Nd$^3+$ and Co$^{2+}$ Binding to Sarcoplasmic Reticulum CaATPase

AN ESTIMATION OF THE DISTANCE FROM THE ATP BINDING SITE TO THE HIGH-AFFINITY CALCIUM BINDING SITES*

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Nd$^3+$ binding to sarcoplasmic reticulum (SR) was detected by inhibition of ATPase activity and directly by a fluorimetric assay. Both methods indicated that Nd$^3+$ inhibited the ATPase activity by binding in the high-affinity Ca$^{2+}$ binding sites. The stoichiometry of binding was about 11 nmol of Nd$^3+$ bound per mg of SR proteins at pNd = 6.8. At higher [Nd$^{3+}$], substantial nonspecific binding occurred. The association constant for Nd$^{3+}$ binding to the high-affinity Ca$^{2+}$ binding sites was estimated to be 2 × 10$^{-9}$ M$^{-1}$.

When the CaATPase was inactivated with fluorescein isothiocyanate (FITC), 5.3 nmol were bound per mg of SR protein. This fluorescent probe is known to bind in the ATP binding site. The stoichiometry of Nd$^{3+}$ binding to FITC-labeled CaATPase was the same, within experimental error, as to the unlabeled CaATPase.

Fluorescence energy transfer between FITC in the ATP site and Nd$^{3+}$ in the Ca$^{2+}$ site was found to be very small. This donor-acceptor pair has a critical distance of 0.93 nm and the distance between the ATP site and the closest Ca$^{2+}$ was estimated to be greater than 2.1 nm. Parallel measurements with FITC-labeled SR and Co$^{2+}$, an acceptor with a critical distance 1.2 nm, suggested the ATP and Ca$^{2+}$ binding sites are greater than 2.6 nm apart.

Knowledge about the number and nature of and the interactions between several cation binding sites on the CaATPase of SR is essential in order to understand the enzymatic mechanism of action. There are two high-affinity Ca$^{2+}$ binding sites on the outside of the SR which are converted to low-affinity sites and exposed to the interior as a result of MgATP binding and hydrolysis. Optimal function of CaATPase requires that K$^+$ and Mg$^{2+}$ also be bound to the enzyme during at least part of the pumping cycle (1).


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The abbreviations used are: SR, sarcoplasmic reticulum; Ln$^{3+}$, members of the lanthanide series; CaATPase, the CaATPase from rabbit skeletal muscle SR; C$_5$E$_5$, dodecyl monoxyethylene ether alcohol (CH$_3$CH$_2$)$_{11}$OCH$_2$CH$_2$OH; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid; FITC, fluorescein isothiocyanate.

scopic techniques and an approach used in many laboratories has been to substitute analogs that have better spectroscopic properties, usually for fluorescence or magnetic resonance measurements. Of particular interest here are the members of the lanthanide series, which have been used with SR to substitute for Ca$^{2+}$ and Mg$^{2+}$ and allow convenient spectroscopic determination of association constants or structural properties of the binding sites (2). This group of ions is known to inhibit ATPase activity (4-10), but experiments can still provide information about the cation binding sites. Although the Ln$^{3+}$ cations have ionic radii that are close to that of Ca$^{2+}$ (11-14), it is becoming clear that they are, not always specific analogs for Ca$^{2+}$ (3, 15), and that one must identify the type of site to which they bind.

In the experiments reported here, Nd$^{3+}$ binding to SR vesicles and to FITC-labeled SR was investigated. FITC specifically labels the ATP binding site of the CaATPase, and FITC and Nd$^{3+}$ are a donor-acceptor pair suitable for fluorescence energy transfer measurements for the distance range of 0.5 to 2 nm. It was found that free Nd$^{3+}$ at low concentrations inhibits the ATPase activity by specifically binding to the high-affinity Ca$^{2+}$ binding sites on the CaATPase. The stoichiometry of Nd$^{3+}$ binding was not changed by labeling with FITC. At higher (>10$^{-7}$ M) concentrations of free Nd$^{3+}$, there was considerable nonspecific binding to the protein and lipids. Experiments were done at low [Nd$^{3+}$] using FITC-labeled SR in order to estimate the distance between the Ca$^{2+}$ and ATP sites. This distance is too great to be measured accurately with this donor-acceptor pair, and is at least 2.1 nm. The distance from FITC to bound Co$^{2+}$ was estimated to be greater than 2.6 nm, confirming the Nd$^{3+}$ results. Thus it appears that high-affinity Ca$^{2+}$ binding sites are not contiguous with the ATP hydrolyzing site. On the contrary, they may be far away from the ATP site.

