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Distances between the functional sites of sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase and the lipid/water interface

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Measurements of fluorescence energy transfer have been performed to determine the distance between the lipid-water interface and the ATP-binding site in the (Ca^{2+} + Mg^{2+})-ATPase from sarcoplasmic reticulum. The calculated distance between the donor, FITC bound to the protein (nucleotide binding-site marker), and the acceptor, rhodamine-5′-isothiocyanyldipalmitoylphosphatidylethanolamine (RITC-DPPE) incorporated in the membrane, was in the range of 34–42 Å. In addition the distance between the high affinity Ca^{2+}-binding sites and the lipid/water interface has been calculated by luminescence energy transfer from Tb^{3+} bound to the Ca^{2+} sites to RITC-DPPE included in the membrane, and it was approx. 10 Å.

Introduction

The (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum is one of the most well-characterized ion transport systems at the molecular level. The enzyme catalyzes the active transport of 2 calcium ions/ATP hydrolyzed (see Refs. 1, 2 for reviews). The primary structure of this enzyme has been deduced very recently, from its complementary DNA sequence [3], and their separate profile structures determined by X-ray and neutron diffraction [4]. However, a very important functional issue, not well clarified yet, is the precise location of the active site in the primary and tertiary structure of the enzyme.

This problem has been very recently addressed by measuring the distances between the functional sites of this ion pump using luminescence energy transfer measurements [5]. This was done taking advantage of the enzyme labelling with fluorescein isothiocyanate, which has been shown to react specifically, in certain experimental conditions, with the ATP binding site of the (Ca^{2+} + Mg^{2+})-ATPase [6,7], and the binding of Tb^{3+} at the Ca^{2+} sites.

We present in this paper our studies on the topography of the functional sites of the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase. We have measured the distances between the ATP binding site, labelled with FITC, and RITC-DPPE incorporated in the membrane, by fluorescence energy transfer, and also the distance between the high-affinity Ca^{2+} binding sites, labelled with Tb^{3+}, and RITC-DPPE situated in the membrane, by luminescence energy transfer.

Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DPPE, dipalmitoylphosphatidylethanolamine; FITC, fluorescein 5′-isothiocyanate; RITC, rhodamine 5′-isothiocyanate; RITC-DPPE, rhodamine-5′-isothiocyanyldipalmitoylphosphatidylethanolamine; TNP-N, trinitrophenylnucleotide.

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Materials and Methods

Chemicals. FITC, RITC and DPPE were purchased from Sigma, Poole, Dorset, U.K., and all other analytical grade from Merck, Darmstadt, F.R.G.

Preparations. Sarcoplasmic reticulum vesicles were prepared from rabbit back and leg muscles as previously described [8]. The $(Ca^{2+} + Mg^{2+})$-ATPase was purified by method 2 of Meissner et al. [9].

FITC labelling. The purified enzyme was labelled with FITC, essentially as described by Pick and Karlish [8]. Briefly, FITC was added to 2.7 mg of protein suspended in 50 mM Tris-HCl, 80 mM KCl, 0.2 M sucrose (pH 8.8) to give a final FITC concentration of 30 μM. The mixture was incubated at 25°C during 20 min. The reaction was stopped by centrifuging through a column of Sephadex G-50 packed in a 1 ml syringe and equilibrated in 20 mM Mops (pH 7.0) and 80 mM KCl. The FITC/ATPase molar ratio obtained was 0.8–1.1 in all experiments, assuming that 100% of the proteins are ATPase in this purified and reconstituted preparation, and that the molecular weight of the ATPase is 109783 [3].

Synthesis of RITC-DPPE. RITC-DPPE was prepared essentially as described by Vanderwerf and Ullman [10]. 50 μl of rhodamine isothiocyanate was added to 5 ml of chloroform/methanol (2:1, v/v), 50 μmol dipalmitoylphosphatidylethanolamine and 500 μmol triethylamine. This mixture was stirred for 4 h at 35°C. When no free DPPE was detected by reaction with ninhydrin on thin-layer chromatography, the solvents were completely removed at low pressure and the dry product was redissolved in a small volume of chloroform. RITC-DPPE was isolated by preparative thin-layer chromatography on silica gel.

