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NMR RELAXATION MEASUREMENTS DETECT FOUR INTERMEDIATE STATES OF ATPase AND TRANSPORT CYCLE OF SARCOPLASMIC RETICULUM Ca^{2+}-ATPase†

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SUMMARY: At least four of the intermediate states of Ca^{2+}-ATPase (and presumably ion transport) can be trapped and characterized using water proton relaxation measurements. Gd^{3+} binds to two occluded Ca^{2+} transport sites on Ca^{2+}-ATPase which have a low accessibility to solvent water. In the presence of the MgATP analogue $Co(NH_3)_4$AMPPCP, a new state for bound Gd^{3+} with one less water of hydration) is observed. In the presence of $Co(NH_3)_4$ATP or ATP, two additional states for bound Gd^{3+} are detected by NMR, the first of which probably represents an intermediate state of ATP hydrolysis. The latter is the most occluded Gd^{3+} site yet observed in these studies and corresponds to the highly occluded E_1-P state observed with CrATP (Vilsen and Andersen, Biochim. Biophys. Acta 898, 313 (1987).

The sarcoplasmic reticulum Ca^{2+}-ATPase[1], which is responsible for active calcium transport across the SR membrane, exists in the SR membrane as a 115,000-dalton protein with associated, essential phospholipid. Calcium transport depends on conformation changes in this protein (de Meis, 1981), 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 Ca^{2+}, which is unable to exchange with Ca^{2+} at either side of the membrane (Sumida and Tonomura, 1974; Inesi et al., 1978; Dupont, 1980; Serpersu et al., 1982), but the nature of the Ca^{2+}-occluded form is largely uncharacterized.

One approach to the details of Ca^{2+} transport is to use a spectroscopically active analogue of Ca^{2+} ion. Our laboratory demonstrated

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Abbreviations: NMe_4^+, tetramethylammonium; PIPES, 1,4-piperazine-diethanesulfonic acid; AMP-PCP, adenyl-5'-yl methylenediphosphate.

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in 1979 that the paramagnetic lanthanide ion, Gd^{3+}, could be used for NMR and EPR studies of the Ca^{2+}-ATPase (Stephens and Grisham, 1979). These studies showed that the ATPase bound two Gd^{3+} ions at high affinity 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 Ca^{2+}-ATPase also binds Gd^{3+} at occluded sites (Klemens et al., 1986). Vilsen and Andersen (1986) subsequently reported occlusion of Ca^{2+} ions in soluble, monomeric SR Ca^{2+}-ATPase, in corroboration of our NMR results with Gd^{3+}.

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

MATERIALS AND METHODS

The Ca^{2+}-ATPase was purified and prepared for spectroscopic studies as described (Klemens et al, 1986). The β,γ-bidentate complexes of Co(III) with ATP and AMP-PCP were synthesized as described (Cornelius et al., 1977). $Co(NH_3)_5PO_4$ was synthesized according to Schmidt and Taube (1963).

Water proton relaxation rate measurements were used to examine the interactions of Gd^{3+} with the ATPase. The 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 +/- 1°C on samples of 100 microliters. The calculations in this paper and the practical application of the theory to structural studies of biomolecules have been reviewed (Mildvan and Engle, 1972).

RESULTS

Formation of a Stable Complex with $Co(NH_3)_4$AMPPCP

In order to form a stable nucleotide complex of the ATPase with Gd^{3+}, the Ca^{2+}-ATPase-Gd^{3+} complex was titrated with $Co(NH_3)_4$AMPPCP (Figure 1A). The sample contained 0.06 mM Ca^{2+}-ATPase and 0.05 mM $GdCl_3$. The enhancement of the longitudinal relaxation rate of water protons decreases to a value of 4.5 at saturating CoAMPPCP. As shown in Figure 1B, in the absence of added CoAMPPCP, there is a small, slow decrease in the observed enhancement of the binary Gd^{3+}-ATPase complex over a period of 200 minutes, 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 ε^* is consistent with the formation of a stable ATPase-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