EXPERIMENTAL PROCEDURES

Proteins and Chemicals—Sarcoplasmic reticulum vesicles were isolated from New Zealand rabbit hindleg muscle by the method of Eletr and Inesi (16). Electrophoresis on polyacrylamide gels in the presence of 1% sodium dodecyl sulfate indicated the vesicles had 80-85% of the protein with Mf = 1.1 × 10$^7$, presumably the CaATPase. Typical ATPase activities at 25 °C were 7-8 µmol of P, min$^{-1}$ mg$^{-1}$, SR prepared this way forms a maximum of 4-5 nmol of phosphorylated enzyme/mg of protein and binds about 8-10 nmol of Ca$^{2+}$ per mg of protein (21, 39). Protein concentrations were determined by the biuret method (17), using isolated SR protein (dry weight) as a standard. In some cases, relative protein concentrations were determined by intrinsic tryptophan fluorescence intensities. Detergent-solubilized CaATPase monomer was prepared using the nontoxic detergent C$_5$E$_5$ as described in Murphy et al. (31). FITC-labeled CaATPase was prepared by a modification of the method of Pick (18, 30). It was found that the Ca$^{2+}$-activated ATPase activity was abolished when 5.3 nmol of FITC were incorporated per mg of SR protein.
Most experiments involving Nd³⁺ were done in the absence of EGTA. In these cases, SR vesicles were first treated with Chelex 100 by a batch method to remove divalent cations and then Mg²⁺ and Ca²⁺ were added to obtain the desired concentrations. Thus, in these cases, [Ca³⁺] was Ca²⁺ added and low free [Ca³⁺] could not be determined accurately. Chemicals were of the highest available commercial grade. Chelex 100 was from Bio-Rad. The ionophore A23187 and the detergent C₁₂E₅ were from Sigma. Ultrapure Nd₂O₃ was from Alfa Chemical Co. and Nd³⁺ was produced by dissolving the oxide in 6 N HCl and neutralizing with Na₂O·CH₃CO₂. Exposure of the Nd³⁺ solutions to glass was limited to the minimum possible. Tb³⁺ and La³⁺ solutions were prepared from ultrapure chloride salts (Alfa).

ATPase Activities—The standard assay conditions were 0.01 mg/ml of SR protein, 2 mM ATP, 5 mM MgCl₂, 0.85 mM EGTA, 1 mM CaCl₂, 0.3 µM A23187, 75 mM KCl, 50 mM MOPS (pH 7.0) at 37 °C. Phosphate production was measured by a phosphomolybdate method (22). Assays that included Nd³⁺ had 50 µM CaCl₂ added and no EGTA. When the Ca²⁺ and Mg²⁺ concentrations were varied, the per cent activity with Nd³⁺ was corrected for the changes in ATPase activity due to changes in [Ca³⁺] or [Mg²⁺]. Assays that included Co²⁺ were done with and without EGTA. Free [Co³⁺] was calculated by iteration using a computer. The association constants used were those compiled by Martell and Smith (26).

Spectroscopy—UV absorption spectra and fluorescence spectra were obtained using a MacPherson model EU-700 spectrophotometer and a Perkin-Elmer model MPF-44B fluorospectrophotometer, respectively. In the fluorescence energy transfer experiments, λₑ was 470 nm and λₐ was 520 nm. Control measurements with buffer and unlabeled SR indicated that light scattering made a negligible contribution to the apparent fluorescence in the absence of polarizers or cut off filters. The quantum yield for FITC-labeled SR was determined by the ratio method using FITC as a standard (23). The overlap integrals for FITC-labeled SR and Nd³⁺ or Co³⁺ were determined by a numerical integration using Simpson’s composite formula (37) done with a Hewlett-Packard HP-85 calculator. The absorption spectra were those of free Nd³⁺ and Co³⁺ because of their low extinction coefficients.