Reconstitution of the $(Ca^{2+} + Mg^{2+})$-ATPase with egg yolk phosphatidylcholine plus RITC-DPPE. The method of detergent dilution essentially as described by Johannson et al. [11] was used. Variable amounts of RITC-DPPE were mixed with egg yolk phosphatidylcholine in chloroform/methanol (2:1, v/v) given a total amount of 1.3 μmol of lipid in all the cases. The mixture was dried under dry nitrogen and further evaporated under vacuum during 2 h. 50 μmol of 1 M KCl, 0.25 M sucrose, 50 mM phosphate buffer (pH 8.0) and 0.5 mg of cholate were added to the dry lipid and thorough mixed to obtain a clear solution. Then, it was sonicated for 1 min in a bath sonicator and chilled on ice. To this lipid solution, 0.135 mg of purified ATPase (labelled with FITC) were added and the mixture was incubated on ice for 90 min. The lipid substitution was stopped by diluting this mixture to 2 ml with 80 mM KCl, 0.1 mM EGTA (pH 7.0) at 4°C. Aliquots of this suspension were used to measure phosphatase activity, protein concentration, and also for the fluorescence energy transfer experiments. In the case of the luminescence energy transfer experiments, where a higher concentration of protein was needed, after the incubation period in the presence of cholate, the labelled and reconstituted membranes were recovered by centrifugation in a discontinuous sucrose gradient as follows: 1 ml of the incubation mixture containing the protein, was placed atop of a centrifuge tube containing 1 ml of 50% sucrose, in 80 mM KCl, 20 mM Mops (pH 7.0) and 2 ml of 25% sucrose in the same buffer. After centrifugation at 4°C, during 4 h at 50000 r.p.m., in a Beckman SW-60Ti rotor, the band formed was recovered, resuspended in 80 mM KCl, 20 mM Mops (pH 7.0), and further centrifuged at 4°C during 40 min, at 38000 r.p.m. The pellet was resuspended in the same buffer and the protein and RITC-DPPE concentration assayed.

Assays. Protein concentration was assayed after the procedure of Lowry et al. [12] using bovine serum albumin as standard. The amount of FITC bound to the protein was estimated by absorbance taking a molar absorbance coefficient of 80000 $M^{-1} \cdot cm^{-1}$ in 0.1 M NaOH, 1% SDS [6].

ATPase activity was assayed as previously described [8]. The *p*-nitrophenylphosphatase activity of the enzyme was used as a control for the FITC-inhibited ATPase, since this activity was measured by adding 3.4 μg of protein to 1 ml of medium containing 10 mM *p*-nitrophenyl phosphate, 0.6 mM $CaCl_2$, 0.6 mM EGTA, 5 mM $MgCl_2$, 0.7 M sucrose and 50 mM Tris-HCl (pH 7.5). The appearance of *p*-nitrophenol was followed at 400 nm and its concentration was calculated using a molar absorbance coefficient of 10000 $M^{-1} \cdot cm^{-1}$ [13].

The RITC-DPPE incorporated into the membrane was estimated by absorbance, using a molar absorbance coefficient of 81 065 $M^{-1} \cdot cm^{-1}$ at 547 nm in ethanol [14].

Fluorescence measurements. Steady-state fluorescence measurements were done in either a Shimadzu RF-540 or in a Perkin-Elmer MPF-44B. The anisotropy parameter (E_A) was calculated according to Fleming et al. [15].

Fluorescence energy transfer between FITC-ATPase acting as donor and RITC-DPPE acting as acceptor, was estimated by recording the emission spectrum of each sample exciting at 480 nm. Quenching efficiencies were calculated from the fluorescence intensities at 520 nm and comparing with a standard sample without RITC-DPPE.

Luminescence energy transfer studies were done in a medium containing 80 mM KCl, 20 mM Mops (pH 7.0), treated with Chelex-100 to eliminate the contaminant Ca^{2+}. Purified (Ca^{2+} + Mg^{2+})-ATPase reconstituted with egg yolk phosphatidylcholine/RITC-DPPE, was added to a final concentration of 0.7 mg/ml and mixed with 1 μM $TbCl_3$ (Ca^{2+} binding sites marker) so that the final ATPase/Tb^{3+} molar ratio was 6.4, assuming again that ATPase is 100% of the total protein and it has a molecular weight of 109 763 [3]. Measurements were carried out in a Perkin-Elmer LS-5 luminescence spectrometer equipped with a magnetic stirred cell, at several concentrations ratios of acceptor-to-donor, by exciting the protein at 295 nm, so that energy transfer occurs from tryptophan to Tb^{3+}, and from Tb^{3+}, acting as donor, to RITC-DPPE, acting as acceptor. The emission of Tb^{3+} luminescence was measured at 550 nm. The decay curves of luminescence of Tb^{3+} bound to the (Ca^{2+} + Mg^{2+})-ATPase were obtained by measuring the luminescence intensity with varying delay times of emission.

