Distances between the Functional Sites of the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase of Sarcoplasmic Reticulum*

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Terrence L. Scott
From the Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114

Luminescence energy transfer measurements have been used to determine the distances between the two high affinity Ca\(^{2+}\) binding-transport sites of the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase of skeletal muscle sarcoplasmic reticulum. The lanthanide Tb\(^3+\) situated at one high affinity Ca\(^{2+}\) site was used as the transfer donor, and acceptors at the other Ca\(^{2+}\) site were the lanthanides Nd\(^3+\), Pr\(^3+\), Ho\(^3+\), or Er\(^3+\). Terbium bound to the enzyme was excited directly with a pulsed dye laser. Analysis of the changes in the terbium luminescence lifetime due to the presence of the acceptor indicates that the distance between the Ca\(^{2+}\) sites is 10.7 Å.

The distance between the Ca\(^{2+}\) sites and the nucleotide-binding catalytic site was determined using Tb\(^3+\) at the Ca\(^{2+}\) sites and either trinitrophenyl nucleotides (TNP-N) or fluorescein 5-isothiocyanate (FITC) in the catalytic site as energy acceptors. The \(R_0\) values for the Tb-acceptor pairs are -30 and -40 Å for TNP-N and FITC, respectively. The distance between Tb\(^{3+}\) at the Ca\(^{2+}\) sites and TNP-ATP at the nucleotide site is ~35 Å and that between the Ca\(^{2+}\) sites and the FITC labeling site is ~47 Å. Considerations of the molecular dimensions of the ATPase polypeptide indicate that while the two Ca\(^{2+}\) sites are close to each other, the Ca\(^{2+}\) sites and the nucleotide site are quite remote in the three-dimensional structure of the enzyme.

The central problem of the mechanism of ion transport systems involves understanding on the molecular level how the scalar energy derived from the hydrolysis of ATP is transduced to the vectorial movement of ions against their electrochemical gradient. While much information has been obtained on the kinetics of specific reaction steps of the ATP hydrolysis cycle, synthesis of structure-function relationships in these transport proteins has only begun to emerge in recent years.

The (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase of sarcoplasmic reticulum is one of the most well-characterized ion transport systems on the molecular level. The enzyme catalyzes the active transport of 2 calcium ions/ATP hydrolyzed (for review, see Ikemoto et al., 1981). Recent work has begun to elucidate the primary structure of portions of the ATPase polypeptide (Allen et al., 1980), but little is known concerning the precise location of the ion transport sites or the catalytic site in the primary structure of the enzyme. Further, the location of these functional regions in the tertiary structure of the pump complex is unknown. Molecular models of the energy transduction mechanism have been proposed, but it is clear that resolution of these alternatives will require a more extensive knowledge of the structural relations between these functional regions.

The work reported here extends initial use of lanthanide ions (Ln\(^{3+}\) or M\(^{3+}\)) as Ca\(^{2+}\) analogs in the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase (Highsmith and Head, 1983; Scott, 1984; Scott, 1985). The luminescence properties of Tb when situated at the high affinity Ca sites have been exploited to measure the distance between the functional sites via luminescence energy transfer. A lower limit of 26 Å for the distance between the Ca\(^{2+}\) and nucleotide sites has been reported (Highsmith and Murphy, 1984). The measurements reported here provide precise measurements of the distances among the functional sites of an ion transport protein, which should stimulate further discussions on the molecular mechanism of biological energy transduction.

**EXPERIMENTAL PROCEDURES**

*Preparations—SR vesicles were prepared essentially as previously described (Ikemoto et al., 1981). The Ca\(^{2+}\)-ATPase was partially purified using 0.2 mg of deoxycholate/mg of SR protein, essentially by the method of Meissner et al. (1973).

Assays—ATP hydrolytic activity was determined as previously described (Scott, 1984). Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard and by absorbance, using A (1%, 280 nm, 1 cm) = 10.0 in 1% sodium dodecyl sulfate (Thorley-Lawson and Green, 1977).

