BINDING OF Eu^{3+} TO CARDIAC SARCOPLASMIC RETICULUM (Ca^{2+} + Mg^{2+})-ATPase-LASER EXCITED Eu^{3+} SPECTROSCOPIC STUDIES

NANDA B. JOSHI AND ADIL E. SHAMOO
Membrane Biochemistry Research Laboratory, Department of Biological Chemistry, University of Maryland, School of Medicine, Baltimore, Maryland 21201

ABSTRACT The binding of Eu^{3+} with Ca^{2+}-stimulated, Mg^{2+}-dependent adenosine triphosphatase ([Ca^{2+} + Mg^{2+}]-ATPase) of cardiac sarcoplasmic reticulum (SR) has been investigated using direct laser excited Eu^{3+} luminescence. Eu^{3+} is found to inhibit both Ca^{2+}-dependent ATPase activity and Ca^{2+}-uptake in a parallel manner. This is attributed to the binding of Eu^{3+} to the high affinity Ca^{2+}-binding sites. The K_i for Ca^{2+}-dependent ATPase is ~50 nM. The $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of Eu^{3+} in cardiac SR shows a peak at 579.3 nm, as compared to 578.8 nm in potassium-morpholino propane sulfonic acid (K-MOPS) pH 6.8. Upon binding with cardiac SR, Eu^{3+} shows an increase in fluorescence intensity as well as in lifetime values. The fluorescence decay of bound Eu^{3+} exhibits a double-exponential curve. The apparent number of water molecules in the first coordination sphere of Eu^{3+} in SR is 2.8 for the short component and 1.0 for the long component. In the presence of ATP, a further increase in fluorescence lifetimes is observed, and the number of water molecules in the first coordination sphere of Eu^{3+} is reduced further to 1.3 and 0.5. The double exponential nature of the decay curve and the different number of water molecules coordinated to Eu^{3+} for both decay components suggest that Eu^{3+} binds to two sites and that these are heterogeneous. The reduction in the number of H_2O ligands in the presence of ATP shows a change in the molecular environment of the Eu^{3+}-binding sites upon phosphoenzyme formation, with a movement of Eu^{3+} to an occluded site on the enzyme.

INTRODUCTION

The regulation of intracellular Ca^{2+} levels is the key to the contraction and relaxation of the cardiac muscle (1). The sarcoplasmic reticulum (SR) plays a major role in Ca^{2+} regulation. The ATP-dependent transport of Ca^{2+} across the SR is mediated by Ca^{2+}-stimulated, Mg^{2+}-dependent adenosine triphosphatase ([Ca^{2+} + Mg^{2+}]-ATPase), a single polypeptide of 110 kD. The active transport of Ca^{2+} by the cardiac SR has been reported to involve a phosphoprotein intermediate and to proceed by a mechanism similar to that of skeletal muscle (1–5). However, the rates of Ca^{2+} transport and concomitant Ca^{2+}-dependent hydrolysis of ATP are considerably lower in cardiac SR than in skeletal SR. A specific protein, phospholamban, is closely associated with the (Ca^{2+} + Mg^{2+})-ATPase of cardiac SR. The Ca^{2+}-dependent ATPase activity and the Ca^{2+}-uptake are activated upon phosphorylation of phospholamban by cAMP-and/or Ca^{2+}-calmodulin-dependent protein kinase (2–5). Most of the previous studies on the cardiac SR have focussed mainly on understanding the role of phospholamban in Ca^{2+} regulation. However, little is known about the Ca^{2+}-binding sites. This report deals with the characterization of the Ca^{2+}-binding sites in the cardiac SR and their molecular environment, using Eu^{3+}, as a first step toward understanding the molecular mechanism of Ca^{2+} regulation. Lanthanide ions are known as Ca^{2+} analogue in a variety of biological systems (6). It has been demonstrated in skeletal SR (Ca^{2+} + Mg^{2+})-ATPase that the Ca^{2+} sites may be effectively probed by Gd^{3+}, Tb^{3+}, Eu^{3+}, and other lanthanides (7–11). The usefulness of the luminescent lanthanides, such as Eu^{3+} and Tb^{3+}, has been greatly extended by the direct laser excitation technique developed by Horrocks and his group (12–15). The technique used in the present study involves direct excitation of the $^7F_0 \rightarrow {}^5D_0$ transition between the nondegenerate levels in Eu^{3+} by means of an intense laser pulse (13). Excitation profiles and luminescence decay, which are highly sensitive to the environment of Eu^{3+}, are used to characterize the distinct Eu^{3+}-binding sites in the system.

MATERIALS AND METHODS

$EuCl_3$ and D_2O (99.9%) were purchased from Alfa-Ventron (Danvers, MA). Tris-ATP was the product of Sigma Chemical Co. (St. Louis, MO). Adenylyl (β-γ methylene) triphosphate (AMP-PCP) and adenylyl-imidotriphosphate (AMP-PNP) were obtained from Boehringer Mannheim Diagnostics, Inc. (Houston, TX). (γ-^{32}P)ATP and $^{45}CaCl_2$ were purchased from New England Nuclear, Boston, MA. All other chemicals were reagent grade.

