

# NMR studies identify four intermediate states of ATPase and the ion transport cycle of sarcoplasmic reticulum $\text{Ca}^{2+}$ -ATPase

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Water proton nuclear relaxation measurements are used to detect and characterize four distinct intermediate states for  $\text{Gd}^{3+}$  bound to  $\text{Ca}^{2+}$  sites of sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase in complexes with ATP analogues. In the absence of nucleotides,  $\text{Gd}^{3+}$  binds to two occluded  $\text{Ca}^{2+}$  transport sites on  $\text{Ca}^{2+}$ -ATPase which have a low accessibility to solvent water. In the presence of the nonhydrolyzable ATP analogue,  $\text{Co}(\text{NH}_3)_4\text{AMPPCP}$ , a new state for bound  $\text{Gd}^{3+}$  (still occluded and with fewer waters of hydration) is observed. In the presence of  $\text{Co}(\text{NH}_3)_4\text{ATP}$  or ATP, two additional states for bound  $\text{Gd}^{3+}$  are detected in the NMR studies. The first of these probably represents an intermediate state for bound  $\text{Gd}^{3+}$  during ATP hydrolysis. The latter is the most occluded  $\text{Gd}^{3+}$  site yet observed in these studies and is probably analogous to the highly occluded  $\text{E}_1\text{-P}$  state observed with CrATP [(1987) *Biochim. Biophys. Acta* 898, 313-322].

## 1. INTRODUCTION

The sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase, which is responsible for active calcium transport across the SR membrane, exists in the SR membrane as a 115 kDa protein with associated, essential phospholipid. Calcium transport depends on conformation changes in this protein [1], but the detailed translocation mechanism is for the most part unresolved. Several authors have demonstrated the existence of a phosphorylated transport intermediate containing occluded calcium ions, i.e. bound  $\text{Ca}^{2+}$ , which is unable to exchange with  $\text{Ca}^{2+}$  at either side of the membrane [2-5], but the nature of the  $\text{Ca}^{2+}$ -occluded form is largely uncharacterized.

One approach to the details of  $\text{Ca}^{2+}$  transport is to use a spectroscopically active analogue of  $\text{Ca}^{2+}$ . Our laboratory demonstrated in 1979 that the para-

magnetic lanthanide ion,  $\text{Gd}^{3+}$ , could be used for NMR and EPR studies of the  $\text{Ca}^{2+}$ -ATPase [6]. These studies showed that the ATPase bound to  $\text{Gd}^{3+}$  at high-affinity  $\text{Ca}^{2+}$  sites, and that these calcium sites were in fact occluded sites, with a very low accessibility of solvent water. More recently, we have shown that the soluble, monomeric  $\text{Ca}^{2+}$ -ATPase also binds  $\text{Gd}^{3+}$  at occluded sites [7]. Vilsen and Andersen [8] subsequently reported occlusion of  $\text{Ca}^{2+}$  in soluble, monomeric SR  $\text{Ca}^{2+}$ -ATPase, in corroboration of our NMR results with  $\text{Gd}^{3+}$ .

We show here that several  $\text{Gd}^{3+}$ -ATPase complexes, which may represent intermediates of ATP hydrolysis and/or  $\text{Ca}^{2+}$  transport, can be isolated and characterized using NMR techniques with appropriate substrate analogues.

## 2. MATERIALS AND METHODS

The  $\text{Ca}^{2+}$ -ATPase was purified and prepared for spectroscopic studies as described [7]. The  $\beta, \gamma$ -bidentate complexes of Co(III) with ATP and AMP-PCP were synthesized as described [9].  $\text{Co}(\text{NH}_3)_5\text{PO}_4$  was synthesized according to Schmidt and Taube [10].

Water proton relaxation rate measurements were used to examine the interactions of  $\text{Gd}^{3+}$  with the ATPase. The

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*Abbreviations:*  $\text{NMe}_4^+$ , tetramethylammonium; Pipes, 1,4-piperazinediethanesulfonic acid; AMP-PCP, adenylyl-5'-yl methylenediphosphate

longitudinal relaxation rate,  $1/T_1$ , of the protons of water was measured between the frequencies of 20 and 85 MHz on a variable frequency, pulsed NMR spectrometer of our own design. Gadolinium is a paramagnetic ion and increases the  $1/T_1$  rate of water protons in its coordination sphere. In the presence of a macromolecule, the effect of the paramagnetic metal ion on the relaxation rate is enhanced due to an increase in the correlation time of the ion-water interaction. All measurements were obtained at  $23 \pm 1^\circ\text{C}$  on samples of  $100 \mu\text{l}$ . The calculations in this paper and the practical application of the theory to structural studies of biomolecules have been reviewed [11].