Assays for Free Nd³⁺—A modification of the adaptation by Miller and Senkfor (24) of the method of Barelza and Sherry (25) for detecting Tb³⁺ was used to determine the free [Nd³⁺]. In this method, SR and bound Nd³⁺ are removed by filtration through Millipore filters or by centrifugation and the filtrate or supernatant is precisely diluted to iteration using a computer. The association constants used were those compiled by Martell and Smith (26).

RESULTS

Inhibition of the ATPase Activity—Nd³⁺ inhibits the ATPase activity of SR. Shown in Fig. 1 is a typical result for the loss of ATPase activity due to increasing added Nd³⁺. Inhibition of SR ATPase activity by similarly high concentrations of added cation has been reported for many of the Ln³⁺ ions (3–10, 14) and for Nd³⁺ in particular (8). If a small excess of EGTA over Nd³⁺ was added 10 min after the Nd³⁺, the activity was restored to >90% of its normal value. Increasing the amount of SR in the assay increased the amount of Nd³⁺ required for a 50% reduction in activity. The averaged results for three [SR]’s are shown in the inset of Fig. 1. Extrapolation to [SR] = 0 gives the [Nd³⁺] which can be used to calculate an apparent association constant for Nd³⁺ and SR. It is 19 µM Nd³⁺ but the free [Nd³⁺] is actually much lower because ATP chelates a significant amount of the added Nd³⁺. The association constant for Nd³⁺ and ATP in the assay buffer was taken as 1 × 10⁵ M⁻¹. This is a reasonable value based on the apparent association constants of ATP for La³⁺, Kₛ = 0.9 × 10⁵ M⁻¹ (5), and Tb³⁺, Kₛ = 1.4 × 10⁵ M⁻¹ (3) under similar conditions, and on the association constant for Nd³⁺ at pH 8.0, Kₛ = 3.5 × 10⁶ M⁻¹ (40). Using 1 × 10⁻⁵ M⁻¹ to determine the free [Nd³⁺] for [SR] = 0, the apparent affinity constant for Nd³⁺ and SR in the assay buffer is 1.1 × 10⁻⁵ M⁻¹. This value is somewhat higher than that obtained for Tb³⁺, 7.1 × 10⁻⁵ M⁻¹ (3). These apparent association constants are estimates and the dependence of the [Nd³⁺] for 50% inhibition on [SR] (Fig. 1) indicates they may be lower limits. That Nd³⁺ is a more effective inhibitor of SR Ca²⁺-activated ATPase activity than is Tb³⁺ is in agreement with the original observations by dos Remedios (8).

The slope of the plot of [Nd³⁺] for 50% inhibition against [SR] (Fig. 1, inset) suggests that over 4000 nmol of Nd³⁺ bind/mg of SR protein. Direct measurements in the absence of ATP indicate that for free [Nd³⁺]’s in the range expected for the assay conditions at all [SR]’s, the bound Nd³⁺/mg of SR is near 10 (see below). The large slope obtained from the inhibition study does not affect any of the results or the conclusions drawn from them; but no adequate explanation for the strong apparent dependence of [Nd³⁺] on [SR] is obvious. When the [ATP] was doubled, or the [A23187] was varied, or the inhibition curves were generated using an ATP-regenerating system to keep [ATP] constant, the free [Nd³⁺] determined from the intercept and the large stoichiometry determined from the slope were little changed. Experiments are in progress to try to explain this observation.

In order to determine the nature of the inhibitory site, Ca²⁺ and Mg²⁺ competition experiments were done. Ca²⁺ protected against inhibition by Nd³⁺, but Mg²⁺ did not (Fig. 2), indicating that the Nd³⁺ inhibits by binding in a Ca²⁺ binding site. This result parallels that obtained with Tb³⁺ (3), and taken together with the apparent association constants, show that the Ln³⁺ inhibition of Ca²⁺-activated ATPase activity is due to binding in the high-affinity Ca²⁺ binding sites. The affinity of these sites for Nd³⁺ obtained from the intercept in Fig. 1 can now be adjusted for the competing free Ca²⁺ present in the solution, using the equation

\[ K_{app} = \frac{K_{app}}{1 + [Ca³⁺][Ca³⁺]} \]

where \( K_{app} = 1.1 \times 10⁵ M⁻¹ \), \( K_{Ca} = 4 \times 10⁶ M⁻¹ \), and [Ca³⁺] = 38 µM. This calculation gives \( K_{app} = 1.7 \times 10⁵ M⁻¹ \) for Nd³⁺.