Luminescence Measurements—Luminescence studies were done in a medium containing 50 mM MOPS-K\(^+\), pH 6.8, 100 mM KCl, 5 mM MgCl\(_2\), and SR vesicles or purified ATPase (0.2-1.0 mg ml\(^{-1}\)) at various concentrations of added TbCl\(_3\). Steady-state luminescence was measured with a Spex Fluorolog fluorometer and time-resolved decay curves were obtained using direct laser excitation and collection apparatus as described (Scott, 1984; Wang et al., 1982). Ln\(^{3+}\)-Ln\(^{3+}\) energy transfer experiments were typically done at 2.0 µM Tb\(^{3+}\), 4.0 µM Ln\(^{3+}\) acceptor, 1.0 mg ml\(^{-1}\) ATPase. Assuming that all the lanthanide ions have approximately the same binding constants, the above concentrations would yield the following distribution of complexes: 0.04, 0.32, and 0.64 for Tb\(_2\)-E, Tb-Ln\(_2\)-E, and Ln\(_2\)-E, respectively. The Tb\(_2\)-E complex has the unperturbed lifetime of 1 ms, the Tb-Ln\(_2\)-E complex the energy transfer shortened lifetime, and the Ln\(_2\)-E complex has no luminescence. The measurements were done at several concentration ratios of acceptor-to-donor and the data reported are for the short lifetime component. The control for the effect of Ln\(^{3+}\) binding per se was the luminescence of Tb-AL-E, where Ln\(^{3+}\) has insignificant spectral overlap with Tb-AL emission.

Overlap integrals were calculated by Simpson quadrature implemented on a PDP 11/44 computer, using spectral measurements at 1.0-nm increments. Acceptor absorption spectra were recorded with either a Perkin-Elmer Lambda 3 spectrophotometer or the Spex instrument. The program was tested by comparison of calculated overlap integrals with published values for the donor-acceptor pairs IAEADANS-TNP-ATP (Tao and Lamkin, 1981) and TbDPA-tdrhodamine B and TbDPA-b-NBD diethanolamine (Thomas et al., 1978) and the agreement was within 2%.

1 The abbreviations used are: Ln\(^{3+}\), ions of the lanthanide series; SR, sarcoplasmic reticulum vesicles; FITC, fluorescein 5'-isothiocyanate; TNP-N, 2'(3')-O-(2,4,6-trinitrophenyl)adenosine nucleotides; IAEADANS, N-(iodoacetetyl)-N'-(6-sulfo-1-naphthyl)ethylenediamine; DPA, dipicolinic acid; NBD, nitrobenzodioxazole; C\(_{12}\)Es, dodecyl octaoxyethylene ether alcohol; Tb-C, troponin C; MOPS, 3-(N-morpholino)propanesulfonic acid.
FITC Labeling—The SR or purified enzyme was incubated with FITC at various ratios at pH 7.5 or 8.0 essentially as described by Pick and Karlish (1980). Unreacted FITC was removed by treatment with Dowex-1. The amount of label incorporated was determined from the absorbance of the fluorescein chromophore, using \( A_{\text{oe}} = 8 \times 10^4 \text{m}^{-1} \text{cm}^{-1} \) (Pick and Karlish, 1980).

FITC and TNP—Nucleotides were purchased from Molecular Probes, and the chloride salts of Tb\(^3+\), Pr\(^3+\), Nd\(^3+\), Ho\(^3+\), and Er\(^3+\) from Alpha Ventron.

RESULTS AND DISCUSSION

In order to measure the Ca\(^{2+}\)-Ca\(^{2+}\) intersite distance, Tb\(^{3+}\) situated at one Ca\(^{2+}\) site was used as the donor, and various lanthanides were used as acceptors at the other Ca\(^{2+}\) site. The spectral parameters used for the calculation of \( R_0 \) and \( R \) values for the Tb-lanthanide pairs are given in Table I. Calculation of \( R_0 \) used the spectral overlap integrals reported by Rhee et al. (1981) and the quantum yield, \( Q \), was determined from the measured lifetime of the Tb-ATPase complex (1.0 ms; Scott, 1984) and the intrinsic lifetime (4.75 ms; Stein and Wurzberg, 1975) as \( Q = 1.0/4.75 \), or 0.21. It is evident from Table I that the site-site distances determined using the four different acceptors agree quite well, with a mean value of 10.7 Å. While this is similar to the Ca\(^{2+}\)-Ca\(^{2+}\) site distances of other Ca\(^{2+}\) binding proteins (Rhee et al., 1981; Snyder et al., 1981; Wang et al., 1982), there is no evidence for a parvalbumin-like helix-coil-helix structure in the hydrophilic portions of the ATPase which have been sequenced (Allen et al., 1980). The presumed 2 Ca\(^{2+}\) binding sites/chain are thus quite close within the tertiary structure of the ATPase. Addition of the detergent C\(_{18}\)E\(_{8}\) did not affect the measured lifetimes or distances, suggesting that energy transfer did not take place intermolecularly.