Cardiac microsomes were prepared from canine heart left ventricle according to the method of Harigaya and Schwartz (16) with minor

Address all correspondence to Dr. Adil E. Shamoo.

modifications (17). Cardiac left ventricular muscle was homogenized in 10 mM $NaHCO_3$ and centrifuged at 8,700 *g* for 20 min. The supernatant was then centrifuged at 10,000 *g* for 20 min. The supernatant was strained through four layers of cheesecloth, followed by centrifugation at 37,000 *g* for 1 h. The pellet was suspended in 20 mM Tris-maleate, 0.6 M KCl, pH 6.8, and centrifuged at 100,000 *g* for 1 h. The pellet containing microsomes was suspended in 0.25 M sucrose. The protein concentration was determined from the absorption at 280 nm in 1% SDS using $A_{1\%}$ = 10.0 (18) and by the method of Lowry et al. (19).

The ATPase activity was measured by the isotopic method as described elsewhere (20). Membrane vesicles were suspended at a concentration of 200 μg protein/ml in 0.4 ml of a reaction mixture containing 100 mM KCl, 10 mM $MgCl_2$, 5 mM NaN_3, 10 mM K-MOPS (pH 6.8), and $CaCl_2$. $CaCl_2$ concentrations were 30 and 60 μM. $EuCl_3$ concentration was varied from 0–1 mM. The reaction was initiated by the addition of 5 mM Tris (γ-^{32}P)ATP and was carried out at 25°C. The reaction was quenched by adding 0.2 ml of 10% trichloracetic acid (TCA). The samples were centrifuged, and the free phosphate liberated in the supernatant was extracted with 8.5% ammonium molybdate and butyl acetate and counted for radioactivity. The Ca^{2+}-independent ATPase activity was measured essentially by the above procedure, but 5 mM EGTA was included instead of $CaCl_2$.

Ca^{2+}-uptake assay was performed by millipore filtration (21). Membrane vesicles were suspended at a concentration of 200 μg protein/ml in 0.4 ml of reaction mixture, essentially the same as described above for the ATPase assay except $^{45}CaCl_2$ was included instead of $CaCl_2$. The concentration of $EuCl_3$ was varied from 0 to 1 mM. The reaction was started by adding 5 mM Tris-ATP and was carried out at 25°C. The reaction mixture was then filtered through 0.2-μ membranes. The membrane vesicles bound to the Millipore filter were washed with the reaction buffer and counted for radioactivity.

Phosphorylation of the SR vesicles was accomplished essentially by the method of Kirchberger et al. (22) with minor modification (17). Membrane vesicles were suspended at a concentration of 250 μg protein/ml in a reaction mixture containing 10 mM K-MOPS at pH 6.8, 100 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3, and 20 μM $CaCl_2$, or 20 μM $EuCl_3$, or 5 mM EGTA. The reaction was started by the addition of 50 μM Tris-(γ-^{32}P)ATP and was carried out at 25°C. The final volume was 0.2 ml. The reaction was terminated by adding 2 ml of a cold mixture of 10% TCA containing 0.1 mM KH_2PO_4 and 0.2 mM Tris-ATP. 0.2 ml of 0.63% bovine serum albumin (BSA) was added as a carrier protein. The samples were kept on ice for 15 min, followed by a centrifugation at 2,000 *g* for 10 min. The pellets were suspended in 0.2 ml of 0.6 N NaOH. The protein was reprecipitated by the addition of 2 ml of 10% TCA, centrifuged, resuspended in NaOH and then washed similarly three times. Finally, the pellet was suspended in 0.2 ml of 0.6 N NaOH and transferred to scintillation vials for counting radioactivity.

The luminescence instrumentation has been described elsewhere (23). Briefly, the exciting light for luminescence measurements was provided by a DL-12 dye laser pumped by a UV-12 pulsed nitrogen laser (Molectron Corp., Sunnyvale, CA). The line width at 580 nm (Rhodamine 6G dye) is ~0.01 nm. The sample housing, the collection optics, and the associated electronics were built by Photochemical Research Associates Inc. (London, Ontario). The emitted photons were selected by a double monochromator (model DH20; Instruments S.A., Inc., Metuchen, NJ) and detected by a cooled broadband photomultiplier tube (Hamamatsu Corp., Middlesex, NJ) in the photon counting mode. Lifetime data were collected on a multichannel sealer (model TN-1750; Tracor Northern, Middleton, WI) and transferred to a MINC-23 minicomputer (Digital Equipment Corp., Marlboro, MA) for storage and analysis. The excitation and the emission spectra were recorded directly on an X-Y plotter via a digital/analog interface built in our laboratory. The emission and the excitation spectra reported here are uncorrected. Analyses of the decay curves were performed on the MINC-23 using a nonlinear least squares fitting method.