Results

Fluorescence energy transfer between FITC bound into the nucleotide binding site, and RITC-DPPE at the lipid/water interface

Labelling of the (Ca^{2+} + Mg^{2+})-ATPase by FITC, under the experimental conditions described in Materials and Methods resulted in a total inhibition of the ATPase activity (see Table I and Refs. 6 and 16), although the enzyme is still able to catalyze the hydrolysis of pseudosubstrates as acetylphosphate or *p*-nitrophenyl phosphate which do not interfere with the FITC binding to the ATP site [16]. Hence the *p*-nitrophenylphosphatase activity was used to monitor that the enzyme was not damaged after reconstitution. Table I shows that the activity was not changed by the process of reconstitution in egg yolk phosphatidylcholine plus RITC-DPPE.

The location of FITC at the catalytic site is supported by competition with ATP binding [16]. It appears that the covalent bound of FITC to the protein occurs through a lysine residue near the T_1 tryptic cleavage site of the ATPase [17].

In this case in which FITC-ATPase acts as donor and RITC-DPPE as acceptor, the geometry model proposed by Koppel et al. [18] was adopted. After this model for membrane-bound chromophores, acceptors are assumed to be randomly distributed in the surface of a planar membrane

TABLE I

ATPase AND *p*-NITROPHENYLPHOSPHATASE ACTIVITIES OF PURIFIED (Ca^{2+} + Mg^{2+})-ATPase USED AS CONTROL, LABELLED WITH FITC AND AFTER RECONSTITUTION IN EGG YOLK PHOSPHATIDYLCHOLINE PLUS RITC-DPPE

The enzymatic activities are given in I.U./mg of protein, at 25°C and pH 7.0.

Enzyme	Activity							
	Purified ATPase	Molar ratio, rhodamine/fluorescein						
		0	46	23	11.5	5.7	2.9	1.4
ATPase	4.1	0.01	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
p-Nitrophenylphosphatase	0.15	0.041	0.039	0.047	0.044	0.041	0.044	0.056

whereas donors might be situated at a distance, h, from the membrane surface, so that h would be positive if the donor is inside the membrane or negative if it is outside, and then the energy transfer efficiency is related to the distance separating the donor and acceptor molecules by the following equation, proposed by Koppel et al. [18]

$$\langle E\rangle^{-1} = \sigma^{-1.1}\left[\frac{0.62}{\pi R_0^2} \times \frac{R-h}{R}\exp(-0.34r_0 + 1.63r_0^2)\right]^{+1.1} + 1 \quad (1)$$

where $\langle E\rangle$ is the ensemble average of energy acceptors, σ is the surface density of energy acceptors, R is the radius of the vesicle, h is the distance between the donor and the membrane surface, R_0 is the distance between donor and acceptor when the energy transfer is 50%, and r_0 is defined as

$$r_0 = r_{min}/R_0 \quad (2)$$

where r_{min} is the distance of closest approach of donor and acceptor. In our case the effect of curvature is neglected since the vesicles are big enough to allow this (> 0.2 μm, as observed by negative contrast in electron microscopy) and hence $(R - h/R) \cong 1$.

Data were fit to a complete theory of energy transfer which takes into account all the possible relative orientations of donor and acceptor and thus variations in the orientation factor, k^2 [18]. Bounds for k^2 were established by calculating k^2 for the case in which the dipolar moments of donor and acceptor are in the plane of the membrane, and also when the dipolar moment of the donor is perpendicular to the plane of the membrane (see Equations 3A and 5A of Ref. 18). Maximum and minimum values for r_0 were obtained in this way (Table II). Using the values given in Table II for the different parameters involved, a series of curves of $\langle E\rangle$ as a function of σ were obtained by computations using Eqn. 1 (see Fig. 1). It can be observed in Fig. 1, that the experimental data of energy transfer between FITC-ATPase and RITC-ATPase, are very well fit to a minimum value of r_{min} of approx. 34 Å and to a maximum value of r_{min} of approx. 42 Å.