TNP nucleotides have been shown to bind to the catalytic site of the ATPase with a binding constant of \( \sim 5 \times 10^6 \text{M}^{-1} \) (Dupont et al., 1982; Watanabe and Inesi, 1982). This is about one order of magnitude higher than that of ATP for this site, making it an excellent nucleotide analog for the catalytic site.

The binding of TNP-nucleotides to the ATPase was measured in order to measure the Ca\(^{2+}\)-Ca\(^{2+}\) intersite distance, Tb\(^{3+}\) situated at one Ca\(^{2+}\) site was used as the donor, and various lanthanides were used as acceptors at the other Ca\(^{2+}\) site. The spectral parameters used for the calculation of \( R_0 \) and \( R \) values for the Tb-lanthanide pairs are given in Table I. Calculation of \( R_0 \) used the spectral overlap integrals reported by Rhee et al. (1981) and the quantum yield, \( Q \), was determined from the measured lifetime of the Tb-ATPase complex (1.0 ms; Scott, 1984) and the intrinsic lifetime (4.75 ms; Stein and Wurzberg, 1975) as \( Q = 1.0/4.75 \), or 0.21. It is evident from Table I that the site-site distances determined using the four different acceptors agree quite well, with a mean value of 10.7 Å. While this is similar to the Ca\(^{2+}\)-Ca\(^{2+}\) site distances of other Ca\(^{2+}\) binding proteins (Rhee et al., 1981; Snyder et al., 1981; Wang et al., 1982), there is no evidence for a parvalbumin-like helix-coil-helix structure in the hydrophilic portions of the ATPase which have been sequenced (Allen et al., 1980). The presumed 2 Ca\(^{2+}\) binding sites/chain are thus quite close within the tertiary structure of the ATPase. Addition of the detergent C\(_{18}\)E\(_{8}\) did not affect the measured lifetimes or distances, suggesting that energy transfer did not take place intermolecularly.

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Fluorescein 5-isothiocyanate has been used as a fluorescent monitor of such binding (Pick and Karlish, 1980). The site of FITC incorporation has been localized at lysine residue 10 of the major tryptic fragment B (Mitchinson et al., 1982).

Since the luminescence amplitude of Tb\(^{3+}\) bound to the ATPase at stoichiometric ratios is very low when excited indirectly via aromatic protein residues by UV light (Highsmith and Head, 1983), Tb emission spectra used for calculation of the spectral overlap integrals were obtained for several different complexes. Tb\(^{3+}\) complexes with dipicolinate (DPA), troponin C (TnC), or the Sr Ca\(^{2+}\)-ATPase at high (50 μM) [Tb\(^{3+}\)] gave qualitatively similar emission spectra upon excitation at 280 nm, albeit with slight differences. The differences in \( R_0 \) values determined using the three emission spectra were insignificant, the extremes differing from the mean by <3%. The absorption spectra of the TNP-N- and FITC-enzyme complexes and the luminescence emission spectrum of DPAs-Tb are shown in Fig. 1. Table II contains the relevant spectral parameters of the two donor-acceptor

![Fig. 1. Spectral overlap of Tb emission and FITC-ATPase and TNP-N-ATPase absorption spectra. Upper panel, solid line, absorption spectrum of TNP-ATP- SR; dotted line, absorption spectrum of FITC-SR. Lower panel, corrected fluorescence emission spectrum of Tb-SR (50 μM TbCl\(_3\), 50 μg/ml SR), excitation at 280 nm. ex. coeff., extinction coefficient.](image-url)

### Table I

<table>
<thead>
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<th>Acceptor</th>
<th>( R_0 )</th>
<th>Efficiency</th>
<th>( R )</th>
</tr>
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<tr>
<td>La(^{3+})</td>
<td>6.63</td>
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<td>10.83</td>
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<tr>
<td>Pr(^{3+})</td>
<td>6.91</td>
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<td>Er(^{3+})</td>
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<td>10.86</td>
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<tr>
<td>Nd(^{3+})</td>
<td>7.97</td>
<td>0.14</td>
<td>10.79</td>
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\( R_{\text{ave}} = 10.72 \pm 0.54 \text{ Å}^a \)

\( ^a \) \( R_0 \) was calculated using \( Q = 0.21, n = 1.40, \chi^2 = 0.67, \) and overlap integrals from Snyder et al., 1981.

\( ^b \) For discussion of uncertainties, see text.

