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EVIDENCE THAT THE ATP BINDING SITE OF SARCOPLASMIC RETICULUM CaATPase HAS A Mg(2+) ION BINDING SUB-SITE

Stefan Highsmith

Department of Biochemistry
School of Dentistry
University of the Pacific
San Francisco, CA 94115

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SUMMARY: The CaATPase of skeletal muscle sarcoplasmic reticulum was specifically labeled in the ATP binding site with fluorescein isothiocyanate under gentle conditions (pH 7•5). Fluorescence energy transfer from the attached fluorescein to Nd^{3+} indicated that a cation binding site was about 1•0 nm away from the fluorescein. Thus it appears that the ATP site includes a cation binding site. At 25°C in 0•5 M KCl, the association constants for Nd^{3+}, Ca^{2+} and Mg^{2+} were $3•3x10^5$ M^{-1}, 84 M^{-1} and 35 M^{-1}, respectively, making it possible that, in vivo, the site binds Mg^{2+}.

Several experimental results suggest that a Mg^{2+} ion binds in or near the ATP binding site of rabbit skeletal muscle sarcoplasmic reticulum CaATPase. For example, Mg^{2+} is required for the phosphorylation of the CaATPase by orthophosphate (1). In addition, the binding of Mg^{2+} to the protein accelerates the hydrolysis the phosphoenzyme (2). It has been shown also that Mg^{2+}-free ADP acts as a substrate for the reversal of the calcium pump to form MgATP (3). Finally, a Mg^{2+} thought to come from MgATP may remain bound to the enzyme after ATP has been hydrolyzed and ADP has dissociated (4). However, none of these data is conclusive and all are compatible with the purported Mg^{2+} binding site being distant from the ATP site.

Two facts make the direct detection of a Mg^{2+} binding site in the ATP site difficult. One is that Mg^{2+} is not easy to detect spectroscopically. The other is that the CaATPase is an intrinsic membrane protein that is unlikely to be crystallized in three dimensions. These difficulties can be circumvented partially by using a Mg^{2+} analog. In the present case, Nd^{3+} ion was used and its distance from the fluorescent probe fluorescein, covalently attached to the CaATPase in the ATP binding site, was determined by the method of fluorescence energy transfer.

MATERIALS AND METHODS

Vesicles rich in CaATPase were prepared from rabbit skeletal muscle (5) and stored at 0°C in 30% (by weight) sucrose solutions for up to 4 days. All

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chemicals were of the highest available commercial grade. Ultrapure Nd_2O_3 was dissolved in 6N HCl and neutralized with NaO_2CCH_3, avoiding contact with glass containers. Ca^{2+} activated ATPase activities were determined at 37°C in 63 mM KCl, 4·2 mM $MgCl_2$, 0.83 mM EGTA, 1 mM $CaCl_2$, 42 mM MOPS (pH 7·0), 200 nM A23187 with 0·01 mg/ml SR protein.

Fluorescein isothiocyanate (FITC) stock solutions in ethanol, used for labeling experiments, were stored for up to 7 days at -20°C in the dark. Fluorescence intensities were measured with a Perkin-Elmer MPF-44B fluorospectrophotometer, irradiating at 490 nm and observing at 520 nm. The amount of FITC bound to the SR was determined from $((F_T - F_D)/F_T) \times [FITC]/mg$ SR protein, where F_T is the fluorescence intensity of the solution without SR present and F_D is the intensity after the SR was added, the solution incubated and quenched, and then centrifuged for 1 hour at 20,000 rpm.

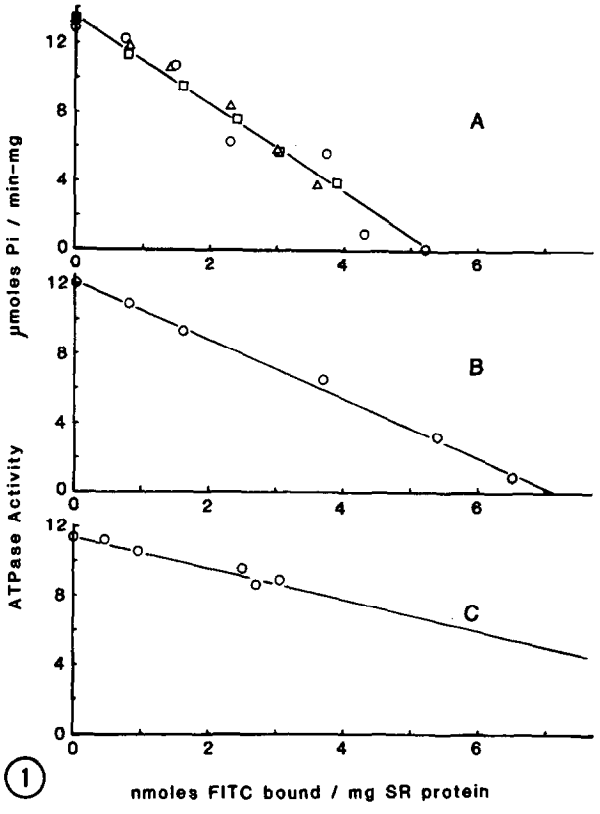
Association constants were calculated from, $K' = K/(1+\Sigma [M]K_M)$, where K is the association constant and K' is the apparent association constant in the presence of competing metal ions, with [M] = free metal ion concentrations and K_M = metal ion association constant.

