**Tb**³⁺ Binding to Ca²⁺ and Mg²⁺ Binding Sites on Sarcoplasmic Reticulum ATPase*

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The interactions of **Tb**³⁺ and sarcoplasmic reticulum (SR) were investigated by inhibition of Ca²⁺-activated ATPase activity and enhancement of **Tb**³⁺ fluorescence.

Ca²⁺ protected against **Tb**³⁺ inhibition of SR ATPase activity. The apparent association constant for Ca²⁺, determined from the protection, was about 6 x 10⁶ M⁻¹, suggesting that **Tb**³⁺ inhibits the ATPase activity by binding to the high affinity Ca²⁺ binding sites. Mg²⁺ did not protect in the 2–20 mM range. The association constant for **Tb**³⁺ binding to this Ca²⁺ site was estimated to be about 1 x 10⁶ M⁻¹. No cooperativity was observed for **Tb**³⁺ binding. No enhancement of **Tb**³⁺ fluorescence was detected.

A second group of binding sites, with weaker affinity for **Tb**³⁺, was observed by monitoring the enhancement of **Tb**³⁺ fluorescence (λₘₓ 285 nm, λₘₜ 345 nm). The fluorescence intensity increased 950-fold due to binding. Ca²⁺ did not compete for binding at these sites, but Mg²⁺ did. The association constant for Mg²⁺ binding was 94 M⁻¹, suggesting that this may be the site that catalyzes phosphorylation of the ATPase by inorganic phosphate.

For vesicles, **Tb**³⁺ binding to these Mg²⁺ sites was best described as binding to two classes of binding sites with negative cooperativity. If the SR ATPase was solubilized in the nonionic detergent C₁₂E₆ (dodecyl nonanoyl ethylene ether alcohol), in the absence of Ca²⁺, only one class of **Tb**³⁺ binding sites was observed. The total number of sites appeared to remain constant. If Ca²⁺ was included in the solubilization step, **Tb**³⁺ binding to these Mg²⁺ binding sites displayed positive cooperativity (Hill coefficient, 2.1). In all cases, the apparent association constant for **Tb**³⁺, in the presence of 5 mM MgCl₂, was in the range of 1–5 x 10⁶ M⁻¹.

The trivalent cations in the lanthanide series are known to inhibit the Ca²⁺-activated ATPase activity of sarcoplasmic reticulum (1–5). The mechanism of the inhibition is usually assumed to be due to binding of Ln³⁺ to Ca²⁺ binding sites. The crystal radii of the lanthanide cations are 96–115 pm, which is similar to that of Ca²⁺ (99 pm) (6). The Ln³⁺ ions have been described as Ca²⁺ analogs (7, 8). Their higher charge density makes them bind more tightly than Ca²⁺. As an example of their relative affinities, EGTA has about a 10⁶ times larger association constant for members of the Ln³⁺ series than it does for Ca²⁺ (9). Nonetheless, for SR, there is no evidence that Ln³⁺ inhibition of ATPase activity is due to binding to a Ca²⁺ binding site. In particular, binding to nonspecific sites or inhibition by **Tb**ATP has not been excluded.

Some of the ions in the Ln³⁺ series have useful spectroscopic properties. Several are paramagnetic and can be used as shift and/or broadening reagents in magnetic resonance measurements (10–12). Others are fluorescent. **Tb**³⁺, Dy³⁺, and Eu³⁺ have millisecond excited state lifetimes and are suitable for use in fluorescent energy transfer experiments and as probes of Ca²⁺ binding sites (13–15). As a beginning of a project to exploit the fluorescent properties of these Ca²⁺ analogs in the solubilized SR ATPase, **Tb**³⁺ binding to vesicular and detergent solubilized SR was investigated.

The results indicate that **Tb**³⁺ inhibits the ATPase activity of SR by binding to the high affinity Ca²⁺ binding sites. The affinity of the SR Ca-ATPase for **Tb**³⁺ is at least 2 orders of magnitude greater than for Ca²⁺. **Tb**³⁺ bound to these sites is nonfluorescent. Additional **Tb**³⁺ binding at lower affinity sites on the enzyme did enhance **Tb**³⁺ fluorescence. Surprisingly, these sites are Mg²⁺ binding sites. Scatchard-type analysis indicated that these Mg²⁺ binding sites interact with another under some conditions. The nature of the interaction was modified by nonionic detergent solubilization and depended on the [Ca²⁺] at the time of solubilization.