### Table II

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>( J )</th>
<th>( R_0 )</th>
<th>Efficiency</th>
<th>( R )</th>
</tr>
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<tbody>
<tr>
<td>TNP-ATP</td>
<td>2.43 \times 10^{14}</td>
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<td>35.2</td>
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<tr>
<td>FITC</td>
<td>1.32 \times 10^{13}</td>
<td>40.2</td>
<td>0.27</td>
<td>47.4</td>
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\( ^a \) Overlap integrals represent the average \( J \) of the Tb-DPA, Tb-TnC and Tb-SR emission spectra with the acceptor-\( E \) complexes.

\( ^b \) \( R_0 \) calculated using \( Q = 0.21, n = 1.40, \chi^2 = 0.67. \)
pairs; calculated $R_0$ values are $\sim 50$ and $\sim 40$ Å for TNP nucleotides and FITC, respectively. In the absence of energy transfer acceptors, the lifetime of Tb$^{3+}$ bound to the high affinity Ca$^{2+}$ sites of the SR Ca$^{2+}$-ATPase is $1 \text{ ms}$ in aqueous media (Scott, 1984). The use of lifetime analysis circumvents the difficulties incurred when steady-state intensity values are measured, since these are subject to other effects and optical artifacts.

The fact that the fluorescein probe is at a greater distance (Table II) than the TNP-N is understandable on the assumption that it is the fluorescein moiety of FITC that mimics the terbium donor, since emission occurs from an excited $5D_4$ state which has degenerate excited levels resulting in isotropic symmetry and similarly for absorption by the lanthanide acceptors. In this case, $\chi^2$ is restricted to values close to 0.67. In the case of the TNP-N and FITC acceptors, for an isotropic donor and a polarized acceptor, $0.33 < \chi^2 < 1.33$ (Dale and Eisinger, 1975). An additional uncertainty lies in the value of $n$, the refractive index, where $1.33 < n < 1.50$ (O'Hara et al., 1981). Taking into account the extremes of these uncertainties in the calculation of $R_0$, for Tb-E-TNP-N, $30.4 < R < 41.33$ Å and for Tb-E-FITC, $40.4 < R < 55.2$ Å.

Due to the long luminescence lifetime of Tb when bound to the ATPase, it is possible that significant intermolecular energy transfer could occur by lateral diffusion of neighboring acceptor-ATPase complexes into the transfer distance range of the Tb donor during the excited state. The mean path length, $\bar{\chi}$, for diffusion in two dimensions during a time $t$ is $(2Dt)^{1/2}$ (Estep and Thompson, 1979). For $t = 1 \text{ ms}$ and $D = 10^{-8} - 10^{-9}$ cm$^2$ s$^{-1}$ (Peters et al., 1974; Eddie and Fambrough, 1973; Schlessinger et al., 1976), $5 < \bar{\chi} < 140$ Å. For $\bar{\chi} > R_0/3$, diffusion may affect the transfer process (Estep and Thompson, 1979), and the unit cell dimensions for the ATPase and its surrounding lipids are $\sim 100 \times 100$ Å (Napolitano et al., 1983; Taylor et al., 1984). Intermolecular energy transfer due to either diffusion or oligomeric interaction would shorten the apparent distance between the sites. For these reasons, the intramolecular distance between the catalytic and ion transport sites may be even larger than the measured values. However, as above for the Ca$^{2+}$-Ca$^{2+}$ intersite distance, the addition of detergent had no effect on the measured distances, suggesting that the effects of intermolecular energy transfer are minimal. It has been suggested previously, based on other evidence, that the catalytic and transport sites are well-separated in the tertiary structure of the enzyme (Shamoo et al., 1976; Scott and Shamoo, 1982).

Present views of the molecular dimensions of the ATPase monomer suggest a cylinder with an aequous portion of $\sim 40$–60 Å diameter and $\sim 60$–Å length and an intramembranous portion 40 Å in diameter and 40 Å in length (Taylor et al., 1984; Napolitano et al., 1983; Castellani and Hardwicke, 1983). The overall length is $\sim 100$ Å and thus the ionic-catalytic site distance would span $35$–$50\%$ of the total length of the ATPase molecule.

A recent model or the Ca$^{2+}$ pump implies that the transported Ca$^{2+}$ and the nucleotide phosphate would come into contact during energy transduction (Dupont, 1983), which according to the distances measured here would require extensive motion of one or both sites to provide contiguity during the transport cycle. While it remains for further work to understand how energy coupling occurs between two sites which are so distant, it should be noted that the ATP and ouabain binding sites of the (Na$^+$ + K$^-$)-ATPase are $70$–$80$ Å apart on opposite sides of the bilayer and yet interact during the transport cycle (Cantley et al., 1982).

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**REFERENCES**