RESULTS

FITC Labeling - The high pH (≈10) used in the original FITC labeling studies (6) seemed potentially hazardous to the CaATPase for some conditions; so the specificity of labeling at lower pH was investigated. Results for the pH range 6·0 to 8·5 are shown in Fig. 1. In each case, SR samples were incubated for 2 hours at 23°C with varying amounts of FITC and then assayed for ATPase activity and bound FITC. In all cases a straight line gave a good fit to the data plotted as activity vs nmoles FITC bound/mg SR protein. For pH's as low as 7·5, complete inactivation of the Ca^{2+}-activated ATPase activity (corrected for basal activity) occured when 5·1±0·3 nmoles of FITC were bound. At lower pH, there appeared to be more bound FITC required to completely inhibit the ATPase activity. This could be due to non-specific covalent attachment of FITC to protein and lipid amino groups or to non-covalent partitioning of the FITC into the lipid portion of the vesicles. For pH greater than or equal to 7·5, the stoichiometry is very close to that of phosphoenzyme formation (7,8) and confirms to high specificity of FITC labeling for alkaline conditions (6,9).

At pH 7·5, MgATP is a potent inhibitor of FITC inactivation (Fig. 2). This confirms results obtained at higher pH's (6,9) indicating that FITC binds in the ATP binding site. Subsequent experiments were done using SR vesicles that were 80 to 90% inactivated by incubation with FITC at pH 7·5, 100 mM KCl, 5 mM $MgCl_2$, 0·10 mM $CaCl_2$, 10 mM MOPS, 2 mg/ml SR protein, 25°C and then purified on a bio-gel P-10 size exclusion column eluted with the required buffer.

Fluorescence Energy Transfer - It has been shown that FITC and Nd^{3+} are a suitable pair for energy transfer (10,11). Assuming a value of 2/3 for the orientation factor, the critical distance is 0·93 nm. Thus, this pair can be



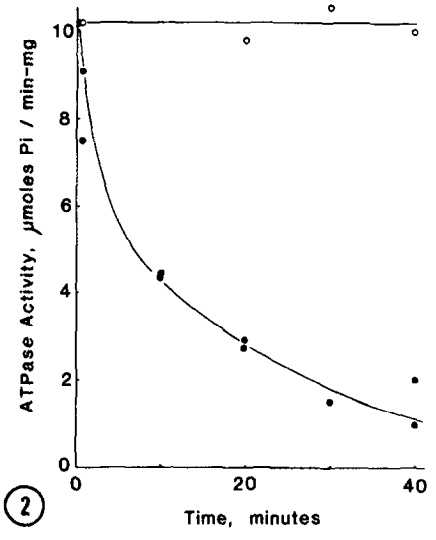


Figure 1.- Labeling SR with FITC. Solutions of SR 2·0 mg/ml in 100 mM KCl, 5 mM $MgCl_2$, 0·10 mM $CaCl_2$, 20 mM MOPS were incubated with varying amounts of FITC (0 to 20 µM) at 25°C. After 2 hours, aliquots were taken and the ATPase activity at pH 7·0, 37°C was assayed and the fraction of the original FITC that had become bound to the SR vesicles was determined. Results are shown for pH's 8·5 (△), 8·0 (○) and 7·5 (□) in panel A; for pH 7·0 in panel B; and for pH 6·0 in panel C.