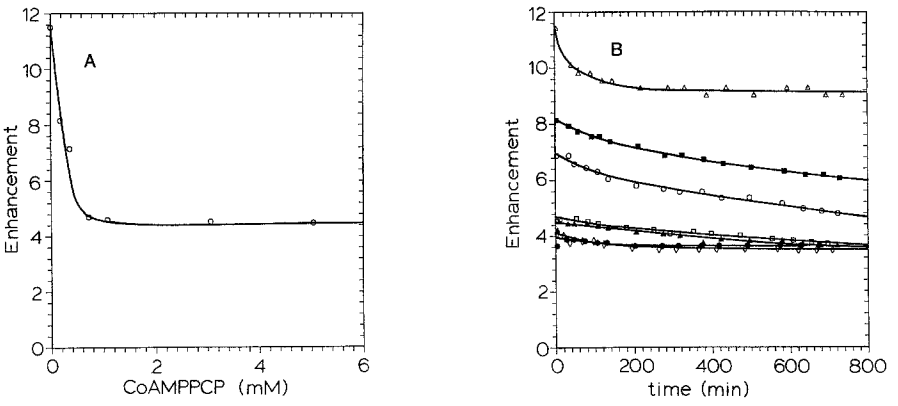

FIGURE 1: Effect of CoAMPPCP on water proton relaxation by ATPase-bound Gd^{3+}. Initial values are shown in A and the time course is shown in B. Concentrations of CoAMPPCP: 0.00 mM (Δ), 0.18 mM (□), 0.36 mM (o), 0.72 mM (□), 1.09 mM (Δ). 3.06 mM (◊), 5.04 mM (•).

coordinated to Gd^{3+} or a change in the dipolar correlation time for the Gd^{3+}-H_2O interaction. This point is addressed below.

Sequential Formation of Multiple Complexes With ATP and $Co(NH_3)_4ATP$

In contrast to the results obtained with the nonhydrolyzable CoAMPPCP, titrations of the ATPase-Gd^{3+} binary complex with either ATP or $Co(NH_3)_4ATP$ resulted in a series of time-dependent changes in the observed enhancement. Figure 2 shows the behavior observed in titrations with ATP. A rapid, concentration-dependent decrease in the observed enhancement (Fig. 2B) is followed by a slower increase and then a decrease in ε^*. The same pattern is observed at all ATP concentrations employed (0.025 mM to 0.658 mM). As the ATP concentration is increased, the enhancement measured immediately

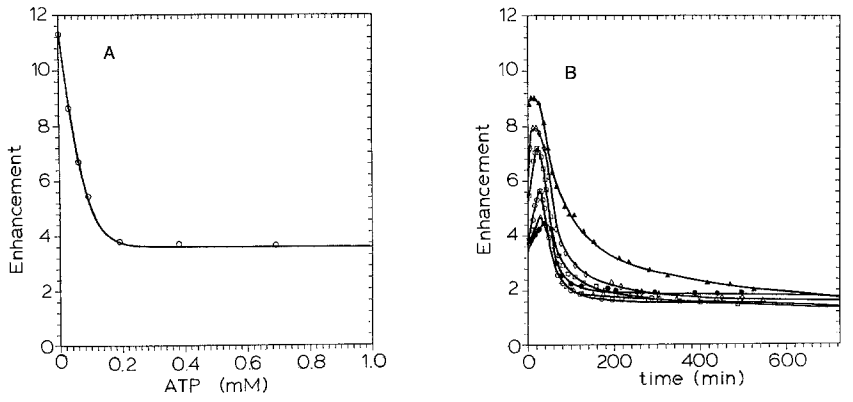

FIGURE 2: Effect of ATP on enhancement of water relaxation by ATPase-Gd^{3+}. Initial values in A and time course in B. Concentrations of ATP: 0.025 mM (Δ), 0.060 mM (◊), 0.094 mM (□), 0.188 mM (o), 0.376 mM (Δ), 0.658 mM (o).

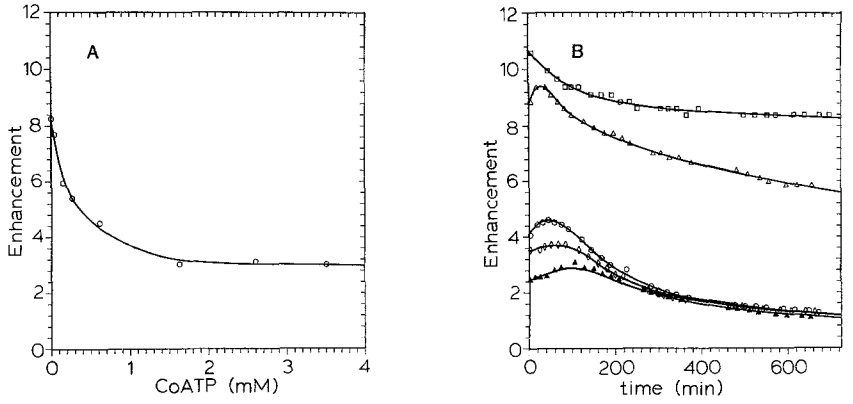

FIGURE 3: Effect of CoATP on enhancement of water relaxation by ATPase-Gd^{3+}. Initial values in A and time course in B. Concentrations of CoATP: 0.00 mM (□), 0.04 mM (Δ), 0.77 mM (○), 1.53 mM (◇), 3.06 mM (Δ).