The apparent association constant for SR Ca ATPase and Tb³⁺ was inadvertently given as 8.3 × 10⁵ M⁻¹ instead of 7.1 × 10⁵ M⁻¹ (3).
the ATPase activity assay medium restored the activity inhibited by Nd³⁺ (Panel A), Mg²⁺ did not protect (Panel B). The conditions are given in the legend to Fig. 1, [SR] = 0.01 mg/ml, [Nd³⁺] = 60 μM. Per cent activities are corrected for inhibition due to high [Ca²⁺] or Mg²⁺.

Binding of Nd³⁺ to sites on the SR vesicles, it is highly probable that nonspecific binding also occurs. The net negative charge of the CaATPase, other proteins, and the lipids, and the high positive charge density of the Nd³⁺ assure binding will occur even in the presence of millimolar levels of Mg²⁺. Using a modification of an assay for Ln³⁺ (24) the free and bound Nd³⁺ were determined for increasing total [Nd³⁺] (see "Experimental Procedures"). At [Nd³⁺] above about 10⁻⁶ M, there is a large and rapidly increasing amount of Nd³⁺ bound to the vesicles (Fig. 4). The data for measurements made with low [Nd³⁺] are shown in the inset. All experiments were done in solutions containing 0.1 mg/ml SR, 5 mM MgCl₂, 0.1 mM CaCl₂, 1 mM MOPS, 0.5 mM KCl (pH 7.0), 25 °C.

It is clear from the shape of the curve in Fig. 4 and number of Nd³⁺ ions bound per mg of SR that substantial nonspecific binding occurs when Nd³⁺ is present in excess. Even more Nd³⁺ binds at higher concentrations, but the enzymatic activity was irreversibly lost after 10 min incubation at pH Nd > 4, and this concentration range was not investigated. At low [Nd³⁺] (Fig. 4, inset), close to 10 nmol of Nd³⁺/mg of protein. The average value for Nd³⁺/SR below pH Nd = 5.8 was 10.4 ± 4 nmol/mg of protein. Table I shows the results for the stoichiometry determined many times at a single low [Nd³⁺] in solutions containing 10 μM added Ca²⁺. The result for SR vesicles, 10.6 ± 3.6 nmol/mg, is near the value for high-affinity Ca²⁺ binding sites, and along with the Ca²⁺ specific reversal of the Nd³⁺ inhibition of the ATPase activity (Fig. 2), suggests that at low free [Nd³⁺], specific binding to the high-affinity Ca²⁺ binding sites is obtained.

Attempts to detect the reversal of the binding of Nd³⁺ by
including high [Ca\textsuperscript{2+}] in the buffer were not successful because Ca\textsuperscript{2+} interferes with the Nd\textsuperscript{3+} assay (24). In particular, the signal to noise ratio dropped to unusable levels at [Ca\textsuperscript{2+}] \geq 5 \times 10^{-4} \text{ M}. For this assay, Tb\textsuperscript{3+} gives a much larger signal than Nd\textsuperscript{3+} at all Ca\textsuperscript{2+} concentrations, so Ca\textsuperscript{2+} reversal of Tb\textsuperscript{3+} binding was attempted. Tb\textsuperscript{3+} also inhibits SR ATPase activity (3, 8, 46). Fig. 5 shows the results for Ca\textsuperscript{2+} reversal of Tb\textsuperscript{3+} binding at low [Tb\textsuperscript{3+}] to freshly prepared SR vesicles. Ca\textsuperscript{2+} appears to reverse the binding of about half the Tb\textsuperscript{3+}. This is consistent with the observed lack of cooperative Tb\textsuperscript{3+} binding when it inhibits ATPase activity (3), and suggests that Tb\textsuperscript{3+} and Ca\textsuperscript{2+} can simultaneously bind to the CaATPase. However, in ATPase assay buffers, the Ca\textsuperscript{2+} reversal of Tb\textsuperscript{3+} inhibition is complete (3, 46) and the Ca\textsuperscript{2+} binding appears to be cooperative (46). When older vesicles (\geq 3 days) were used, the stoichiometry of Tb\textsuperscript{3+} binding at low [Ca\textsuperscript{2+}] was higher and the Ca\textsuperscript{2+} reversal was weaker. Nonetheless, the results in Fig. 5 suggest that Tb\textsuperscript{3+} binding to the high-affinity Ca\textsuperscript{2+} sites is not cooperative and that half the sites have a very high affinity under equilibrium conditions, without ATP.