### 3. RESULTS

#### 3.1. Formation of a stable complex with $\text{Co}(\text{NH}_3)_4\text{AMPPCP}$

In order to form a stable nucleotide complex of the ATPase with  $\text{Gd}^{3+}$ , the  $\text{Ca}^{2+}$ -ATPase- $\text{Gd}^{3+}$  complex was titrated with  $\text{Co}(\text{NH}_3)_4\text{AMPPCP}$  (fig.1A). The sample contained  $0.06 \text{ mM}$   $\text{Ca}^{2+}$ -ATPase and  $0.05 \text{ mM}$   $\text{GdCl}_3$ . The enhancement of the longitudinal relaxation rate of water protons decreases to a value of 4.5 at saturating CoAMPPCP. As shown in fig.1B, in the absence of added CoAMPPCP, there is a small, slow decrease in the observed enhancement of the binary  $\text{Gd}^{3+}$ -ATPase complex over a period of 200 min, after which the observed enhancement is constant. When CoAMPPCP is added, the observed enhancement decreases rapidly to a value which is either constant (at high levels of CoAMPPCP) or decreases only very slowly for several hours. The rapid decrease in the observed enhancement on addition of CoAMPPCP followed by a relatively constant value of  $\epsilon^*$  is consistent with the formation of a stable ATPase- $\text{Gd}^{3+}$ -CoAMPPCP complex with a water relaxation enhancement factor of approximately 3.6. The reduced enhancement factor may represent either a reduction in the number of fast exchanging water molecules coordinated to  $\text{Gd}^{3+}$  or a change in the dipolar correlation time for the  $\text{Gd}^{3+}$ - $\text{H}_2\text{O}$  interaction. This point is addressed below.

#### 3.2. Sequential formation of multiple complexes with ATP and $\text{Co}(\text{NH}_3)_4\text{ATP}$

In contrast to the results obtained with the non-hydrolyzable CoAMPPCP, titrations of the ATPase- $\text{Gd}^{3+}$  binary complex with either ATP or  $\text{Co}(\text{NH}_3)_4\text{ATP}$  resulted in a series of time-dependent changes in the observed enhancement. Fig.2 shows the behavior observed in titrations

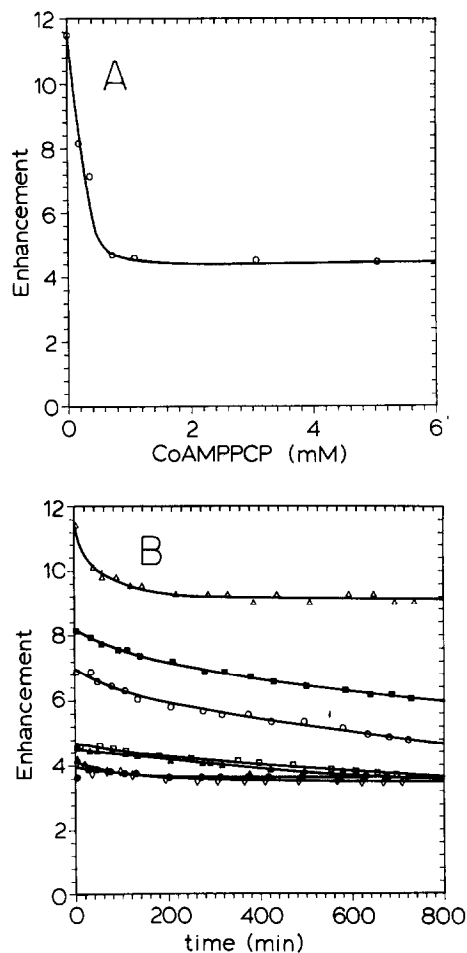


Fig.1. Effect of CoAMPPCP on water proton relaxation by ATPase-bound  $\text{Gd}^{3+}$ . Initial values are shown in A and the time course is shown in B. Concentrations of CoAMPPCP:  $0.00 \text{ mM}$  ( $\Delta$ ),  $0.18 \text{ mM}$  ( $\blacksquare$ ),  $0.36 \text{ mM}$  ( $\circ$ ),  $0.72 \text{ mM}$  ( $\square$ ),  $1.09 \text{ mM}$  ( $\blacktriangle$ ),  $3.06 \text{ mM}$  ( $\diamond$ ),  $5.04 \text{ mM}$  ( $\bullet$ ).