following the addition of ATP decreases, and the maximum enhancement measured during the slow phase is decreased. The values of ε^* following the rapid initial decrease on ATP addition are plotted vs. ATP concentration in Figure 2A. The limiting value of ε^* at high ATP is 3.6, similar to that obtained at high levels of CoAMPPCP in Figure 1A, consistent with the formation of similar complexes in these two cases. The final enhancement of approximately 1 could either represent enzyme-bound Gd^{3+} with a low enhancement factor or Gd^{3+} 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 $Co(NH_3)_4ATP$. As shown in Figure 3B, a rapid, concentration-dependent decrease in the observed enhancement is followed by a slower increase and then a decrease in ε^*. The limiting value of ε^* at high levels of CoATP is somewhat lower than that observed for 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 ε^* on ATP addition was analyzed in terms of the following mechanism:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \qquad (1)$$

Values for k_1 and k_2 were determined by fitting the data of Figures 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^{-1} and the best fit for k_2 is 0.0065 min^{-1}. The point of maximum enhancement in the slow phase occurs at longer times as the concentration of CoATP is increased.

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.,

Table I

Analysis of the Frequency Dependence of T_{1p}

Complex	τ_c (s x 10^9)	q
ATPase-Gd_{site1}	1.91	3.11
ATPase-Gd_{site2}	2.12	1.66
ATPase-Gd_{site1}-$Co(NH_3)_4$AMPPCP	1.62	1.06
ATPase-Gd_{site1}-$Co(NH_3)_5PO_4$	4.89	0.42
ATPase-Gd_{site1}-$Co(NH_3)_4$ATP	9.90	0.25

Ca^{2+}) on the Ca^{2+}-ATPase (Stephens and Grisham, 1979; Klemens et al., 1986). 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 τ_c, the correlation time for the Gd^{3+}-nucleus dipolar interaction. For enzyme complexes, τ_c itself is dominated by τ_s, the spin-lattice relaxation time for the electrons of Gd^{3+}. τ_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 τ_c will be large. τ_c is normally determined by a study of the frequency dependence of the metal-induced relaxation rate, $1/fT_{1p}$ (Mildvan and Cohn, 1970). The results of such a study for the complexes studied in this paper are shown in Table I. The τ_c values for the Ca^{2+}-ATPase obtained here (Table I) and in our previous studies (Stephens and Grisham, 1979; Klemens et al., 1986) are unusually long compared to those observed in other Gd^{3+}-protein complexes (Cottam et al., 1974; Dwek and Richards, 1971). The complex with the longest value of τ_c (i.e., τ_s) is the Gd^{3+}-ATPase-$Co(NH_3)_4$ATP complex formed at long incubation times. The value of τ_c observed in this case (1 x 10^{-8} sec) 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 (1987) of highly occluded Ca^{2+} in an E_1-P state formed after long incubations with $Cr(H_2O)_4$ATP.

In the limit of fast exchange, the Solomon-Bloembergen equation which describes the Gd^{3+}-H_2O dipolar interaction (Mildvan and Cohn, 1970) 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:

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

In this equation, ω_I is the nuclear resonance frequency and ω_S is the electron resonance frequency. Having determined values for τ_c, we can use equations 3 and 4 to calculate q, the values of which are shown in Table I. These values of q are based on an assumed value of 3.1 Å for the Gd^{3+}-H_2O proton distance (Dwek, 1973). The decrease of q from 3 to 1 upon addition of $Co(NH_3)_4$AMPPCP 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 $Co(NH_3)_4$ATP and $Co(NH_3)_5PO_4$.