**FITC Labeling**—When vesicular CaATPase was incubated at pH 7.5 with a 15-fold molar excess of FITC, the ATPase activity was lost as the FITC became covalently attached to the enzyme. As shown in Fig. 6, saturation of the attached probe molecules occurred within 30 min (inset) and 5.3 \pm 0.4 nanomoles of bound FITC/mg of SR protein caused complete inhibition of CaATPase activity (the basal activity was 0.53 \text{ nmol min}^{-1} \text{ mg}^{-1}). It has been shown that ATP protects the enzyme. As shown in Fig. 6, saturation of the attached probe molecules occurred within 30 min (inset) and 5.3 \pm 0.4 nanomoles of bound FITC/mg of SR protein caused complete inhibition of CaATPase activity (the basal activity was 0.53 \text{ nmol min}^{-1} \text{ mg}^{-1}). It has been shown that ATP protects against FITC labeling and results indicate FITC binds in the ATP site (20, 42). FITC-labeled SR was prepared freshly the day of an experiment by incubating SR vesicles and excess FITC for 30 min in 100 mM KCl, 5 mM MgCl\textsubscript{2}, 10 mM MOPS (pH 7.5, KOH), 100 \mu M CaCl\textsubscript{2} at 25 °C, and then removing excess FITC by size exclusion chromatography. In agreement with others (19, 20), all the FITC was on the CaATPase, and suggests that Tb\textsuperscript{3+} and Ca\textsuperscript{2+} can simultaneously bind to the CaATPase. However, in ATPase assay buffers, the Ca\textsuperscript{2+} reversal of Tb\textsuperscript{3+} inhibition is complete (3, 46) and the Ca\textsuperscript{2+} binding appears to be cooperative (46). When older vesicles (\geq 3 days) were used, the stoichiometry of Tb\textsuperscript{3+} binding at low [Ca\textsuperscript{2+}] was higher and the Ca\textsuperscript{2+} reversal was weaker. Nonetheless, the results in Fig. 5 suggest that Tb\textsuperscript{3+} binding to the high-affinity Ca\textsuperscript{2+} sites is not cooperative and that half the sites have a very high affinity under equilibrium conditions, without ATP.

**Fluorescence Energy Transfer**—The suitability of fluorescence and Nd\textsuperscript{3+} as a donor-acceptor pair is shown in Fig. 7 for SR labeled with 5.3 nmol of FITC/mg of SR protein (fluorescence spectrum) and free Nd\textsuperscript{3+} (absorbance spectrum). The overlap integral was 7.23 \times 10^{-18} \text{ cm}^{-2} \text{ M}^{-1} when calculated using the equation (29)

\[
J_o = \frac{\int F(\lambda)\epsilon(\lambda)\lambda d\lambda}{\int F(\lambda)d\lambda}
\]

where \(F(\lambda)\) and \(\epsilon(\lambda)\) are the fluorescence intensity of the donor and the molar extinction coefficient of the acceptor, respectively, and \(\lambda\) is the wavelength. \(J_o\) and other relevant constants and parameters are listed in Table II. The critical distance \(R_o\) is 0.93 nm when calculated from the equation (29)

\[
R_o = (J_oQ_kn_oK^2)^{1/6} \times 9.7 \times 10^2 \text{ nm}
\]

where \(Q_k\) is the quantum yield, \(n_o\) is the refractive index of the medium between the donor and the acceptor, and \(K^2\) is the orientation factor. The approximately spherical symmetry of the acceptor, Nd\textsuperscript{3+}, makes the assumption of \(K^2 = 2/3\) much safer than in cases where both acceptor and donor dipole orientations are unknown (29, 30). Given that there is considerable local freedom of motion for the probe FITC attached to SR (38) and the symmetry of the Nd\textsuperscript{3+}, the