TABLE II

VALUES OF PARAMETERS USED IN THE CALCULATIONS OF ENERGY TRANSFER

Donor	Acceptor	J_{DA} [a] ($\times 10^{13}$) ($M^{-1}\cdot cm^3$)	Q_D [b]	k^2 [c]	R_0 [d] (Å)
FITC	RITC	1.30	0.160	0.41–0.91	35–40
Tb^{3+}	RITC	1.80	0.210	0.67	41.9

[a] J_{DA} is the overlap integral for the normalized emission and absorption spectra of the donor and acceptor, respectively.
[b] Q_D is the quantum yield of the donor in the absence of acceptor.
[c] k^2 is the orientation factor for the donor-acceptor dipoles.
[d] $R_0 = (J_{DA}k^2Q_Dn^{-4})^{1/6} \times 9.7 \times 10^3$ Å, where n is the refractive index, assumed to be 1.4 [19].

Luminescence energy transfer between Tb^{3+} bound to the Ca^{2+}-binding site and RITC-DPPE at the lipid/water interface

Tb^{3+} has been used as a Ca^{2+} analog with luminescence properties. It has been shown that Tb^{3+} competes with Ca^{2+} for the high-affinity Ca^{2+} binding-transport sites of the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum [20].

The luminescence amplitude of Tb^{3+} bound to the ATPase at stoichiometric ratios is relatively very low when excited indirectly via aromatic protein residues by ultraviolet light [5–20] if it is compared to the enhancement obtained when

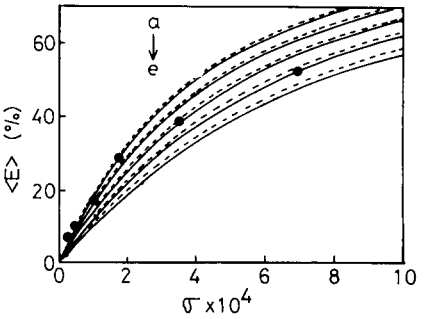

Fig. 1. Variation of energy transfer efficiency, $\langle E\rangle$, with surface density, σ, of acceptors for the FITC-RITC pair. Experimental (●) and theoretical data are shown. The continuous lines show the data of $\langle E\rangle$ calculated taking $r_0 = 35$ Å, at the following distances of closest approach of donor and acceptor, r_{min}: a, 30; b, 32; c, 34; d, 36; and e, 38 Å. The dashed lines show the data of $\langle E\rangle$ calculated taking $R_0 = 40$ Å, at the following values of r_{min}: a, 38; b, 40; c, 42; d, 44; and e, 46 Å. Protein concentration was 4.7 mg/ml.

bound to other molecules like dipicolinic acid or troponin C [5]. However, it was necessary to use this route of excitation in order to obtain the resonance energy transfer to RITC, and in the experimental conditions used here, the signal obtained was good enough to obtain reproducible measurements. Note that the Tb^{3+} concentration was kept low enough (1 μM) to maintain the ATPase/Tb^{3+} molar ratio at 6.4, and that under these conditions Tb^{3+} is bound to the Ca^{2+} sites [20,21]. On the other hand, binding to phospholipids would be very unlikely since ATPase has been reconstituted in egg yolk phosphatidylcholine.

In order to calculate the energy transfer between Tb^{3+} and RITC, the luminescence decay curves of Tb^{3+} in the presence and in the absence of RITC were measured. The fluorescence intensity $F(t)$ of the donor in an infinite plane at time t following a very short light flash will be given (see Refs. 22–24) by

$$F(t) = F(0)\exp(-t/\tau_D)\exp[-\sigma S(t)] \quad (3)$$

$$S(t) = \int_{r_{min}}^{\infty} \{1-\exp[-(t/\tau_D)(R_0/r)^6]\} 2\pi r dr \quad (4)$$

where $\exp[-\sigma S(t)]$ is the energy transfer term, $F(0)$ is the initial fluorescence intensity, σ is the surface density of energy acceptors, τ_D is the lifetime of the donor, r is the distance from the donor to an acceptor, and r_{min} is the distance of closest approach of donor and acceptor.

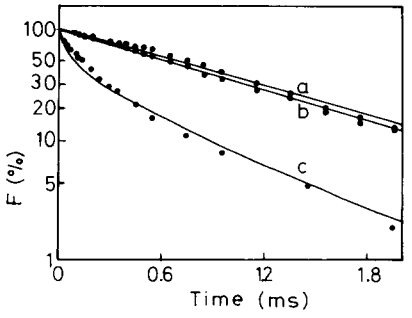

Fig. 2. Exponential decay curves of Tb^{3+} luminescence bound to the reconstituted ATPase including RITC-DPPE in the membrane. The calculated values (———) and the experimental data (●) were obtained at the following values of surface density, σ, of acceptors: a, 0; b, $8\cdot10^{-6}$, and c, $2.04\cdot10^{-4}$. Tb^{3+} and protein concentrations were 1 μM and 0.7 mg/ml, respectively.

