Lithium-7 Nuclear Magnetic Resonance, Water Proton Nuclear Magnetic Resonance, and Gadolinium Electron Paramagnetic Resonance Studies of the Sarcoplasmic Reticulum Calcium Ion Transport Adenosine Triphosphatase[†]

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ABSTRACT: The interactions of gadolinium ion, lithium, and two substrate analogues, β,γ-imido-ATP (AMP–PNP) and tridentate CrATP, with the calcium ion transport adenosine triphosphatase (Ca^{2+}–ATPase) of rabbit muscle sarcoplasmic reticulum have been examined by using $^7Li^+$ NMR, water proton NMR, and Gd^{3+} EPR studies. Steady-state phosphorylation studies indicate that Gd^{3+} binds to the Ca^{2+} activator sites on the enzyme with an affinity which is ~10 times greater than that of Ca^{2+}. $^7Li^+$, which activates the Ca^{2+}–ATPase in place of K^+, has been found to be a suitable nucleus for probing the active sites of monovalent cation-requiring enzymes. $^7Li^+$ nuclear relaxation studies demonstrate that the binding of Gd^{3+} ion to the two Ca^{2+} sites on Ca^{2+}–ATPase increases the longitudinal relaxation rate ($1/T_1$) of enzyme-bound Li^+. The increase in $1/T_1$ was not observed in the absence of enzyme, indicating that the ATPase enhances the paramagnetic effect of Gd^{3+} on $1/T_1$ of $^7Li^+$. Water proton relaxation studies also show that the ATPase binds Gd^{3+} at two tight-binding sites. Titrations of Gd^{3+} solutions with Ca^{2+}–ATPase indicate that the tighter of the two Gd^{3+}-binding sites (site 1) provides a higher enhancement of water relaxation than the other, weaker Gd^{3+} site (site 2) and also indicate that the average of the enhancements at the two sites is 7.4. These data, together with a titration of the ATPase with Gd^{3+} ion, yield enhancements, ϵ_B, of 9.4 at site 1 and 5.4 at site 2. Analysis of the frequency dependence of $1/T_1$ of water indicates that the electron spin relaxation τ_s of Gd^{3+} is unusually long (2×10^{-9} s) and suggests that the Ca^{2+}-binding sites on the ATPase experience a reduced accessibility of solvent water. This may indicate that the Ca^{2+} sites on the Ca^{2+}–ATPase are buried or occluded within a cleft or channel in the enzyme. The analysis of the frequency dependence is also consistent with three exchangeable water protons on Gd^{3+} at site 1 and two fast exchanging water protons at site 2. Addition of the nonhydrolyzing substrate analogues, AMP–PNP and tridentate CrATP, to the enzyme–Gd^{3+} complex results in a decrease in the observed enhancement, with little change in the dipolar correlation time for Gd^{3+}, consistent with a substrate-induced decrease in the number of fast-exchanging water protons on enzyme-bound Gd^{3+}. From the effect of Gd^{3+} on $1/T_1$ of enzyme-bound Li^+, Gd^{3+}–Li^+ separations of 7.0 and 9.1 Å are calculated. On the assumption of a single Li^+ site on the enzyme, these distances set an upper limit on the separation between Ca^{2+} sites on the enzyme of 16.1 Å.

Detailed information on the structure of the Ca^{2+}–ATPase[1] would add substantially to our understanding of the mechanism of calcium transport in muscle sarcoplasmic reticulum (SR). The Ca^{2+}–ATPase exists in the SR membrane as a 100000-dalton protein with associated, essential phospholipid. The ATPase is activated by Mg^{2+}, Ca^{2+}, and a monovalent cation, such as K^+ (Carvalho & Leo, 1967). Despite numerous studies on this system (Hasselbach & Makinose, 1963; Martonosi & Feretos, 1964; Inesi, 1972; Baskin, 1977), the detailed mechanism of catalysis and transport has not been established. In addition to the three types of metal ion binding sites, there appear to be two types of ATP-binding sites as well as both acid-labile and acid-stable sites for inorganic phosphate. Several aspects of the binding sequence for substrate and cations have been established (Meissner, 1973; Froelich & Taylor, 1975), but the active-site structure and the nature of site–site interactions have remained largely unexplored.