with ATP. A rapid, concentration-dependent decrease in the observed enhancement (fig.2B) is followed by a slower increase and then a decrease in  $\epsilon^*$ . The same pattern is observed at all ATP concentrations employed ( $0.025$ – $0.658 \text{ mM}$ ). As the ATP concentration is increased, the enhancement measured immediately following the addition of ATP decreases, and the maximum enhancement measured during the slow phase is decreased. The values of  $\epsilon^*$  following the rapid initial decrease on ATP addition are plotted versus ATP concentration in fig.2A. The limiting value of  $\epsilon^*$  at high ATP is 3.6, similar to that obtained at high levels of

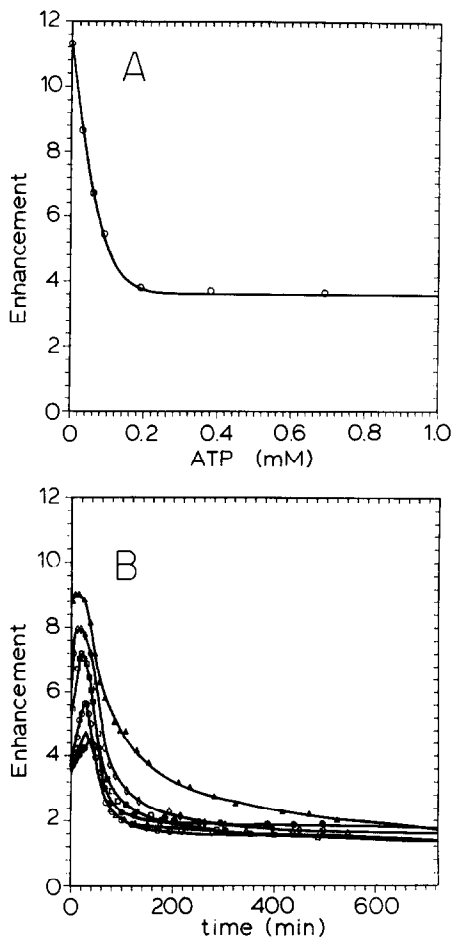


Fig.2. Effect of ATP on enhancement of water relaxation by ATPase-Gd<sup>3+</sup>. Initial values in A and time course in B. Concentrations of ATP: 0.025 mM (▲), 0.060 mM (◊), 0.094 mM (□), 0.188 mM (○), 0.376 mM (Δ), 0.658 mM (●).

CoAMPPCP in fig.1A, consistent with the formation of similar complexes in these two cases. The final enhancement of approx. 1 could either represent enzyme-bound Gd<sup>3+</sup> with a low enhancement factor or Gd<sup>3+</sup> which has been dissociated from the enzyme. Methods to distinguish these possibilities are described below.

Similar behavior, with a somewhat slower time course, is also observed for Co(NH<sub>3</sub>)<sub>4</sub>ATP. As shown in fig.3B, a rapid, concentration-dependent decrease in the observed enhancement is followed by a slower increase and then a decrease in  $\epsilon^*$ . The limiting value of  $\epsilon^*$  at high levels of CoATP is somewhat lower than that observed for

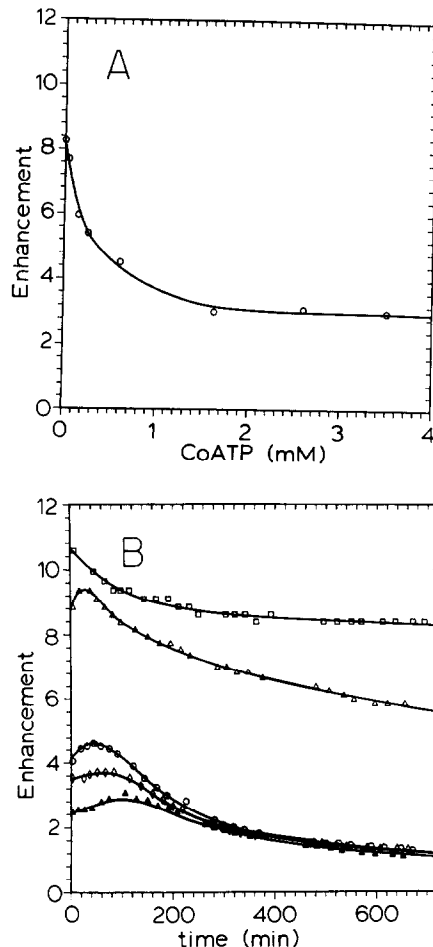


Fig.3. Effect of CoATP on enhancement of water relaxation by ATPase-Gd<sup>3+</sup>. Initial values in A and time course in B. Concentrations of CoATP: 0.00 mM (□), 0.04 mM (Δ), 0.77 mM (○), 1.53 mM (◊), 3.06 mM (▲).