**EXPERIMENTAL PROCEDURES**

Proteins and Chemicals—Sarcoplasmic reticulum vesicles were isolated from New Zealand rabbit hind leg muscle by the method of Eletr and Inesi (16). Electrophoresis on polyacrylamide gels in the presence of 1% sodium dodecyl sulfate indicated that the vesicles had 85% of the protein with a Mᵦ = 1 x 10⁶, presumably the (Ca⁺⁺, Mg⁺⁺)-ATPase. Typical ATPase activities were 7–8 µmol of P₁ min⁻¹ mg⁻¹. SR was prepared this way forms a maximum of about 4 nmoles of phosphorylated enzyme/mg of protein and transports about 8 nmoles of Ca²⁺/mg of enzyme. Protein concentrations were determined by the biuret method (17), using bovine serum albumin as a standard without correction. In some cases, relative protein concentrations were determined by intrinsic tryptophan fluorescence intensities.

Experiments involving **Tb**³⁺ were done in the absence of EGTA. In these cases, SR vesicles were first treated with Chelex 100 to remove divalent cations and then Mg²⁺ and Ca²⁺ were added to obtain the desired concentrations. Thus, in these cases, [Ca²⁺] was Ca²⁺ added and low free [Ca²⁺] could not be determined accurately.

SR phospholipids were isolated from SR vesicles by CHCl₃/CH₃OH extraction and resuspended as synthetic vesicles (18). Additional ether extraction of lipoproteins from the SR phospholipids did not affect the results.

Chemicals were of the highest available commercial grade. Chelex 100 was from Bio-Rad. The ionophore A23187 and the detergent

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The abbreviations used are: Ln³⁺, members of the lanthanide series other than **Tb**³⁺; C₁₂E₆, dodecyl nonanoyl ethylene ether alcohol (CH₃(CH₂)₁₁(OCH₂CH₂)₉OH); Mₚ₃, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid.
RESULTS AND DISCUSSION

ATPase Inhibition—Tb3+ inhibits SR vesicle ATPase activity. The loss of activity as a function of added [Tb3+] is shown in Fig. 1 for [SR] between 0.01 and 0.1 mg/ml. The free [Tb3+] is much lower due to chelation by ATP. Also shown, in the inset, is the [Tb3+] required for 50% inhibition versus the [SR]. One point is included from the original observation of Tb3+ inhibition of SR Ca-ATPase activity by dos Remedios (3). The inhibition could be completely reversed by including enough EGTA in the assay to chelate the Tb3+, but not the Ca2+. No Tb3+ fluorescence enhancement was detected for conditions that were completely inhibited. To eliminate the large inner filter effect due to ATP, measurements of Tb3+ fluorescence were made at 330 nm from solutions irradiated at 295 nm. Tb3+ fluorescence was measured at 545 nm from solutions irradiated at 295 nm. A 430 nm cut-off filter was used to minimize any contribution from light scattering. The temperature was 25 ± 0.1 °C. A titration took from 10 to 20 min.

Fluorescence Measurements—All measurements were made on a Perkin-Elmer MPF-44B fluorospectrophotometer equipped with a DCSU-2 microprocessor. Tryptophan fluorescence was measured at 330 nm from solutions irradiated at 295 nm. Tb3+ fluorescence was determined from the decrease in ATP absorbance at 260 nm due to Tb3+ binding.

ATPase Activities—The standard assay conditions were 0.01 mg/ml of SR protein, 5 mM MgCl2, 0.83 mM EGTA, 1 mM CaCl2, 0.8 nM A23187, 75 mM KCl, 50 mM MOPS-KOH, pH 7.0, at 37 °C. Phosphate production was measured by a phosphomolybdate method (19). Assays that included Tb3+ had no ETGA and 50 μM CaCl2 added. Assays done in the presence of C2E9 usually did not contain A23187. The affinity of ATP for Tb3+ in the assay buffer (minus A23187 and SR) was estimated from free [Tb3+] determined from the inhibition of activity for the lowest [SR] were taken from the literature (3).

Conditions are as given in the legend to Fig. 1 except that [Tb3+] = 70 μM. [Mg2+] and [Ca2+] are total added concentrations. For each divalent cation concentration, the activity with Tb3+ was divided by the activity without Tb3+ to obtain the per cent activity. The activity without Tb3+ was typically 6–7 μmol of Pi min-1 mg-1.