The number of water molecules *q* in the first coordination sphere of Eu^{3+} bound to protein was calculated as described by Horrocks and Sudnick (12, 13) using the equation $q = 1.05$ ms (KH_2O-KD_2O). These authors have shown an uncertainty of ±0.5 in the estimation of the number of H_2O molecules by this procedure. The lifetimes were measured in H_2O-D_2O buffer mixtures with different mole fractions of H_2O. The decay constant $k = 1/\tau$ was plotted as a function of χ, the mole fraction of H_2O. The decay constant *k* in 100% D_2O was estimated by extrapolation.

The concentrations of free Eu^{3+}, Ca^{2+}, and Mg^{2+} were calculated using algorithms of Goldstein using a MINC-23 minicomputer (24). The association constant for Eu^{3+} to ATP was 6×10^6 M^{-1} (25).

In all of the luminescence measurements, 20 μM Eu^{3+} and 2 mg protein/ml SR were used. These concentrations were chosen to keep a 2:1 mol ratio of Eu^{3+} to enzyme. It is reported that 40% of the protein content of cardiac SR is (Ca^{2+} + Mg^{2+})-ATPase (2, 20). Taking the molecular weight of (Ca^{2+} + Mg^{2+})-ATPase to be 100 KDa, the 2 mg protein/ml is therefore approximately equivalent to a 10 μM enzyme concentration.

K_i was calculated, using the Dixon plot for competitive inhibition (26). The reciprocal of Ca^{2+}-dependent ATPase activity was plotted for various concentrations of free Eu^{3+}.

RESULTS

Eu^{3+} Inhibition of ATPase Activity and Ca^{2+} Uptake

To demonstrate that Eu^{3+} can compete with Ca^{2+} at the Ca^{2+}-activator sites of cardiac SR ATPase, we have studied the effect of Eu^{3+} on Ca^{2+}-dependent ATPase activity and Ca^{2+}-uptake. It was found that Eu^{3+} inhibits both Ca^{2+}-dependent ATPase and Ca^{2+}-uptake in a parallel manner as shown in Figs. 1 and 2. In the presence of 60 μM $CaCl_2$, the concentration of Eu^{3+} required for 50% inhibition of both functions was 0.6 μM. The inhibition of the ATPase activity by Eu^{3+} in the presence of 30 and 60 μM $CaCl_2$ concentrations is shown in Fig. 1. From this figure, it is clear that the concentration of Eu^{3+} required for 50% inhibition of ATPase varies with $CaCl_2$ concentration. In the inset, the Dixon plot for competitive inhibition is displayed (26). K_i was calculated as described in the Materials and Methods section. The K_i value obtained using these data is 50 nM. Our studies on the inhibition of the ATPase activity by Eu^{3+} are consistent with the lanthanide inhibition of the skeletal muscle enzyme (9–11). Inhibition of the Ca^{2+}-uptake (Fig. 2), which has not been reported previously, further supports the existence of a competition between Eu^{3+} and Ca^{2+} for the Ca^{2+} sites.

Phosphoenzyme Formation in the Presence of 20 μM Eu^{3+}

We have also investigated the phosphoenzyme (EP) formation in the presence of 20 μM Eu^{3+}; the data are shown in Fig. 3. These studies were performed in conjunction with our spectroscopic measurements using 20 μM Eu^{3+}. Fig. 3 shows the time course of EP formation in the presence of 20 μM Eu^{3+}. For comparison, the data obtained in the presence of 20 μM $CaCl_2$ and 5 mM EGTA are also shown. These results clearly show the formation of EP in the presence of 20 μM Eu^{3+}, although the EP formation is slower when compared with that occurring with Ca^{2+}.