Figure 2. - ATP Protection. Solutions of SR (1·0 mg/ml) in 100 mM KCl, 5 mM $MgCl_2$, 0·5 mM EGTA, 20 mM MOPS (pH 7·5) were incubated at 25°C with 20 µM FITC and either 5 mM (○) or 0 mM (●) ATP. Aliquots were taken at increasing times and assayed at 35°C in the presence of 0.10 mM free Ca^{2+}.

used to accurately measure distances in the range of 0·5 to 2 nm. When FITC-labeled CaATPase in SR vesicles was titrated with Nd^{3+} there was a decrease in fluorescence intensity. The mid-point of this transition in solutions containing 5 mM $MgCl_2$ and 0·10 mM $CaCl_2$ was $3{\cdot}5 \times 10^{-6}$ M Nd^{3+} (Fig. 3) and had a Hill coefficient of about 1. At saturation, the fluorescence was 0·60 of its original value. Also shown in Fig. 3 are results for Tb^{3+} and La^{3+}, ions similar to Nd^{3+} in size and charge density, which do not absorb in the 400 to 700 nm range. The lack of fluorescence decrease in the presence of these ions demonstrates that the Nd^{3+} effect is fluorescence energy transfer rather than quenching due to a protein conformational change. The fluorescence decrease caused by the addition of 3×10^{-5} M Nd^{3+} was completely reversed by a small

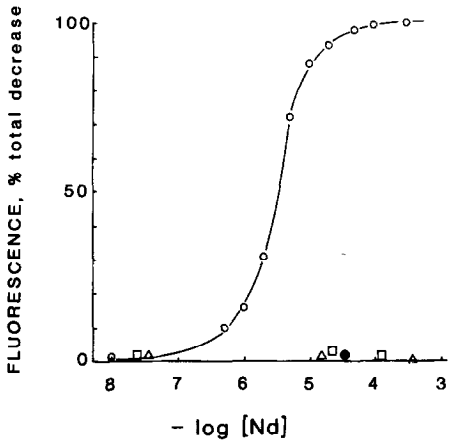


Figure 3. - Nd^{3+}Quenching of FITC Fluorescence. Solutions of 0·05 mg/ml FITC-labeled SR vesicles in 0·50 M KCl, 5 mM $MgCl_2$, 0·10 mM $CaCl_2$, 1 mM MOPS (pH 7·0) were titrated at 25°C with $NdCl_3$ (○). The fluorescence intensity at 520 nm (λ ex = 490 nm) decrease by 40%, but was restored when EGTA was added (●). Other Ln^{3+} ions, La^{3+} (□) and Tb^{3+} (△) did not reduce the FITC fluorescence.

molar excess of EGTA (Fig.3) and the CaATPase activity was also restored (10). Adding Nd^{3+} to free FITC (no SR) in the concentration range shown in Fig. 3 did not reduce the fluorescence intensity.

Both Mg^{2+} and Ca^{2+} protected against Nd^{3+} binding, as detected by fluorescence energy transfer from FITC to Nd^{3+}. Increasing amounts of Mg^{2+} or Ca^{2+} present during a titration displaced the curve shown in Fig. 3 to the right without changing its shape. The average Hill coefficient for all data was 1·3±0·3. The midpoints of the Nd^{3+}-induced fluorescence transition are plotted as a function of added Mg^{2+} or Ca^{2+} in Fig. 4. These data were analyzed using a model of competing cations for a single class of sites having no cooperativity and assuming the added concentration equaled the free concentration. The association constants for binding to the CaATPase in 500 mM KCl, 1·0 mM MOPS (pH 7·0) with 0·10 mg/ml SR protein at 25°C were $K_{Nd} = 3{\cdot}3 \times 10^5$ M^{-1}, $K_{Mg} = 35\ M^{-1}$ and $K_{Ca} = 84\ M^{-1}$.

Distance Estimates - The apparent distance between a single donor-acceptor pair can be calculated from (13)

$$r_{app} = R_o \left(\frac{1-E}{E} \right)^{1/6}$$

where E is the observed efficiency of fluorescence energy transfer. From the above results r_{app} is 1·0 nm. However, the number of acceptors per donor may be greater than 1. The transition in Fig. 3 suggests a single class of sites, but it is known that non-specific binding by Nd^{3+} occurs in the 10^{-5} M range