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 Figure 4, with the states detected here with Gd^{3+} and ATP analogues superimposed on the more traditional mechanism. The complex formed here with $Co(NH_3)_4$AMPPCP likely represents E_1CaATP. The initial drop in ε^* upon addition of either ATP or $Co(NH_3)_4$ATP (Figures 2 and 3) are also manifestations of the formation of this same complex. The decrease in q by 2 upon binding of $Co(NH_3)_4$AMPPCP 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 P-31 NMR studies showing direct coordination of the terminal phosphate (γ-P) of bound $Co(NH_3)_4$AMPPCP to Gd^{3+} at calcium transport sites of the ATPase (Klemens, 1987), and is consistent with luminescence studies showing that CrATP binds within 10 Å of the calcium transport sites of the Ca^{2+}-ATPase (Hermann et al., 1986). The increase with time, then decrease, in ε^* observed in Figures 2 and 3 may represent the formation of complexes analogous to E_1CaADP·P_i and E_1Ca-P, respectively. Consistent with this is the observation by Vilsen and Andersen that the highly occluded Ca^{2+} complex formed by the ATPase with $Cr(H_2O)_4$ATP is an analogue of E_1-P. The values of τ_c and q determined in the present study indicate that the sequential formation of these intermediates in the Ca^{2+}-ATPase pathway involve increasingly occluded and desolvated Gd^{3+} (i.e., Ca^{2+}) ions. This is to be expected for a transport system which must move Ca^{2+} ions across the sarcoplasmic reticulum membrane, and in Figure 4, the final state observed by NMR is indicated by movement of the bound Gd^{3+} deeper into

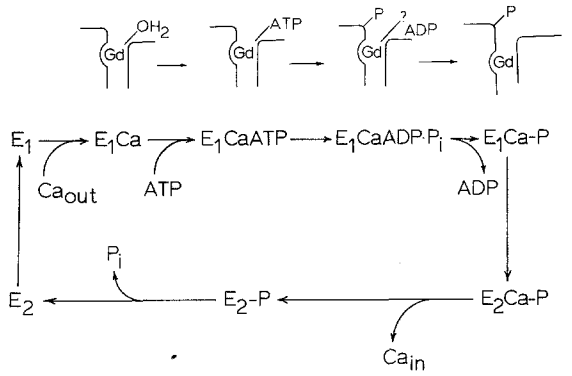

FIGURE 4: A proposed mechanism for the Ca^{2+}-ATPase including the events of ATP binding and E-P formation. The states observed with Gd^{3+} in the present study are shown superimposed on the more traditional mechanism.

the transport channel. The NMR data presented here permit for the first time a quantitative characterization of the sequential formation of the intermediate states of Ca^{2+}-ATPase and ion transport.

REFERENCES

Cornelius, R., Hart, P. and Cleland, W. (1977) Inorg. Chem. 16, 2799.
Cottam, L., Valentine, K., Thompson, B. and Sherry, A. (1974) Biochemistry 13, 3532.
deMeis, L. (1981) The Sarcoplasmic Reticulum: Transport and Energy Transduction, Wiley (New York).
Dupont, Y. (1980) Eur. J. Biochem. 109, 231-238.
Dwek, R. (1973) Nuclear Magnetic Resonance in Biochemistry, Clarendon Press (Oxford).
Dwek, R. and Richards, R. (1971) Eur. J. Biochem. 21, 204.
Hermann, T., Gangola, P. and Shamoo, A. (1986) Eur. J. Biochem. 158, 555-560.
Inesi, G., Kurzmack, M. and Verjovski-Almeida, S. (1978) Ann. N.Y. Acad. Sci. 307, 224-227.
Klemens, M., Ph.D. dissertation, University of Virginia, 1987.
Klemens, M., Andersen, J. and Grisham, C. (1986) J. Biol. Chem. 261, 1495-1498.
Mildvan, A. and Cohn, M. (1970) Adv. Enzymol. 33, 1-70.
Mildvan, A. and Engle, J. (1972) Methods in Enzymology, 26C, 654-682.
Schmidt, W. and Taube. H. (1963) Inorg. Chem. 2 , 698-705
Serpersu, E., Kirch, U. and Schoner, W. (1982) Eur. J. Biochem. 122, 347-354.
Stephens, E. and Grisham, C. (1979) Biochemistry 18, 4876-4885.
Sumida, M. and Tonomura, Y. (1974) J. Biochem. 75, 283-297.
Vilsen, B. and Andersen, J. (1986) Biochim. Biophys. Acta 855, 429-431.
Vilsen, B. and Andersen, J. (1987) Biochim. Biophys. Acta 898, 313-322.