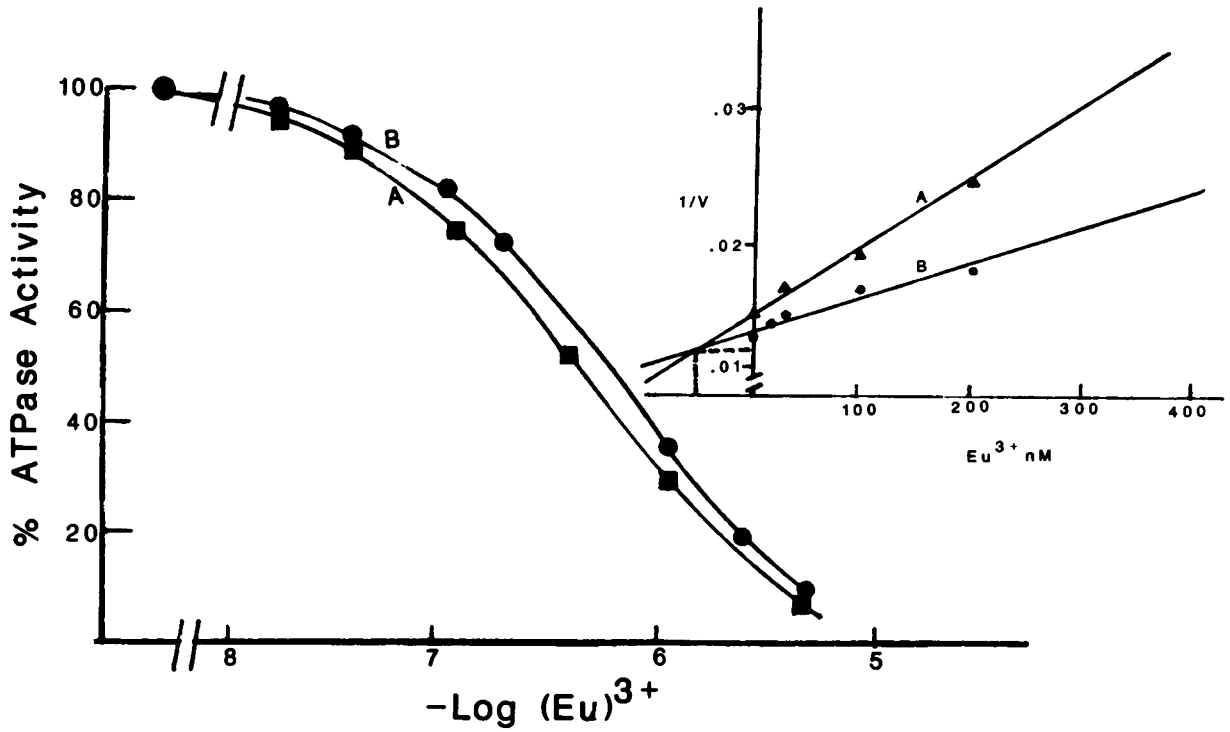

FIGURE 1 Ca^{2+}-dependent ATPase activity of (Ca^{2+} + Mg^{2+})-ATPase of cardiac SR for various concentrations of Eu^{3+}. 200 μg protein/ml SR vesicles were suspended in 0.4 ml of a reaction mixture containing 10 mM K-MOPS (pH 6.8), 100 mM KCl, 10 mM $MgCl_2$, 5 mM NaN_3, and (*A*) 30 μM $CaCl_2$, and (*B*) 60 μM $CaCl_2$. Eu^{3+} concentration was varied from 0 to 1 mM. The reaction was started by adding 5 mM Tris (γ^{-32}P)ATP and was quenched by adding 0.2 ml of 10% TCA at the required time. The free Eu^{3+} concentrations shown in the abscissa were calculated as described in the Materials and Methods section. (*Inset*) reciprocal of ATPase activity ($1/V$) vs. free Eu^{3+} plot for (*A*) 30 μM $CaCl_2$ and (*B*) 60 μM $CaCl_2$.

Fluorescence Emission and Excitation Spectra

The fluorescence spectra of Eu^{3+} in the absence and presence of the SR preparation were recorded using 390 nm ($^7F_0 \rightarrow {}^5L_6$) excitation wavelengths and are shown in

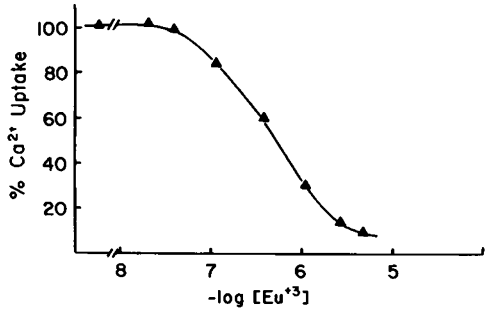

FIGURE 2 Ca^{2+}-uptake by cardiac SR in the presence of various concentrations of Eu^{3+}. Membrane vesicles were suspended at a concentration of 200 μg protein/ml in 0.4 ml of a reaction mixture containing 10 mM K-MOPS (pH 6.8), 100 mM KCl, 10 mM $MgCl_2$, 5 mM NaN_3, 5 mM Tris-ATP, and 60 μM $^{45}CaCl_2$. Eu^{3+} concentration was varied from 0 to 1 mM. The reaction was started by adding 5 mM Tris-ATP and the reaction mixture was filtered through 0.2-μ membrane at the required time. The membrane vesicles bound to the millipore filter were washed with the reaction buffer. Free Eu^{3+} concentrations given in the abscissa were calculated as described in Materials and Methods section.