CoAMPPCP and ATP (2.5 compared to 3.6). The overall time course, on the other hand, is very similar to that seen with ATP. The slow increase, then decrease, in  $\epsilon^*$  on ATP addition was analyzed in terms of the following mechanism:



Values for  $k_1$  and  $k_2$  were determined by fitting the data of figs 2B and 3B to a sequential mechanism as in eqn 1. At a concentration of CoATP of 0.776 mM, for example, the best fit for  $k_1$  is 0.02 min<sup>-1</sup> and the best fit for  $k_2$  is 0.0065 min<sup>-1</sup>. The point of maximum enhancement in the slow

phase occurs at longer times as the concentration of CoATP is increased.

### 3.3. Characterizing intermediates with occluded calcium sites

We have previously shown that the nuclear relaxation properties of water can be used to detect and characterize occluded sites for  $Gd^{3+}$  (i.e.,  $Ca^{2+}$ ) on the  $Ca^{2+}$ -ATPase [6,7]. The relaxation rates of water protons coordinated to  $Gd^{3+}$ , which is in turn bound to  $Ca^{2+}$  sites on the ATPase, are dependent on  $\tau_c$ , the correlation time for the  $Gd^{3+}$ -nucleus dipolar interaction. For enzyme complexes,  $\tau_c$  itself is dominated by  $\tau_s$ , the spin-lattice relaxation time for the electrons of  $Gd^{3+}$ .  $\tau_s$  is in turn dependent on the rate of transient distortions of the  $Gd^{3+}$  coordination geometry due to collisions with solvent molecules. If the  $Gd^{3+}$  site is occluded, transient distortions due to water encounters will be infrequent and the experimental  $\tau_c$  will be large.  $\tau_c$  is normally determined by a study of the frequency dependence of the metal-induced relaxation rate,  $1/fT_{1p}$  [12]. The results of such a study for the complexes studied in this paper are shown in table 1. The  $\tau_c$  values for the  $Ca^{2+}$ -ATPase obtained here (table 1) and in our previous studies [6,7] are unusually long compared to those observed in other  $Gd^{3+}$ -protein complexes [13,14]. The complex with the longest value of  $\tau_c$  (i.e.,  $\tau_s$ ) is the  $Gd^{3+}$ -ATPase- $Co(NH_3)_4ATP$  complex formed at long incubation times. The value of  $\tau_c$  observed in this case ( $1 \times 10^{-8}$  s) is 30–200 times greater than those for typical  $Gd^{3+}$ -protein complexes and reflects what is apparently the most highly occluded  $Gd^{3+}$  site observed to date. This is consistent with the report by Vilsen and Andersen [15] of highly occluded  $Ca^{2+}$  in an  $E_1$ -P state formed after long incubations with  $Cr(H_2O)_4ATP$ .

In the limit of fast exchange, the Solomon-Bloembergen equation which describes the  $Gd^{3+}$ - $H_2O$  dipolar interaction [12] is

$$1/fT_{1p} = 2[H_2O]/([Gd^{3+}]T_{1p}) = (896/r)^6 q [f(\tau_c)] \quad (3)$$

where  $q$  is the number of water protons in the inner coordination sphere of  $Gd^{3+}$ ,  $r$  is the  $Gd^{3+}$ -water proton distance, and  $f(\tau_c)$  is the correlation function, which is given by:

$$f(\tau_c) = 3\tau_c/(1 + \omega_I^2\tau_c^2) + 7\tau_c/(1 + \omega_S^2\tau_c^2) \quad (4)$$

Table 1

Complex	Analysis of the frequency dependence of $T_{1p}$	
	$\tau_c$ (s $\times 10^9$ )	$q$
ATPase- $Gd_{site 1}$	1.91	3.11
ATPase- $Gd_{site 2}$	2.12	1.66
ATPase- $Gd_{site 1}$ - $Co(NH_3)_4AMPPCP$	1.62	1.06
ATPase- $Gd_{site 1}$ - $Co(NH_3)_5PO_4$	4.89	0.42
ATPase- $Gd_{site 1}$ - $Co(NH_3)_4ATP$	9.90	0.25