**Fig. 6. FITC labeling.** Aliquots were taken from a solution of 1 mg/ml SR, 100 mM KCl, 20 mM MOPS (pH 7.5), 5 mM MgCl\textsubscript{2}, 0.1 mM CaCl\textsubscript{2} and 100 \mu M FITC at 25 °C and quenched by diluting 100-fold in ice-cold buffer at pH 7. Total activities were determined under "Experimental Procedures." The amount of bound FITC (inset) was determined by centrifugation to separate the free and bound FITC, which were quantitated by fluorescence intensity (\(\lambda_{\text{ex}} 490, \lambda_{\text{em}} 520\)).

**Fig. 7. FITC-Nd\textsuperscript{3+} donor-acceptor spectra.** The fluorescence (---) of FITC-labeled SR vesicles and the extinction coefficient of Nd\textsuperscript{3+} (-----) are shown for the region where there is overlap. Fluorescence is in arbitrary units, extinction coefficient is in cm\textsuperscript{-1} M\textsuperscript{-1}, and wavelength is in nanometers.
Distance between ATP and Ca\(^{2+}\) Sites on SR CaATPase

**TABLE II**

Constants for F.E.T. calculations with FITC, Nd\(^{3+}\), and Co\(^{2+}\)

\(J_0\) was calculated as described in the text, \(Q_0\) was determined for SR labeled with 5.3 nmol of FITC/mg of SR protein, \(K^2\) is assumed, \(n_r\) is the refractive index of alanine, \(R_\text{c}\) was calculated as described under "Experimental Procedures."

| Cation     | \(\text{[Cation]}\) | \(\text{[C}]\text{mM}\) | \(E_\text{r}\) | \(r_\text{c}\) | \(r_\text{a}\) |
|------------|----------------------|----------------|-----------|-----------|-----------|
| La\(^{3+}\) | 0.50                 | 0.007          | 1.2       | 1.2       | 1.2       |
| Nd\(^{3+}\) | 0.50                 | 0.034          | 1.7       | 1.9       | 1.9       |
| Nd\(^{3+}\) | 0.50                 | 0.010          | 2.1       | 2.4       | 2.4       |
| Co\(^{2+}\) | 0.50                 | 0.021          | 2.3       | 2.6       | 2.6       |

**TABLE III**

Efficiencies and distances

Efficiencies were determined from decreases in FITC fluorescence intensity at 520 nm. The SR CaATPase was labeled as described in the text. The conditions were as given in Table I. (Cation) is free concentration, \(r_1\) (\(n = 1\)) is for the case where one Ca\(^{2+}\) site is closer to the FITC than the other and \(r_2\) \((n = 2)\) are for two equivalent and equidistant Ca\(^{2+}\) sites (see text for fuller description). \(E_\text{r}\) is the experimental efficiency.

| Cation     | [Cation] | [C]mM | \(E_\text{r}\) | \(r_1\) | \(r_2\) |
|------------|----------|-------|-------------|-------|-------|
| La\(^{3+}\) | 0.50     | 0     | 0.007       | 1.2   | 1.2   |
| Nd\(^{3+}\) | 0.50     | 0     | 0.034       | 1.7   | 1.9   |
| Nd\(^{3+}\) | 0.50     | 0     | 0.010       | 2.1   | 2.4   |
| Co\(^{2+}\) | 0.50     | 0     | 0.021       | 2.3   | 2.6   |

The efficiencies of fluorescence energy transfer from FITC to Nd\(^{3+}\) and Co\(^{2+}\) are given in Table III. The \(r_1\) values are lower limits, but it is clear that the ATP and Ca\(^{2+}\) sites are at least 2 nm and more likely greater than 2.6 nm apart. La\(^{3+}\) is not an acceptor for FITC, and the small change observed for La\(^{3+}\) indicates that the change in fluorescence due to any conformational change in replacing Ca\(^{2+}\) with Ln\(^{3+}\) is small.