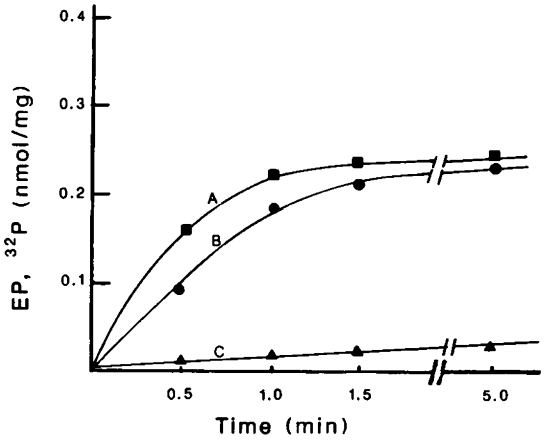

FIGURE 3 Time course of phosphoenzyme (EP) formation of (Ca^{2+} + Mg^{2+})-ATPase of cardiac SR. EP formation was measured with 250 μg/ml cardiac SR in 0.2 ml of a reaction mixture containing 10 mM K-MOPS (pH 6.8), 100 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3, and (*A*) 20 μM $CaCl_2$, (*B*) 20 μM $EuCl_3$, and (*C*) 5 mM EGTA. The reaction was started by adding 50 μM Tris-(γ-^{32}P)ATP and was terminated at the required time by adding 2 ml cold 10% TCA containing 0.2 mM ATP and 0.1 mM KH_2PO_4. 0.2 ml of 0.63% BSA was used as carrier protein. After incubation on ice, the samples were centrifuged and washed three times.

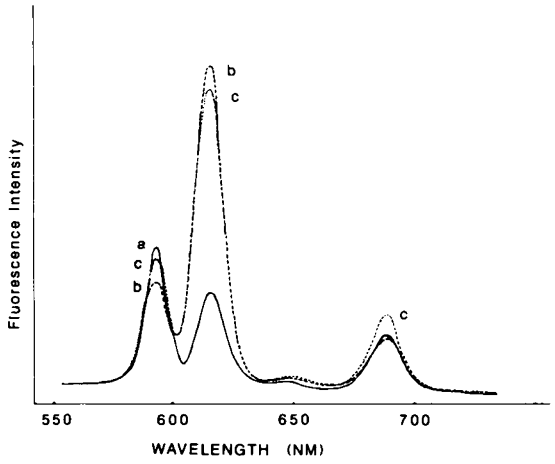

FIGURE 4 Emission spectra of Eu^{3+} in cardiac SR. The medium contained in 10 mM K-MOPS, 100 mM KCl, and 5 mM $MgCl_2$ (pH 6.8). Concentrations of SR and Eu^{3+} were 2 mg protein/ml and 20 μM, respectively. ATP used was 50 μM. The excitation wavelength was 394 nm ($^7F_0 - {}^5L_0$). (*a*) Eu^{3+} without SR, (*b*) Eu^{3+} with SR, and (*c*) Eu^{3+} with SR in the presence of 50 μM ATP.

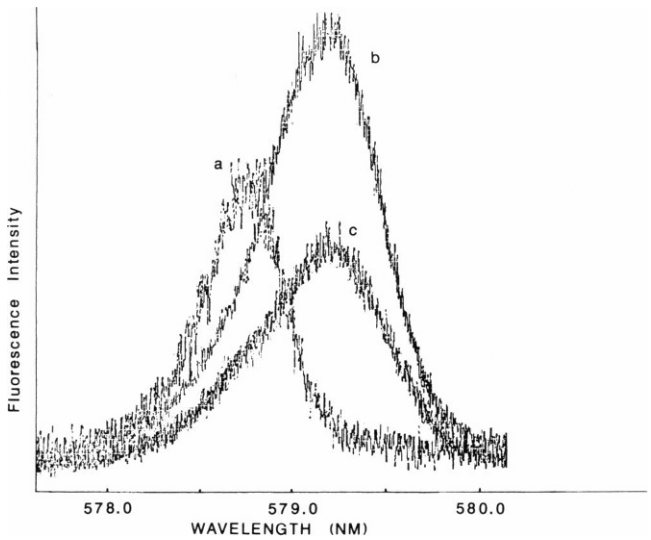

FIGURE 5 Excitation spectra for $^7F_0 \rightarrow {}^5D_0$ transitions of Eu^{3+} in cardiac SR. The medium contained 10 mM K-MOPS, 100 mM KCl, and 5 mM $MgCl_2$ (pH 6.8). SR concentration was 2 mg protein/ml, whereas Eu^{3+} was 20 μM. The concentrations of ATP was 50 μM. Excitation spectra were recorded from 578–580.5 nm for $^7F_0 \rightarrow {}^5D_0$ transition by monitoring the emission intensity for $^5D_0 \rightarrow {}^7F_2$ transition at 618 nm. (*a*) Eu^{3+} without SR, (*b*) Eu^{3+} with SR, and (*c*) Eu^{3+} with SR in the presence of 50 μM ATP.

Fig. 4. The fluorescence spectrum of Eu^{3+} in K-MOPS (pH 6.8) exhibited three main emission bands at 593 nm ($^5D_0 \rightarrow {}^7F_1$), 618 nm ($^5D_0 \rightarrow {}^7F_2$), and 690 nm ($^5D_0 - {}^7F_4$), and one weak band at 650 nm ($^5D_0 \rightarrow {}^7F_3$). In the presence of the enzyme source, a fivefold enhancement of total fluorescence intensity was observed. In addition, the relative intensity of the 618 nm ($^5D_0 \rightarrow {}^7F_2$) band changed dramatically. This is most likely due to the change in the ligand environment around the Eu^{3+} ion (13). This phenomenon is due to a hypersensitive characteristic associated with the $\Delta J = 2$ transition.

