCD-4-labeled ATPase reconstituted into bilayers of DOPC at a lipid-protein molar ratio of 215:1. As reported by Chadwick and Thomas [13] and Pick and Weiss [15], the fluorescence emission spectrum has a maximum at 417 nm, characteristic of an *N*-acylurea. Fig. 2 also shows that when the labeled ATPase is reconstituted into lipid bilayers containing FITC-PE, Forster energy transfer occurs between the NCD-4 and fluorescein groups, as shown by a reduction in intensity of NCD-4 emission and an increased fluorescein emission at 518 nm. The lack of overlap between the emission spectra of NCD-4 and fluorescein means that, in the composite spectra, the intensity at 417 nm is due solely to the NCD-4-labeled ATPase. As shown in Figs. 2 and 3, the intensity of NCD-4 fluorescence decreases with increasing mole fraction of FITC-PE in the bi-

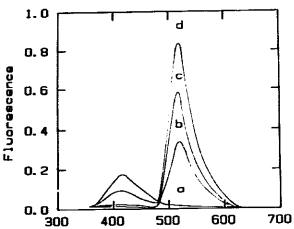

Fig. 2. Fluorescence emission spectra showing energy transfer from NCD-4-labeled ATPase to FITC-PE in reconstituted systems containing DOPC and mole fractions of FITC-PE (a) 0, (b) 0.1, (c) 0.21 and (d) 0.65. The molar ratio of total lipid to ATPase was 210:1. Fluorescence excitation was at 338 nm.

layers. Addition of 0.1 mM vanadate ion to the labeled ATPase causes no change in fluorescence intensity and, as shown in Fig. 3, also results in no change in the fluorescence quenching caused by FITC-PE. Chadwick and Thomas [14] and Pick and Weiss [15] have shown that addition of millimolar concentrations of Ca^{2+} to the labeled ATPase results in a significant reduction in fluorescence, but, again as shown in Fig. 3, addition of 2 mM Ca^{2+} to the labeled ATPase results in no change in the quenching caused by FITC-PE.

If the ATPase is labeled with NCD-4 in the presence of 2 mM Ca^{2+}, the extent of labeling is reduced indicating the presence of both Ca^{2+}-protectable and non-protectable sites [13,15]. Fluorescence quenching by FITC-PE is, however, identical to that observed for the ATPase labeled in the absence of Ca^{2+} (Fig. 3).

To test the accessibility of the NCD-4 labels on the ATPase, and to try to resolve the various labeling sites

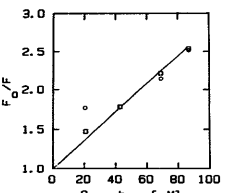

Fig. 4. Stern-Volmer plot of F_0/F against the concentration of added 5-doxylstearic acid for the ATPase labeled with NCD-4 in the absence (□) or presence (○) of 2.0 mM Ca^{2+}.

on the ATPase, we studied quenching of fluorescence by 5-doxylstearic acid. In the simplest cases of dynamic (collisional) or static quenching, fluorescence quenching fits the Stern-Volmer relationship

$$F_0/F = 1 + K_D[Q] \quad (6)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher respectively, $[Q]$ is the quencher concentration and K_D is a constant. As shown in Fig. 4, quenching of the fluorescence of the ATPase labeled with NCD-4 in the presence or absence of Ca^{2+} are experimentally indistinguishable and both give reasonable fits to the Stern-Volmer relationship.

Discussion

$(Ca^{2+} + Mg^{2+})$-ATPase reacts with NCD-4 incorporating two mols of NCD-4 per mol of ATPase into Ca^{2+}-protected sites and one or more mols of NCD-4 into non-specific sites [13–15]. Pick and Weiss [15] reported that there were slight differences in the fluores-