In order to calculate R_0, Q_D (the donor quantum yield) was assumed to be 0.21 [5]; k^2 was supposed to be 2/3 [5] since in this case emission occurs from an excited $^5D_4$ state which has degenerate excited levels resulting in isotropic symmetric and the acceptor, i.e. RITC-DPPE, has a very low value of anisotropy (0.09).

Through numerical integration the theoretical decay curves of $F(t)$ for different values of σ were calculated (Fig. 2). The best fitting corresponded to a value of 10 Å for r_{min} (Fig. 2).

Discussion

Electron microscopy studies on the structure of the (Ca^{2+} + Mg^{2+})-ATPase showed long ago, a globule attached to the membrane by a narrow stalk [25], and that there are transmembrane portions of the protein was evident from freeze-fracture micrographs [26]. Besides, it was concluded from peptide-sequence modelling of the sarcoplasmic reticulum calcium pump protein that about 60% of the total amino acid content is external to the hydrocarbon core of the sarcoplasmic reticulum lipid bilayer [27]. From the studies on the molecular dimensions of the ATPase monomers it has been suggested that it would be a cylinder with an aqueous portion of about 40–60 Å diameter and about 60 Å length and an intramembranous portion of 40 Å in diameter and 40 Å in length [28–30], with an overal length of approx. 100 Å.

More detailed pictures on the structure of the ATPase and its organization in the membrane have emerged very recently as a result of the total amino acid sequencing from its complementary DNA sequence and X-ray and neutron diffraction studies. In the sequencing work carried out by MacLennan and co-workers [3] it was found that the ATPase may be divided in four portions. The one formed by the extramembranous cytoplasmic domains is the biggest, with about 516 amino acid residues (60%) of the total amino acid, including the Ca^{2+}-binding stalk with about 100 residues (11.5%) links the cytoplasmic region with the intramembraneous portion of about 200 residues (23%), and finally a small lumen portion of about 50 residues including the shorter segments linking

the intramembranous segments between them.

On the other hand, it was concluded from X-ray and neutron diffraction studies by Herbette et al. [4] that about 50% of the protein volume is external to the lipid bilayer, with 40% located in the hydrocarbon core (excluding the headgroup regions), with an asymmetry in the distribution of the protein which is in agreement with the model suggested from amino acid sequence.

After the results presented in this paper it seems that the ATP binding-site where FITC is supposed to bound, will be located at about 34–42 Å of the lipid-water interface where rhodamine (RITC-DPPE) is situated. Hence this site would be relatively far away from the membrane. On the other hand, the Ca^{2+}-binding site where Tb^{3+} is bound has been found to be at only 10 Å from rhodamine (RITC-DPPE) and hence it must be situated very close to the lipid/water interface.

Two different estimates of the distance between the ATP and high-affinity Ca^{2+} sites have been previously done. Highsmith and Murphy [32] concluded that the lower limit for this distance would be 26 Å whereas Scott [5] found a distance of about 30 Å between the Ca^{2+} sites and TNP-N binding site and about 40 Å between the Ca^{2+} sites and the FITC-binding site. Assuming the distances calculated in this paper and by triangulating our estimate for the distance between Tb^{3+} sites and FITC site would be of about 35–43 Å, which is in very close agreement with those previous reports, especially with that of Scott [5], which also used Tb^{3+} and FITC. A quantitative comparison between our results and the distances that could be estimated from the primary sequence data [3] is more difficult, since to derive a tertiary structure from the amino acid sequence is not a straightforward thing. Nevertheless MacLennan et al. [3] certainly located the Ca^{2+}-binding stalk relatively close to the membrane whereas the ATP- or FITC-binding site is situated well outside the membrane.

The location of the high-affinity Ca^{2+}-binding sites near the membrane and hence close to the tryptophans may explain why Ca^{2+} binding produces changes in protein intrinsic fluorescence [31]. However, it must be interesting to study how the ATP-binding site and the Ca^{2+}-binding site may interact during the catalytic process, or they seem to be located far apart, as was shown by Scott [5].

Future experiments using fluorescence energy transfer and other techniques may clarify this and other interesting problems concerning the (Ca^{2+} + Mg^{2+})-ATPase.

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