In this equation,  $\omega_I$  is the nuclear resonance frequency and  $\omega_S$  is the electron resonance frequency. Having determined values for  $\tau_c$ , we can use eqns 3 and 4 to calculate  $q$ , the values of which are shown in table 1. These values of  $q$  are based on an assumed value of 3.1 Å for the  $Gd^{3+}$ - $H_2O$  proton distance [16]. The decrease of  $q$  from 3 to 1 upon addition of  $Co(NH_3)_4AMPPCP$  is consistent with the displacement of one fast-exchanging water molecule due to binding of the substrate analogue. The value of  $q$  drops to 0.25 and 0.42 in the complexes with  $Co(NH_3)_4ATP$  and  $Co(NH_3)_5PO_4$ .

## 4. DISCUSSION

Appropriate choices of substrate analogues have permitted the isolation and observation of several intermediates in the  $Ca^{2+}$ -ATPase cycle, and NMR studies with  $Gd^{3+}$  complexes have allowed the characterization of these intermediates. A simple mechanism consistent with the NMR data is shown in fig. 4, with the states detected here with  $Gd^{3+}$  and ATP analogues superimposed on the more traditional mechanism. The complex formed here with  $Co(NH_3)_4AMPPCP$  probably represents  $E_1Ca$ -ATP. The initial drop in  $\epsilon^*$  upon addition of either ATP or  $Co(NH_3)_4ATP$  (figs 2 and 3) are also manifestations of the formation of this same complex. The decrease in  $q$  by 2 upon binding of  $Co(NH_3)_4AMPPCP$  is consistent with the displacement of a coordinated water molecule on bound  $Gd^{3+}$  by the substrate analogue, which in turn would imply direct coordination of the substrate analogue to the bound  $Gd^{3+}$ . This has been confirmed by  $^{31}P$  NMR studies showing direct coordination of the terminal phosphate ( $\gamma$ -P) of bound  $Co(NH_3)_4AMPPCP$  to  $Gd^{3+}$  at calcium transport sites of the ATPase [17], and is consistent with luminescence studies showing that  $CrATP$  binds

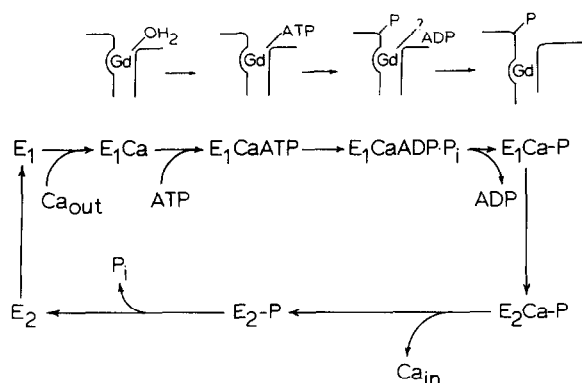


Fig.4. A proposed mechanism for the  $\text{Ca}^{2+}$ -ATPase, including the events of ATP binding and E-P formation. The states observed with  $\text{Gd}^{3+}$  in the present study are superimposed on the more traditional mechanism.

within 10 Å of the calcium transport sites of the  $\text{Ca}^{2+}$ -ATPase [18]. The increase with time, then decrease, in  $\epsilon^*$  observed in figs 2 and 3 may represent the formation of complexes analogous to  $\text{E}_1\text{CaADP}\cdot\text{P}_i$  and  $\text{E}_1\text{Ca-P}$ , respectively. Consistent with this is the observation by Vilsen and Andersen that the highly occluded  $\text{Ca}^{2+}$  complex formed by the ATPase with  $\text{Cr}(\text{H}_2\text{O})_4\text{ATP}$  is an analogue of  $\text{E}_1\text{-P}$ . The values of  $\tau_c$  and  $q$  determined in the present study indicate that the sequential formation of these intermediates in the  $\text{Ca}^{2+}$ -ATPase pathway involve increasingly occluded and desolvated  $\text{Gd}^{3+}$  (i.e.,  $\text{Ca}^{2+}$ ). This is to be expected for a transport system which must move  $\text{Ca}^{2+}$  across the sarcoplasmic reticulum membrane, and in fig.4, the final state observed by NMR is indicated by movement of the bound  $\text{Gd}^{3+}$  deeper into the channel. The NMR data presented here permit for the first time a quantitative characterization of the sequential formation of these intermediates.

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