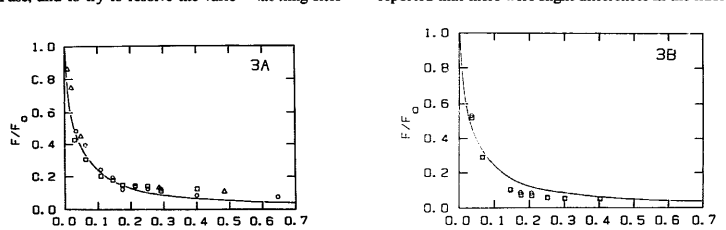

Fig. 3. Fluorescence quenching (F/F_0) of NCD-4-labeled ATPase reconstituted into lipid mixtures at a molar ratio of total lipid/ATPase of 210:1, as a function of the mole fraction of FITC-PE, where F_0 is the fluorescence intensity in the absence of FITC-PE. (A) (○, □) ATPase labeled with NCD-4 in the absence of Ca^{2+} and fluorescence recorded either in the absence of Ca^{2+} (○) or in buffer containing 2 mM Ca^{2+} (□); (△) ATPase labeled with NCD-4 in the presence of 2 mM Ca^{2+} and fluorescence recorded in the absence of Ca^{2+}. (B) ATPase labeled with NCD-4 in the absence of Ca^{2+} and fluorescence recorded either in the absence (○) or presence (□) of 0.1 mM vanadate. Solid lines are theoretical calculations based on the model of Gutierrez-Merino et al. [5], with $d = 5$ and $h = 20$ Å.

cence-emission spectra for the NCD-4 label at the Ca^{2+}-protected and non-specific sites and suggested that one of the Ca^{2+}-protectable sites was relatively hydrophobic. In agreement with the results of Chadwick and Thomas [13], we found that after tryptic cleavage of the labeled ATPase, all the NCD-4 was found on the 24 kDa, A_2 tryptic fragment (data not shown). It has also been shown that the DCCD binding sites are on the 24 kDa tryptic fragment [25]. It has been suggested that this fragment contains the Ca^{2+} binding sites [6]: it contains clusters of negatively charged residues both close to the end of the suggested trans-membranous α-helices (that is, close to the lipid/water interface) and more distant from the membrane surface. These results therefore suggests that NCD-4, like DCCD, labels the ATPase at or close to the Ca^{2+} binding sites [13–15,25,35].

In this paper, we use resonance energy transfer between the NCD-4 sites and FITC-PE to estimate the distance between the sites on the ATPase and the lipid/water interface. Distances measured by resonance energy transfer are always inaccurate both because of uncertainties over the correct value for the orientation factor K^2 and because of uncertainties as to the exact meaning, in molecular terms, of distances measured between bulky fluorescence groups. For energy transfer to lipids labeled in the headgroup region, it seems that assuming a value of 0.66 for K^2 will introduce little error, as described in Materials and Methods. The presence of more than one labeling site on the ATPase could considerably complicate the interpretation of these energy-transfer experiments. If the labeled sites were located at very different distances from the lipid/water interface, the quenching profiles would not fit a simple theory of transfer, because of the sixth-power distance-dependence of efficiency of transfer, as will be demonstrated below. Further, if the sites labeled by NCD-4 were very widely separated, then it would be expected that fluorescence quenching caused by FITC-PE would be different in the presence or absence of Ca^{2+}, since addition of Ca^{2+} to the labeled ATPase quenches the fluorescence from the Ca^{2+}-protectable sites [15]. Similarly, fluorescence quenching caused by FITC-PE would be different for the ATPase labeled in the presence and absence of Ca^{2+}, since the distribution of labeled groups on the ATPase would be different under these different labeling conditions. In fact, as shown in Fig. 3, the pattern of quenching is unaffected by addition of Ca^{2+} to the labeled ATPase and quenching is identical for the ATPase labeled in the presence or absence of 2 mM Ca^{2+}, arguing very strongly that all the labeled sites must be located at similar distances from the lipid/water interface.

In our analysis of energy transfer, we assume, as we did in our analysis of the corresponding data for the ATPase labeled with FITC [7], that all ATPase molecules are equivalent in the bilayer; that is, we assume that no large patches of ATPase molecules are formed from which lipid molecules are excluded. In an analysis of energy transfer for bacteriorhodopsin reconstituted at different lipid/protein ratios, Hasselbacher et al. [26] presented an analysis of the case where large patches of protein molecules were formed, with protein molecules in the center of the patches being far from lipid molecules and so unable to take part in energy transfer to the lipids. This is not the case for the ATPase under the conditions of our experiments. We have recently presented evidence that the ATPase is present in the membrane in aggregates, but that the form of these aggregates is of rows of monomers or dimers of the ATPase, the rows of ATPase molecules being separated by phospholipid [34]. All ATPase molecules would then be in contact with phospholipid. This has been shown directly by East and Lee [16] in studies of the quenching of the tryptophan fluorescence of the ATPase by brominated phospholipids. The non-random distribution of the ATPase molecules could produce a small error in separation distances calculated from energy-transfer measurements, but the error will in our experiments be a constant one, since the lipid/protein ratio was held constant, so that the state of aggregation of the ATPase will also be a constant.