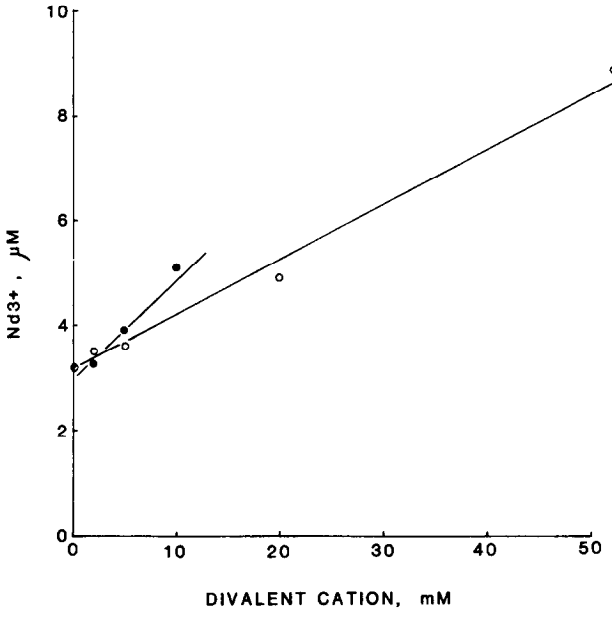


Figure 4. - Divalent Cation Protection. FITC-labeled SR vesicles were titrated as described in the legend for Fig. 3, except that the divalent cation concentrations were varied. The $[Nd^{3+}]$ at the mid-point of the fluorescence decrease is shown for increasing $[Ca^{2+}]$ (●) in the presence of 5 mM $MgCl_2$ and for increasing $[Mg^{2+}]$ (○) in the presence of 0·1 mM $CaCl_2$. The decrease obtained in 50 mM $MgCl_2$ was not reversible with EGTA.

(10). In the most favorable case, only one Nd^{3+} is an acceptor and the actual distance, r, between donor and acceptor is $r = r_{app} = 1{\cdot}0$ nm. No Nd^{3+} is closer than this to the FITC. The other extreme case is one with all the bound Nd^{3+} ions equally effective as acceptors. This would be true if they were equidistant from the FITC. This case is unlikely, but easily calculated and gives the farthest distance that the primary Nd^{3+} acceptors can be from the FITC. In this case, $r = n^{1/6} r_{app}$, where n is the number of acceptors (10). The value of n is estimated to be 9·5 Nd/CaATPase, taking $1{\cdot}15 \times 10^5 = M_r$ of the CaATPase, 65 nmoles Nd^{3+} total/mg SR protein (10), 70% of the SR protein as CaATPase, and correcting for the Nd^{3+} bound in the high-affinity Ca^{2+} binding sites (10,11). For this case $r = 1{\cdot}5$ nm. This distance is undoubtedly larger than that of the nearest Nd^{3+}; but it is useful because it demonstrates that even in the extreme case, the bound Nd^{3+} is close to the FITC on the CaATPase.

There are three primary sources of error to be considered for the calculated distances. The first is the random error of the measurement itself, which is about ±10%. The second source is unaccounted for inter-CaATPase quenching interactions. The small value of R_o (0·93 nm) compared to the dimensions of the CaATPase (about 3·5 nm (19)) make this source of error negligible. The third and major source is the uncertainty in the value assumed

for the orientation factor K^2, used to calculate R_o. The large error that could be introduced by assuming $K^2 = 2/3$ is greatly reduced by the fact that the Nd^{3+} ion is virtually symmetrical (14) and its absorption dipoles are randomly oriented with respect to the FITC emission dipole. A conservative estimate of the total error associated with the fluorescence energy transfer derived distance in this case is ±20%.

DISCUSSION

The results from the labeling experiments show that for pH = 7•5 or greater, there are 5•1 nmoles of FITC incorporated per mg of SR protein, very close to the number of phosphorylatable sites (7, 8). Higher pH does not improve the specificity of labeling. Labeling under conditions of pH = 7•0 or less, leads to incorporation of FITC greater than the number of phosphorylatable sites. The optimal condition appears to be pH 7•5 where the extraneous FITC is minimal and the solution is nearly neutral (Fig. 1).

The distance between the FITC and the Nd^{3+} ion or ions is less than 1•5 nm and probably about 1•0 nm, as estimated from the energy transfer measurements (Fig. 3). It is thought that FITC binds to the CaATPase in the ATP binding site (6) and the ATP protection against labeling at pH 7•5, shown here (Fig. 2), supports that conclusion. The size of the ATP binding site, estimated by assuming it is complementary in shape and size to ATP, is about 1•8 nm by 0•6 nm. The close proximity of Nd^{3+} and FITC, even for the case that gives the largest distance, strongly suggests that the Nd^{3+} binding site(s) should be considered part of the nucleotide binding active site of the CaATPase.

The site that binds Nd^{3+} also binds Mg^{2+} and Ca^{2+} (Fig. 4). This site is clearly not a high-affinity Ca^{2+} binding site, which has been shown to bind Nd^{3+} and other lanthanides with a much higher affinity (10, 15-17). The affinity for Mg^{2+} is compatible with that measured for Mg^{2+} binding to catalyze phosphoenzyme formation from orthophosphate (18), and for the site thought to bind Mg^{2+} derived from the substrate MgATP (4). In any case, Nd^{3+} is not the natural ligand for the site and the affinity for Ca^{2+} is too low for Ca^{2+} to be bound on the vesicle exteriors under physiological conditions. Thus it is likely that this binding site, which appears to be part of the ATP site, is for Mg^{2+}.

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