The magnitude of the apparent association constant (K′) for Tb3+ binding to the inhibiting site in the presence of Ca2+ was estimated from free [Tb3+] values calculated assuming that ATP is the only significant chelator of Tb3+. This is a reasonable assumption, since the assay conditions were 0.01–0.2 mg/ml of SR, 50 μM CaCl2, 5 mM MgCl2, 75 mM KCl, 50 mM MOPS-KOH, 0.83 mM A23187, and 2 mM ATP. The association constant for ATP and Tb3+ in the assay buffer was determined to be 1.4 × 10^5 M⁻¹ (see "Experimental Procedures"), in reasonable agreement with the value of 0.9 × 10^5 M⁻¹ obtained for the similar ion (La3+) binding to ATP under similar conditions (2). The apparent free [Tb3+] that gives 50% inhibition of the ATPase activity still increases with [SR] (Fig. 3). The free [Tb3+] determined from the intercept is 1.4 × 10⁻¹ M, which corresponds to an apparent association constant (K′ = 8.3 × 10⁶ M⁻¹), assuming a single class of sites without cooperativity. The slope indicates that about 8.3 nmol of Tb3+/mg of SR are binding very tightly. Data from the inhibition of activity for the lowest [SR] were analyzed in more detail in an attempt to detect cooperativity in the Tb3+ binding. A Scatchard plot was linear, supporting the assumption that Tb3+ binds without cooperativity (Fig. 4). The slope indicated K′ = 4.9 × 10⁶ M⁻¹, in agreement with the value obtained from the intercept. However, the high values for K′ show that ATP is not a good buffer for Tb3+ (a buffer of necessity, not choice); so it should be emphasized

The loss of Ca2+-activated ATPase activity of SR vesicles when Tb3+ is present. Assays were done at 37 °C in solutions containing 2 mM ATP, 5 mM MgCl2, 50 μM CaCl2, 0.83 mM A23187, 75 mM KCl, 50 mM MOPS-KOH, pH 7.0, and varying amounts of added TbCl3. The SR concentrations were 0.01 (O), 0.08 (Δ), and 0.12 (□) mg/ml, respectively. The inset shows the [Tb3+] that gave 50% inhibition of activity for each [SR] plus one point (○) for 0.05 mg/ml of SR and one point (●) taken from the literature (3).

The free [Tb3+] that gave 50% inhibition of Ca2+-activated ATPase activity of SR vesicles for increasing concentrations of SR. Conditions are given in the legend to Fig. 1.
that \( K'_f \) is an apparent association constant, and perhaps a lower limit.

The data for \( Tb^{3+} \) inhibition and \( Ca^{2+} \) protection can be shown to be consistent with \( Tb^{3+} \) binding in the high affinity \( Ca^{2+} \) binding site. If one assumes it is the high affinity site and uses an association constant for \( Ca^{2+} \) of \( K_{Ca} = 2 \times 10^8 \text{ M}^{-1} \) \( (20-22) \) in the equation

\[
K'_f = \frac{K_f}{1 + [Ca^{2+}]K_{Ca}}
\]

to calculate the association constant \( (K_f) \) for \( Tb^{3+} \) binding, \( K_f = 8.3 \times 10^8 \text{ M}^{-1} \). If this \( K_f \) is then used to adjust the \( K'_o \) for \( Ca^{2+} \) (determined from \( Ca^{2+} \) protection) in the equation

\[
K'_o = \frac{K_o}{1 + [Tb^{3+}]/K_f}
\]

\( K_o = 6.4 \times 10^6 \text{ M}^{-1} \). This is in good agreement with the well established values for \( Ca^{2+} \) binding to the high affinity binding sites and is internally consistent, which justifies assuming \( Tb^{3+} \) binds in that site. Other assumptions \( (viz. \) it is a low affinity \( Ca^{2+} \) site with \( K_o = 10^9 \text{ M}^{-1} \) do not give results that are internally consistent.