Information regarding metal ion and substrate binding as well as coordination schemes and dissociation constants for binary and ternary enzyme complexes may be obtained from magnetic resonance studies of the purified enzyme (Mildvan & Cohn, 1970). It will be the purpose of this paper to examine and characterize the Ca^{2+}–ATPase and its complexes, using gadolinium [Gd(III)] and lithium (Li^+) as spectroscopically appropriate substitutes for Ca^{2+} and K^+ and using CrATP and AMP–PNP as substrate analogues. Gadolinium and several other lanthanide ions have been used in recent years to characterize Ca^{2+} (and in some cases Mg^{2+}) binding sites on proteins and enzymes, using a variety of techniques (Dwek & Richards, 1971; Epstein et al., 1974; Valentine & Cottam, 1973; Cottam et al., 1974; Geraldes & Williams, 1977). In particular, Dwek & Richards (1971) and Cottam and coworkers (Valentine & Cottam, 1973; Cottam et al., 1974) have employed nuclear relaxation measurements of both metal-bound water protons and substrate nuclei to characterize the interaction of Gd^{3+} with several enzyme systems.

Recently, we have demonstrated that ^7Li is an appropriate nucleus for probing monovalent cation sites and active-site structures in enzymes (Hutton et al., 1977; Grisham, 1978; Grisham & Hutton, 1978). ^7Li NMR signals are especially easy to observe, due to a high sensitivity (0.293 relative to protons), a high natural abundance (92.58%), and relatively

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[1] Abbreviations used: Ca^{2+}–ATPase, calcium ion transport adenosine triphosphatase; PRR, proton relaxation rate; NMe_4, tetramethylammonium; Pipes, 1,4-piperazinediethanesulfonic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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small quadrupolar effects on lithium relaxation. We have used $^7Li^+$ NMR to characterize K^+ sites in pyruvate kinase (Hutton et al., 1977) and sheep kidney ($Na^+ + K^+$)-ATPase (Grisham, 1978; Grisham & Hutton, 1978). Since Li^+ ion has been shown (Duggan, 1977) to activate Ca^{2+}-ATPase, we have examined Li^+ as a cation probe in this system as well. In a similar manner, the substitution-inert Cr(III) complexes of ATP and other nucleotides, whose preparation and partial characterization have been described by Cleland and coworkers, have been found to be particularly suitable probes of nucleotide-site structure in a variety of enzymes (DePamphilis & Cleland, 1973; Gupta et al., 1976a,b; Balakrishnan & Villafranca, 1978).

Experimental Procedure

Materials

Ca^{2+}-ATPase was prepared from the hind leg and back muscle of freshly killed rabbits. Sarcoplasmic reticulum was isolated according to Ebashi & Lipmann (1962), and the Ca^{2+}-ATPase was further purified by using the deoxycholate extraction procedure of Warren et al. (1974). The enzyme thus prepared was judged to be more than 95% pure by sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis.

Tridentate CrATP was prepared according to Cleland and co-workers (DePamphilis & Cleland, 1973, and private communication). β,γ-Imido-ATP was either purchased from Sigma Chemical Co. or synthesized according to Yount et al. (1971). Gadolinium trichloride was purchased from Alfa Inorganics, and LiCl was Baker Analyzed Reagent. All other chemicals were the purest grade commercial products available.

Methods

Enzyme Assays. The Ca^{2+}-ATPase was assayed either by the continuous method (Barnett, 1970) or by measuring the amount of inorganic phosphate liberated from ATP in a 10-min period. The enzyme assay mixture in either case contained 20 mM imidazole, pH 7.2 (25 °C), 10 mM KCl, 3 mM $MgCl_2$, 2 mM ATP, and 0.1 mM $CaCl_2$. In the continuous assay, the assay mixture also contained 1.5×10^{-4} M NADH, 1.5×10^{-3} M phosphoenolpyruvate, 20 units/mL pyruvate kinase, and 2 units/mL lactic dehydrogenase. The rate of disappearance of NADH was followed spectrophotometrically at 340 nm. In the phosphate assays, inorganic phosphate was determined by the method of Chen et al. (1956). Protein was determined by using a microbiuret method (Goa, 1953) with crystalline bovine serum albumin as a standard. Enzyme concentration was calculated by assuming a protein molecular weight of 100 000.

Magnetic Resonance Measurements. EPR and PRR measurements were used to study the interaction of Gd^{3+} with Ca^{2+}-ATPase and with substrates and buffers. The interaction of CrATP and AMP-PNP and the formation of ternary complexes of enzyme, Gd^{3+}, and substrates were studied by PRR measurements. The interactions of Li^+ with Gd^{3+} or CrATP were characterized by $^7Li^+$ NMR.