As shown in Fig. 3, the experimental data on the quenching of NCD-4-labeled ATPase by FITC-PE fits well to the theoretical approach of Gutierrez-Merino et al. [7]. As shown elsewhere [7], when the protein donor is located far from the membrane surface, a wide range of pairs of d and h values (Fig. 1) will give equally good fits to the data, with the d and h values chosen so that the distance between the proton donor and the annular shell of lipids surrounding the ATPase is maintained constant. Thus, Fig. 3 shows fits to the model with d and h values of 5 and 20 Å, respectively, but equally good fits to the data can be obtained with, for example, d and h values of 1 and 26 Å, 10 and 9 Å or 15 and 1 Å, respectively.

The experimental data can also be fitted equally well using the approach of Koppel et al. [22], with a distance h between the protein donor and the membrane surface of 20 Å giving a calculated quenching plot identical to that shown in Fig. 3 (Fig. 5). The approximate equations of Dewey and Hammes [23] also fit the data well at low fractions of labeled lipid (below 0.1) with a distance h of between 15 and 20 Å. The observation that three separate methods of calculation give very similar distances suggests that the approximations and assumptions in each of the models used are relatively unimportant.

As described above, more than one site on the ATPase is labeled by NCD-4. We can use the approach of Koppel et al. [22] to explore the likely effect of multiple labeling on the accuracy of the distances we measure.

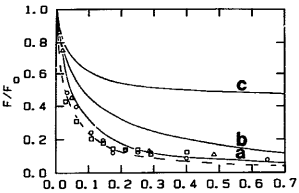

Fig. 5. Simulation of fluorescence quenching using the approach of Koppel et al. [22]. The experimental data (○, □, △) are as in Fig. 3. Broken line, theoretical calculation with $h = 20$ Å. Solid lines, theoretical calculations assuming two sites labeled on the ATPase with equal fluorescence intensities, with one site having $h = 20$ Å and the second site having values of h (Å) of (a), 30, (b), 40 and (c) 50.

For the sake of argument, assume that two sites are labeled on the ATPase, with equal fluorescence intensity. In the absence of any energy transfer between the labels at these two sites, the quenching of each site by energy transfer to FITC-PE will follow Eqn. 1. Fig. 5 illustrates the fluorescence-quenching profiles that would be expected if one site were located a distance of 20 Å from the membrane surface and the other was located at 30, 40 or 60 Å from the surface. It is clear that the calculated profiles, when the second site is located more than approx. 10 Å from the first site, will not fit the experimental data. The fit to the experimental data is better for one or two sites located 20 Å from the surface (Fig. 5, broken line) then for one site located 20 Å from the surface and the other 30 Å from the surface (Fig. 5, curve a). We conclude, therefore, that the likely average position of the sites labeled by NCD-4 is 20 Å from the membrane surface, plus or minus 10 Å.

To probe the accessibility of the NCD-4 sites on the ATPase, we studied quenching with the hydrophobic quencher 5-doxylstearic acid. As shown in Fig. 4, the spin-labeled fatty acid quenches NCD-4 fluorescence. The observations that quenching fits a simple Stern-Volmer relationship and that it is indistinguishable for the ATPase labeled in the presence and absence of Ca^{2+} suggest that all labeling sites are equally accessible to the spin label. Although the mechanism of quenching by nitroxide-free radicals is not yet established, it seems to require a collision between the fluorescent group and the nitroxide, in which the two groups come closer than about 10 Å [27,28]. This would then suggest that the NCD-4 sites on the ATPase are not buried within the ATPase, but are within a few Å of the surface of the protein. With a value for d of between about 1 and 5 Å, the NCD-4 sites would then be located about 20–25 Å above the lipid/water interface of the membranes. With such a location for the NCD-4 sites, it is surprising that a hydrophobic fatty acid spin label quenches fluorescence at all. In part, this could reflect the polar nature of the nitroxide group, which results in relatively poor localisation of the nitroxide in the hydrophobic interior of the lipid bilayer: for example, it has been shown that doxylstearic acids have considerable effects on the nuclear magnetic relaxation times for ^{13}C nuclei in the lipid headgroup region of the bilayer [29]. We have also suggested that fatty acids can bind directly to the ATPase at sites other than those at the lipid/protein interface [30] and quenching might follow from binding at such sites.