To study the binding sites of cardiac SR ATPase, we chose the $^7F_0 \rightarrow {}^5D_0$ transition of Eu^{3+} because it possesses certain unique advantages. Both the ground state ($^7F_0$) and the excited state ($^5D_0$) are nondegenerative (13) and cannot be split by a ligand field. Thus, this transition must generate a single unsplit line for a given Eu^{3+} site. The excitation spectrum for the $^7F_0 \rightarrow {}^5D_0$ transition of Eu^{3+} was scanned from 578 nm to 580.5 nm, by monitoring the emission intensity for $^5D_0 \rightarrow {}^7F_2$ transition of Eu^{3+} at 618 nm. The excitation spectra for the $^7F_0 \rightarrow {}^5D_0$ transition of Eu^{3+} with and without the SR preparation are given in Fig. 5. The excitation spectrum of Eu^{3+} in 10 mM K-MOPS (pH 6.8) with SR exhibited a maximum peak at 579.3 nm, whereas in the absence of SR the peak was at 578.8 nm. The width at half height of the composite band of the Eu^{3+}-SR complex is 0.7 nm as compared with 0.5 nm for the Eu^{3+} aqua ion. The excitation spectrum of the Eu^{3+}-SR complex also showed an additional feature on the low energy side that was absent in the case of the Eu^{3+} aqua ion.

Fluorescence Decay Times

The fluorescence decay curve of the Eu^{3+}-SR complex was multi-exponential, which indicates the presence of more than one emitting species in the system. The decay curves were analyzed by the method of least squares analysis. Table I shows the parameters obtained for single, double, and triple exponentials. We rejected a one component fit on the basis of the χ^2 analysis. A three-component fit was also not acceptable in view of the large uncertainty in the lifetime data and low value of fractional intensity of the third component. Therefore, the best fit could be obtained for two exponentials. The two decay components obtained were 302 ± 5 μs and 847 ± 15 μs with the fractional intensities of 0.62 and 0.38, respectively. The two decay components observed represent two distinct Eu^{3+}-binding sites on the protein. In K-MOPS, Eu^{3+} showed a single exponential with a lifetime of 114 ± 5 μs. We have also measured the lifetimes of the Eu^{3+}-SR complex using 579.0 and 579.6 nm excitation wavelengths, but no significant difference in the lifetime values was observed. The lifetime data given in Table II are the average of three measurements for the excitation at 579.3 nm.

Effect of ATP and ATP Analogue

The effect of ATP on the fluorescence intensity and the lifetimes of Eu^{3+} bound to SR was studied. In the presence of ATP, we observed a decresae in the fluorescence intensity with no change in the peak position, whereas the lifetime values showed an increase. Fig. 6 shows the decay curve of Eu^{3+} bound to SR in the presence of ATP. The decay curve is a double exponential and the lifetimes are 502 ± 16 μs and 1193 ± 48 μs. The decay times of both components showed an increase in the presence of ATP.

TABLE I

Sample	No. of exponentials	Decay time (τ)	Amplitudes (α_i)	Fractional intensity	χ^2
		μs			
Eu^{3+} in cardiac SR	Single	477.19 ± 6.5	1.0	1.0	42
	Double	τ_1 = 302 ± 5	0.70	0.62	0.92
		τ_2 = 847 ± 15	0.14	0.38	
	Triple	τ_1 = 278 ± 12	0.62	0.54	0.86
		τ_2 = 704 ± 132	0.21	0.43	
		τ_3 = 1,683 ± 1,892	0.006	0.03	

Analysis of single, double, and triple exponential curves of the fluorescence decay of Eu^{3+} in cardiac SR. The medium contained 10 mM K-MOPS, 100 mM KCl, and 5 mM $MgCl_2$ (pH 6.8). The concentration of SR was 2 mg protein/ml and the concentration of Eu^{3+} was 20 μM. The excitation wavelength was 579.3 nm and emission was monitored at 618 nm. The closeness of the fit for single, double, and triple exponentials were analyzed using a nonlinear least squares algorithm.

However, in the presence of the ATP analogues AMP-PNP and AMP-PCP, no significant change in the fluorescence lifetime was observed.

Control experiments with ATP in the absence of SR were performed. ATP is known to bind with Eu^{3+} (25). The excitation maximum of the Eu-ATP complex was at 579.0 nm, and the decay curve was a single exponential with a lifetime of 165 ± 5 μs.