Enhancement of \( Tb^{3+} \) Fluorescence—At higher \( [Tb^{3+}] \), fluorescence peaks were observed at 490, 545, 580, and 640 nm in the presence of \( Sr \) vesicles irradiated at 295 nm. This type of behavior is expected if \( Tb^{3+} \) binds near enough to an aromatic amino acid for fluorescence energy transfer from the amino acid to the \( Tb^{3+} \) to occur. Equimolar concentrations of vesicles composed of \( Sr \) phospholipids had a much smaller fluorescence enhancement \((<2\%)\). The fluorescence intensities at 545 nm for \( Sr \) vesicles and \( Sr \) lipid vesicles are plotted in Fig. 5 as a function of added \( Tb^{3+} \). The conditions \((5 \text{ mM MgCl}_2, 10 \text{ mM MOPS-KOH}, \text{pH 7.0}) \) were chosen to minimize \( Tb^{3+} \) binding to phospholipids or to soluble anions. Using the association constant determined below, the fluorescence of \( Tb^{3+} \) was estimated to increase 950-fold. If 1 mM EGTA was added to a solution of \( Tb^{3+} \) and \( Sr \), the fluorescence was reduced to that of the EGTA in the absence of \( Sr \) within a few minutes. The lack of \( La^{3+} \) penetration to the interior of \( Sr \) vesicles \((5) \), the absence of exposed proteins other than the \( Ca^{2+}-ATPase \) on the vesicle exterior \((23) \), and the rapid reversal of \( Tb^{3+} \) binding in the presence of EGTA all suggest that \( Tb^{3+} \) fluorescence enhancement is due to binding to sites on the portion of the \( Ca^{2+}-ATPase \) on the exterior of the vesicles. These sites are in addition to the high affinity \( Ca^{2+} \) sites that are saturated by \( Tb^{3+} \) at much lower \([Tb^{3+}]\).

A Scatchard-type analysis \((24) \) of the fluorescence enhancement due to binding to the vesicles indicated there were at least two classes of sites (Fig. 6). The downward curvature is compatible with independent sites or with negatively cooperative sites. The points obtained with 5 mM \( MgCl_2 \) present can be fit adequately, assuming two classes of sites of equal number with association constants of \( 3.2 \times 10^5 \text{ M}^{-1} \) and \( 0.65 \times 10^4 \text{ M}^{-1} \). The excess of \( Tb^{3+} \) over \( Sr \) makes the total \([Tb^{3+}] = \text{free} \ [Tb^{3+}] + \text{bound} \ [Tb^{3+}] \) considered the relatively low affinity of \( Ln^{3+} \) cations for phospholipids \( (K_o = 1.25 \times 10^4 \text{ M}^{-2} \) for 2 lipid + \( Ln^{3+} \Rightarrow Ln(\text{lipid})_2 \) \( (25) \).

Also shown in Fig. 6 is the fact that \( Ca^{2+} \) (up to 1 mM) did not affect \( Tb^{3+} \) binding. Surprisingly, since \( Tb^{3+} \) is often considered to be a \( Ca^{2+} \) analog, \( Mg^{2+} \) was an effective inhibitor of \( Tb^{3+} \) binding to the sites that enhance fluorescence at 545 nm (Fig. 6). Using the \([Tb^{3+}] \) for 50% increase in fluorescence to estimate average apparent association constants for \( Tb^{3+} \) inhibition and \( Ca^{2+} \) protection can be calculated from \( Ca^{2+} \) protection (Fig. 6).

\[
K'_o = \frac{K_o}{1 + [Mg^{2+}]K_{Ca}}
\]
The data for \([\text{Mg}^{2+}]\) between 0 and 15 mM were well fit, giving \(K_{m} = 94 \pm 9\ \text{mM}\). This value for \(K_{m}\) is near that for \(\text{Mg}^{2+}\) binding in the sites that catalyze phosphorylation of the Ca-ATPase by inorganic phosphate (26-28), suggesting that \(\text{Tb}^{3+}\) may bind to those sites in this case.

**Detergent Solubilization—**Dispersion of the SR Ca-ATPase in micelles of the nonionic detergent \(\text{C}_{12}\text{E}_{9}\) (29) did not affect the net total fluorescence enhancement, suggesting that the number of bound \(\text{Tb}^{3+}\) ions did not change. The background fluorescence for SR lipid vesicles in \(\text{C}_{12}\text{E}_{9}\) was increased, indicating some interaction between the \(\text{Tb}^{3+}\) and the detergent. However, since the \([\text{Tb}^{3+}]\) for 50\% of the increase due to binding SR protein in \(\text{C}_{12}\text{E}_{9}\) was only a few per cent less than it is for the vesicle suspension, \(\text{Tb}^{3+}\) binding to either phospholipids or to the detergent appears to be stoichiometrically negligible compared to the free \(\text{Tb}^{3+}\). Purification of the Ca-ATPase-detergent complex by size exclusion chromatography did not change the magnitude of the fluorescence enhancement, but required times that lead to inactivation of the Ca-ATPase under some of the conditions used, so titrations were done immediately after solubilization.