The suggested position of the NCD-4 sites can be compared with the location of other sites on the ATPase. From measurements of energy transfer between FITC-labeled ATPase and lipids labeled with rhodamine isothiocyanate, we estimated that the FITC group was about 80–100 Å above the plane of the rhodamine groups, the exact value depending on the value of d, the distance between the site and the circumference of the ATPase. Because of the hydrophobic nature of the rhodamine group, it is likely that the lipid headgroup is folded back, so that the rhodamine label is located close the glycerol backbone region of the lipid bilayer [7]. Since the FITC group is thought to occupy the ATP binding site, the ATP binding site would then be located about 80–100 Å above the plane of the glycerol backbone groups. Teruel and Gómez-Fernández [10] carried out fluorescence energy-transfer experiments very similar to those of Gutierrez-Merino et al. [7] and, analyzing their data by the approach of Koppel et al. [22], obtained a much smaller separation distance of 34–42 Å. This difference, in fact, arises from the use of a quantum yield of 0.16 for FITC-labeled ATPase, determined by Teruel and Gómez-Fernández [22], compared to values of 0.65 and 0.60 determined by Gutierrez-Merino et al. [7] and Highsmith and Murphy [8], respectively. With a quantum yield of 0.65 and the corresponding R_0 value of 55 Å [7], the data of Teruel and Gómez-Fernández [10] give a value of h of 80 Å using the approach of Koppel et al. [22]; analysis of the data of Gutierrez-Merino et al. [7] using the approach of Koppel et al [22] gives a value of h of 78–85 Å.

If the FITC and NCD-4 sites are 80 and 20 Å, respectively above the membrane surface, then the separation between the FITC and NCD-4 sites will be approx. 60 Å. Since the fluorescein group is less hydrophobic than the rhodamine group, it is likely that the fluorescein group of the labeled phosphatidylethanolamine is located in the headgroup region of the bilayer, rather than in the glycerol backbone region; if this is the case, then the FITC-NCD-4 separation will be somewhat less than 60 Å. Since Scott [9] has reported that the two Ca^{2+} binding sites on the ATPase are located 11 Å apart and 47 Å from the FITC site, this is consistent with the idea that NCD-4 labels the ATPase

at or close to the Ca^{2+} binding sites [13,15]. This estimate of the position of the Ca^{2+} binding sites on the ATPase can also be compared to estimates made on the basis of charge effects on Ca^{2+} binding to the ATPase [31]. In these experiments it was shown, for example, that binding of 0.2 mM of the positively charged drug methdilazine to the ATPase-containing membrane decreased the p*Ca* value giving half-maximal activity from 6.53, in the absence of drug, to 6.1 in its presence, at pH 7.2, corresponding to an increase of 13.3 mV in the potential at the Ca^{2+} binding sites on the ATPase. Under the ionic concentration conditions of the experiment, it is possible to calculate from the binding constant of methdilazine to the lipid bilayer that 0.2 mM methdilazine will generate a positive surface potential of 49 mV on the lipid portion of the membrane. The potential will decrease with increasing distance from the surface of the membrane and it was estimated that the Ca^{2+} binding sites must be 20–30 Å from the surface for the potential at the sites to be 13.3 mV [31]. This estimate is pleasingly close to that made from the energy-transfer experiments reported here.

We conclude, therefore, that the sites labeled by NCD-4 on the ATPase are located approx. 20 Å above the plane of the lipid/water interface, and that this is also very likely to be the location of the Ca^{2+} binding sites. In terms of the model for the ATPase presented by Brandl et al. [6], this would locate the Ca^{2+} binding sites on that part of the Ca^{2+} binding domain contained in the A_2 tryptic fragment, not close to the lipid/water interface, but at the interface between the calcium binding domains and the transduction domain. A large cluster of glutamate residues occur in this region [6].

The kinetics of the ATPase have been interpreted in terms of a two-conformation model, with one conformation, E1, having two Ca^{2+} binding sites of high affinity exposed on the outer surface of the SR and the other conformation, E2, having two Ca^{2+} binding sites of low affinity exposed to the inner surface. The conformation change between E1 and E2 is the transport event (see Ref. 32). Vanadate ions bind to the ATPase and have been proposed to stabilise the ATPase in the E2 conformation, probably by acting as an analogue of orthophosphate [33]. Previously, we have shown that addition of vanadate to FITC-labeled ATPase results in no movement of the FITC group relative to the lipid/water interface, suggesting that the ATP binding site does not move significantly with respect to the lipid/water interface in the E1–E2 transition [7]. Pick and Weiss [15] have shown that vanadate binds to the ATPase labeled with NCD-4 and reverses the effects of Ca^{2+}, so that vanadate probably also stabilised the E2 conformation of the labeled ATPase. The observation that addition of vanadate to the labeled ATPase results in no significant change in energy transfer between the NCD-4 groups on the ATPase and labeled lipid (Fig. 3) then suggests that the Ca^{2+} binding sites on the E1 form of the ATPase also do not move significantly with respect to the lipid/water interface in the E1–E2 transition.

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