TABLE II

Medium	Excitation peak position	Lifetimes	Nos. of H_2O
	nm	*μs*	
K-MOPS	578.8	114	9
K-MOPS + ATP	579.0	165	6
Cardiac SR	579.3	302	2.8
		847	1.0
Cardiac SR + AMP-PCP	579.3	300	2.8
		868	1.0
Cardiac SR + AMP-PNP	579.3	302	2.8
		900	0.9
Cardiac SR + ATP	579.3	502	1.3
		1,193	0.5

Effect of ATP and ATP analogues (AMP-PCP and AMP-PNP) on the fluorescence parameters and the number of H_2O molecules coordinated to the Eu^{3+} ion. The medium contained 10 mM K-MOPS, 100 mM KCl, and 5 mM $MgCl_2$ (pH 6.8). SR and Eu^{3+} concentrations were 2 mg protein/ml and 20 μM, respectively. Concentration of ATP, AMP-PCP, and AMP-PNP was 50 μM. The excitation wavelength used is given in the second column, and the emission was monitored at 618 nm. Lifetime data shown in the third column are the averages of three measurements. The number of H_2O molecules coordinated to Eu-SR were estimated as described in the text using q = 1.05 (KH_2O − KD_2O). KH_2O and KD_2O are the decay constants in H_2O and 100% D_2O, respectively. To determine KD_2O, the lifetime data were measured for various H_2O-D_2O buffer mixtures and KD_2O was obtained by linear extrapolation to 100% D_2O (Fig. 7).

Number of Coordinated H_2O Molecules

The number of water molecules coordinated to the Eu^{3+}-SR complex was calculated as described in the Materials and Methods section. Fluorescence lifetimes of the Eu^{3+}-SR complex in H_2O-D_2O buffer for various mole fractions (χ) of H_2O were measured. The reciprocal of lifetime, i.e., the decay constant $k = 1/\tau$, for various mole fractions of H_2O (χ) was plotted as shown in Fig. 7. The value of the decay constant in 100% D_2O was determined by linear extrapolation to 100% D_2O, the decay constant obtained by this procedure was 0.78 ms^{-1} for the short and 0.28 ms^{-1}

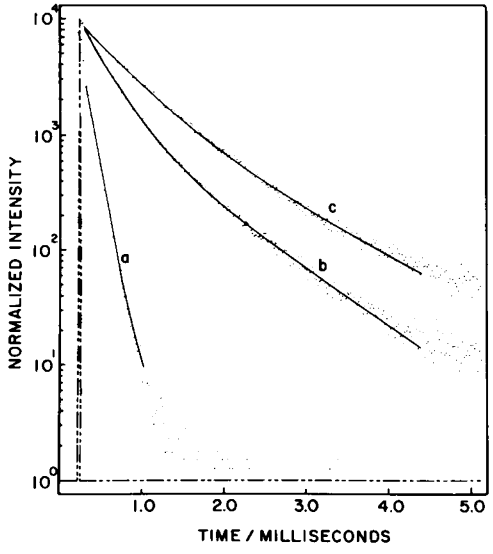

FIGURE 6 Luminescence decay curves of Eu^{3+} in cardiac SR. The medium contained 10 mM K-MOPS, 100 mM KCl, and 5 mM $MgCl_2$ (pH 6.8). SR concentration was 2 mg/ml whereas Eu^{3+} was 20 μM. Measurements were performed using an excitation wavelength of 579.3 nm and an emission wavelength of 618 nm. (*a*) Eu^{3+} without SR, (*b*) Eu^{3+} with SR, and (*c*) Eu^{3+} with SR in the presence of 50 μM ATP.

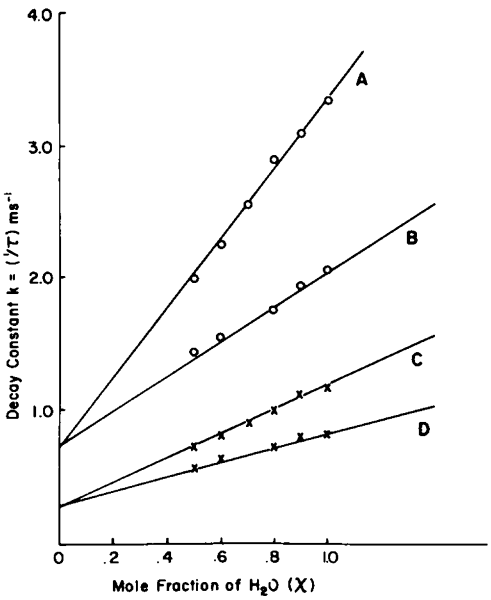

FIGURE 7 Luminescence decay constants $k = 1/\tau$ of the Eu^{3+}-SR complex for various mole fractions (χ) of H_2O. The medium contained 10 mM K-MOPS, 100 mM KCl, and 5 mM $MgCl_2$ (pH 6.8), and different percentages of D_2O. SR concentration was 2 mg/ml and Eu^{3+} was 20 μM. Measurements were performed using the 579.3 nm excitation and 618 nm emission wavelengths. Short lived component: (*A*) without ATP, (*B*) with ATP. Long lived component: (*C*) without ATP, (*D*) with ATP. *Solid lines*, the linear least-squares fit.