**CONCLUSIONS**

The reversible inhibition of Ca\(^{2+}\)-activated ATPase activity by Nd\(^{3+}\) appears to be due to binding to the high-affinity Ca\(^{2+}\) binding sites. The apparent association constant for Nd\(^{3+}\) and the CaATPase, in the presence of ATP, is \(2.2 \times 10^9\) M\(^{-1}\) or greater. This behavior is similar to that observed for the structurally similar Tb\(^{3+}\) (3, 46), and the difference between the two is in semiquantitative agreement with the results of dos Remedios (41). Nd\(^{3+}\) binding to the high-affinity Ca\(^{2+}\) binding sites also was detected in the absence of ATP, and the stoichiometry was similar to that of Ca\(^{2+}\) binding. The ratio of Nd\(^{3+}\) to FITC was close to 2, in good agreement with that of Ca\(^{2+}\) and FITC (3) or Ca\(^{2+}\) and E-P (39). These data and those for Gd\(^{3+}\) (2) and Tb\(^{3+}\) (3) binding suggest that all the Ln\(^{3+}\) ions will bind to the high-affinity Ca\(^{2+}\) binding sites with association constants \(>10^9\) M\(^{-1}\). Thus conditions are known that allow SR vesicles to be specifically labeled at the high-affinity Ca\(^{2+}\) binding sites with Ln\(^{3+}\) cations.

High concentrations of Ca\(^{2+}\) reversed the binding of Nd\(^{3+}\) in the ATPase assay medium, and competed with Tb\(^{3+}\) binding for equilibrium conditions without ATP. The data suggest that Nd\(^{3+}\) and Tb\(^{3+}\) bind strongly to high-affinity Ca\(^{2+}\) binding sites. Labeling with FITC has a negligible effect on the binding of these cations, just as it does on Ca\(^{2+}\) binding (42).

The results for Co\(^{2+}\) are less clear cut. Although it seems likely that Co\(^{2+}\) binds in the high-affinity Ca\(^{2+}\) sites, given the large \(K_{\text{app}}\) and the results for Tb\(^{3+}\) (3) and Nd\(^{3+}\), exclusive binding in the high-affinity sites has not been demonstrated. However, the effect of any additional binding of Co\(^{2+}\) on the efficiency of fluorescence energy transfer would be to increase the efficiency, if the Co\(^{2+}\) were close to the FITC, or not change the efficiency if the Co\(^{2+}\) were far (>3 nm). This makes the distances calculated from the Co\(^{2+}\) data lower limits.
site (18, 20, 27, 28, 42). The binding of Ca²⁺ is unaffected by FITC labeling (41, 42) and the stoichiometry of Nd³⁺ binding is not changed (Table I). The ratio of Nd³⁺ to FITC suggests they are binding with high specificity.

The fluorescence energy transfer measurements for the Ca²⁺-transporting ATPase labeled with FITC and Nd³⁺ indicate that high-affinity Ca²⁺ binding sites are at least 2.1 nm away from the ATP binding site and the Nd³⁺ results suggest they are further than 2.6 nm away (Table III). Model building of ATP suggests its longest dimension is 1.7 nm. Thus it appears that during Ca²⁺ transport the action of MgATP binding and hydrolysis in the ATP site must be transmitted a distance through the protein to change the Ca²⁺ sites. Preliminary reports of steady-state fluorescence and excited state lifetime measurements support this conclusion (43, 44), which is consistent with a recent hypothesis that for coupled vectorial transport the ATP site and the Ca²⁺ sites need to be separated (45).