The results for a \(\text{Tb}^{3+}\) titration of SR solubilized in \(\text{C}_{12}\text{E}_{9}\) were found to depend upon the presence or absence of \(\text{Ca}^{2+}\) at the time of solubilization. In either case, the SR vesicles were first treated with Chelex 100 (Na\(^+\)) to remove all divalent cations. Next, 10 mM MOPS-KOH, pH 7.0, 5 mM \(\text{MgCl}_2\) and 0 or 0.1 mM \(\text{CaCl}_2\) were added. Then \(\text{C}_{12}\text{E}_{9}\) in the respective buffer was added to obtain 20 mg/ml of detergent. The solution was then titrated with \(\text{Tb}^{3+}\). The total net fluorescence enhancement at 545 nm was the same for solubilization with or without \(\text{Ca}^{2+}\) present, but the results obtained by Scatchard analysis were different.

When \(\text{Ca}^{2+}\) was omitted from the solubilization step, there was a single class of sites (Fig. 7) with an association constant of \((4.9 \pm 1.9) \times 10^4\ \text{M}^{-1}\), close to the value for the higher affinity site determined for vesicles at the same \([\text{Mg}^{2+}]\). Adding \(\text{Ca}^{2+}\) up to 1 mM after solubilization without \(\text{Ca}^{2+}\) and incubating up to 30 min had no effect. \(\text{Mg}^{2+}\) inhibited \(\text{Tb}^{3+}\) binding with about the same apparent association constant as observed for the vesicle case (Fig. 7B). It seems that without \(\text{Ca}^{2+}\) present, \(\text{C}_{12}\text{E}_{9}\) converts all the \(\text{Mg}^{2+}\) binding sites to a single type that appears similar with regard to affinity for \(\text{Tb}^{3+}\) to the higher affinity class of sites on the vesicles.

![Fig. 7. Scatchard plots of \(\text{Tb}^{3+}\) fluorescence (\(\lambda_{ex}\) 280 nm, \(\lambda_{em}\) 340 nm) data for titrations of SR solubilized with \(\text{C}_{12}\text{E}_{9}\) not containing \(\text{CaCl}_2\). SR that had been treated with Chelex 100 was then solubilized in 5 mM \(\text{MgCl}_2\), 10 mM MOPS-KOH, pH 7.0, and 20 mg/ml of \(\text{C}_{12}\text{E}_{9}\). The concentrations of \(\text{CaCl}_2\) and \(\text{MgCl}_2\) were adjusted and the solutions were titrated with \(\text{Tb}^{3+}\). A, 5 and 0 mM \(\text{MgCl}_2\) (○); 0.1 (●) or 0.5 (△) mM \(\text{CaCl}_2\). B, no and 0 CaCl\(_2\) (○) or 20 mM MgCl\(_2\) (●).](https://www.jbc.org/)
to involve negative cooperativity or independent sites with different affinities. Taken altogether, these results suggest that the interaction between the high affinity Ca\(^{2+}\) binding sites depends on the nature of the cation bound and is capable of wide variation.

The binding sites that enhance Tb\(^{3+}\) fluorescence are clearly Mg\(^{2+}\) binding sites. Tb\(^{3+}\) is often thought to be a Ca\(^{2+}\) analog, because of their similar crystal radii. Mg\(^{2+}\) is much smaller, and the results shown here indicate that Tb\(^{3+}\) and probably all the lanthanide tervalent cations are not specific for Ca\(^{2+}\). Curious because it shows striking changes, depending on the Mg\(^{2+}\) site dependence on the nature of the cation bound and is capable of wide variation.

Site-site interactions between the Mg\(^{2+}\) binding sites were observed for Tb\(^{3+}\) binding. The nature of this interaction is curious because it shows striking changes, depending on the conditions. Positive, negative, and no cooperativity were observed for Tb\(^{3+}\) binding when the [Ca\(^{2+}\)] and lipid composition were varied. Ca\(^{2+}\) seemed to be essential for the cooperative site-site interactions to occur. Loss of the cooperative interactions between the Ca\(^{2+}\) sites, due to Ca\(^{2+}\) deprivation, has also been observed for SR vesicles (33); but that loss was reversible. In the present case, Ca\(^{2+}\) appears to act as a link of communication between the two Mg\(^{2+}\) binding sites. Once removed in the presence of detergent, the link does not seem to be re-established. When the linkage is present, the lipid environment appears to modify the nature of the interaction between the sites. The natural phospholipids promote negative cooperativity, and the detergent C\(_{12}\)E\(_9\) promotes positive cooperativity, at least for Tb\(^{3+}\) binding.

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