for the long component of decay. The decay constant in 100% H_2O was obtained directly from the lifetime measurements. The number of H_2O molecules estimated was 2.8 for the short component and 1.0 for the long component of decay. Similar measurements were performed in the presence of 50 μM each of AMP-PCP, AMP-PNP, and ATP to estimate the number of H_2O molecules coordinated in their presence. In the presence of ATP, the decay constants of Eu^{3+}-SR complex for various mol fractions of H_2O are plotted in Fig. 7. The plots for AMP-PCP and AMP-PNP are not shown. In the presence of ATP, the number of water molecules coordinated to the Eu^{3+}-SR complex fell to 1.3 and 0.5 for the short and long decay components. However, in the presence of AMP-PCP and AMP-PNP, the nonhydrolyzable analogues of ATP, no significant reduction of the number of H_2O molecules coordinated to the Eu^{3+}-SR complex was observed. These data are shown in Table II. The number of H_2O molecules given in Table II are estimated with an uncertainty of ± 0.5 (12).

DISCUSSION

The results of the present investigation suggest that Eu^{3+} binds to SR ATPase at two sites that are the two high affinity Ca^{2+}-binding sites. Krasnow (7) studied the binding of Gd^{3+} to cardiac SR and observed that Gd^{3+} binds to the high affinity Ca^{2+}-binding sites. He also observed a decrease in the ATPase activity of SR with Gd^{3+} binding. In the present studies, we observed an inhibition of the Ca^{2+}-dependent ATPase activity as well as the Ca^{2+}-uptake by Eu^{3+}. We conclude that the binding of Eu^{3+} to Ca^{2+}-translocating sites is the reason for the inhibition of the ATPase activity and the Ca^{2+} uptake.

It was not possible to resolve the two binding sites from the excitation spectrum of Eu^{3+} bound to SR. This may be due to a charge similarity on the two Ca^{2+} sites, because the position of excitation maximum represents the total charge on the Eu^{3+} ion (13). The results of the present study showed an excitation peak at 579.3 nm for the Eu^{3+}-SR complex as compared with 578.8 nm for free Eu^{3+}. This red shift of bound Eu^{3+} is ~ 15 cm^{-1}. Sudnick (13) has demonstrated that a red shift of 7.5 cm^{-1} in peak position corresponds to a neutralization of one unit charge. In the present case the red shift of 15 cm^{-1} in the excitation peak for the Eu^{3+}-SR complex as compared with the free Eu^{3+} ion corresponds to a neutralization of two charges.

The double exponential nature of the decay curve of the Eu^{3+}-SR complex definitely suggests that Eu^{3+} binds to two sites, which may reflect the high affinity Ca^{2+}-sites of cardiac SR. The association constant for lanthanide attachment to Ca^{2+} binding ligands is usually much higher than that for Ca^{2+} because of their higher charge within the same ionic radius. In the present case, the inhibition of Ca^{2+}-dependent ATPase by Eu^{3+} with a K_i of 50 nM suggests that Eu^{3+} binds to these sites with high affinity. The lower number of H_2O molecules coordinated to the Eu^{3+}-SR complex, as compared with the free ion, indicates that the metal ion sites are relatively unhydrated. The different values of the number of H_2O molecules for the shorter (2.8) and the longer (1.0) decay components indicate that the molecular environment of these sites is different. Further reduction in the number of H_2O molecules to 1.3 and 0.5 in the presence of ATP for both decay components strongly suggests that a change in the molecular environment of the Ca^{2+} binding is associated with EP formation and the movement of the metal ion to an occluded site in the enzyme. We suggest that the reduction of the number of H_2O molecules coordinated to the Eu^{3+}-SR complex in the presence of ATP is not due to a simple binding process because nonhydrolyzable analogues of ATP, such as AMP-PCP or AMP-PNP, did not cause a significant reduction of H_2O molecules. In preparations from skeletal muscle enzyme using NMR techniques, Stefens and Grisham (8) have reported a significant reduction of the number of H_2O molecules coordinated to Gd^{3+} by AMP-PNP. Presently, we do not know the reason for this apparent discrepancy. However, experiments performed in our laboratory on skeletal SR using Eu^{3+} showed no reduction of H_2O molecules in the presence of AMP-PNP (11). The reduction in the number of H_2O molecules to 1.3 and 0.5 indicates that the ATPase polypeptide provides Eu^{3+} with about eight ligands to replace the H_2O molecules. Therefore, in the case of Ca^{2+}, these ligands could strip the H_2O molecules down to zero since the aqua

Ca^{2+} ion possess eight instead of nine H_2O molecules that are associated with the Eu^{3+} ion. These studies are consistent with the studies on the skeletal muscle enzyme, which demonstrate that the two high affinity sites for Ca^{2+} are heterogeneous in nature and that, in the phosphorylated state of the enzyme, these sites are occluded (8, 11).

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