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REFERENCES
1. deMeis, L. (1981) The Sarcoplasmic Reticulum pp. 113–123, John Wiley and Sons, New York
2. Stephens, E. M., and Grisham, C. M. (1979) Biochemistry 18, 4876–4885
3. Highsmith, S. R., and Head, M. R. (1983) J. Biol. Chem. 258, 6858–6862
4. Chevallier, J., and Butow, R. A. (1971) Biochemistry 10, 2733–2737
5. Krasnow, N. (1972) Biochim. Biophys. Acta 282, 187–194
6. Yamada, S., and Tonomura, Y. (1972) J. Biochem. 72, 417–425
7. dos Remedios, C. G. (1977) J. Biochem. 81, 703–708
8. dos Remedios, C. G. (1977) Nature (Lond.) 270, 750–751
9. Barry, K. J., Bloomquist, E., and Mikkelsen, R. (1979) Arch. Int. Physiol. Biochem. 87, 493–499
10. Chiesi, M., and Inesi, G. (1979) J. Biol. Chem. 254, 10370–10377
11. Pauling, L. (1960) The Nature of the Chemical Bond, pp. 511–519, Cornell University, Ithaca, NY
12. Nieboer, E. (1975) Struct. Bonding 22, 1–47
13. Martin, R. B., and Richardson, F. S. (1979) Quart. Rev. Biophys. 12, 181–209
14. dos Remedios, C. G. (1981) Cell Calcium 2, 29–51
15. Chantler, P. D. (1983) J. Biol. Chem. 258, 4702–4705
16. Eletr, S., and Inesi, G. (1972) Biochim. Biophys. Acta 282, 174–179
17. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) J. Biol. Chem. 177, 751–766
18. Pick, U., and Bassilian, S. (1981) FEBS Lett. 123, 127–130
19. Pick, U. (1981) Eur. J. Biochem. 121, 187–195
20. Pick, U., and Karlish, S. J. D. (1980) Biochim. Biophys. Acta 626, 255–261
21. Nakamura, Y., and Tonomura, Y. (1982) J. Biochem. 91, 449–461
22. Murphy, A. J. (1981) J. Biol. Chem. 256, 12046–12050
23. Parker, C. A., and Rees, W. T. (1960) Analyst 85, 587–600
24. Miller, T. L., and Senkfor, S. (1982) Anal. Chem. 54, 2022–2025
25. Barela, T. D., and Sherry, A. D. (1976) Anal. Biochem. 71, 351–357
26. Martell, A. E., and Smith, R. M. (1974) Critical Stability Constants, Vol. 1, Plenum Publishing Corp., New York
27. Cleore, G., Gronenborn, A. M., Mitchelson, C., and Green, N. M. (1982) Eur. J. Biochem. 128, 113–117
28. Pick, U., and Karlish, S. (1982) J. Biol. Chem. 157, 6120–6126
29. Stryer, L. (1975) Biochim. Biophys. Acta 282, 174–194
30. Dale, R. E., and Eisinger, J. (1981) Critical Stability Constants, Vol. 1, Plenum Publishing Corp., New York
31. Murphy, A. J., Pepitone, M., and Highsmith, S. (1982) J. Biol. Chem. 257, 3551–3554
32. Deleted in proof
33. Pesce, A. J., Rosen, C. G., and Pusby, T. L. (1971) Fluorescence Spectroscopy p. 140, Marcel Dekker, Inc., New York
34. Deleted in proof
35. Deleted in proof
36. Deleted in proof
37. Hamming, R. W. (1962) Numerical Methods for Scientists and Engineers p. 158, McGraw-Hill, New York
38. Speirs, A., Moore, C. H., Bexer, D. H., and Garland, P. B. (1983) Biochem. J. 213, 67–74
39. Inesi, G., Kurzmack, M., Coan, C., and Lewis, D. E. (1980) J. Biol. Chem. 255, 3025–3031
40. Morrison, J. F., and Cleland, W. W. (1983) Biochemistry 22, 5507–5513
41. dos Remedios, C. G. (1981) Cell Calcium 2, 29–51
42. Andersen, J. P., Møller, J. K., and Jørgensen, P. L. (1982) J. Biol. Chem. 257, 8300–8307
43. Highsmith, S. R. (1984) Biophys. J. 45, 4a
44. Scott, T. L. (1984) Biophys. J. 45, 3a
45. Tanford, C. (1983) Annu. Rev. Biochem. 52, 379–409
46. Scott, T. L. (1984) J. Biol. Chem. 259, 4035–4037