The EPR spectra of Gd^{3+} in complexes of the ATPase, substrates, and buffers were taken on a Varian E-109 EPR spectrometer at 9.1 GHz at 100-200-mW microwave power, 10-20-G modulation amplitude, and scan rates of 500-1000 G/min. EPR spectra were accumulated, base line corrected, and stored in a PDP 11VO3 computer with 20K of core memory and dual floppy disks for mass storage. Samples were normally 70-100 μL and were contained in quartz aqueous sample cells (Varian E-248-1 or Wilmad WG-808).

In the PRR measurements, the longitudinal relaxation rate, $1/T_1$, of the protons of water was measured on a variable-frequency, pulsed NMR spectrometer of our own design. The magnet for this instrument was a Varian 4012A electromagnet with a 2100-A power supply which had been modified for solid-state operation. The rf components and pulse programmer were designed and built by SEIMCO, New Kensington, PA, and the frequency source was a Harris PRD 7838 frequency synthesizer. A paramagnetic metal ion, such as Gd^{3+}, increases the longitudinal relaxation rate of water protons in its coordination sphere. When a macromolecule which binds Gd^{3+} is present, the effectiveness of the ion in increasing the relaxation rate is enhanced due to an increase in the correlation time of the ion-water interaction (Eisinger et al., 1962). The enhancement factor ϵ^* has been defined as the ratio of the paramagnetic contribution to the PRR of water in the presence of the enzyme-Gd^{3+} complex to that in the presence of an equal concentration of free Gd^{3+} (Mildvan & Cohn, 1970; Eisinger et al., 1962). The frequency dependencies of $1/T_1$ were determined at 8, 16, 24, 30, 40, 50, and 60 MHz on the SEIMCO-pulsed spectrometer and at 100 MHz on the JEOL PS-100 P/EC-100 Fourier transform spectrometer. All of the PRR measurements were carried out at 23 ± 1 °C on samples of 50-100 μL.

^7Li NMR experiments were performed on the JEOL PS-100 P/EC 100 Fourier transform spectrometer. The ambient probe temperature was 23 ± 1 °C, except as noted. The experiments were carried out in 10-mm tubes using a 5-kHz crystal filter at 25 MHz by lowering the magnetic field from the usual value of 23.5 to 15.2 kG (the *g* value for lithium is 2.171). In this way the standard carbon probe could be used to measure lithium spectra. Unfortunately, lowering the field prohibits the use of the normal deuterium lock and these experiments were done unlocked. Lowering the field 12 h before the experiment afforded a very stable field, with drifts of less than 5 Hz/h being typical. In all cases only one transient was necessary for adequate signal to noise ratios. Spin-lattice relaxation times were measured by using a 180-τ-90 pulse sequence, where the time required for a 90° pulse was 20 μs.

Results

Effect of Gd^{3+} on Phosphoenzyme Formation. If gadolinium ion, Gd^{3+}, is to be useful as a spectroscopic probe of Ca^{2+} sites on Ca^{2+}-ATPase, it must first be demonstrated that Gd^{3+} can compete with Ca^{2+} at these Ca^{2+} activator sites. While it has been reported that lanthanide ions inhibit the overall Ca^{2+}-ATPase reaction (Yamada & Tonomura, 1972), such experiments do not easily lend themselves to a quantitative evaluation of metal-binding properties of the ATPase, since both Mg^{2+} and Ca^{2+} are required for the overall hydrolysis reaction. Sorting out the simultaneous binding of Mg^{2+}, Ca^{2+}, and Gd^{3+} to both the enzyme and ATP in the hydrolysis reaction would be complex. We have chosen instead to examine the effect of Gd^{3+} on formation of the intermediate phosphoenzyme complex, a process which requires only Ca^{2+}. Yamamoto & Tonomura (1968) and Makinose (1969) have shown that in the absence of Mg^{2+}, Ca^{2+} ions will stimulate the formation of a phosphorylated enzyme intermediate (EP) upon incubation of the ATPase with [γ-^{32}P]ATP. Yamada & Tonomura (1972) have shown that the activator constant for Ca^{2+} in the phosphorylation reaction (0.35 μM) is the same as that for ATPase activity, so that phosphoenzyme formation should be a useful measure of calcium binding to the enzyme.

The effect of Gd^{3+} on Ca^{2+} ion induced EP formation is shown in Figure 1. Low levels of Gd^{3+} cause a sharp decrease in EP levels. The data were compared with theoretical curves

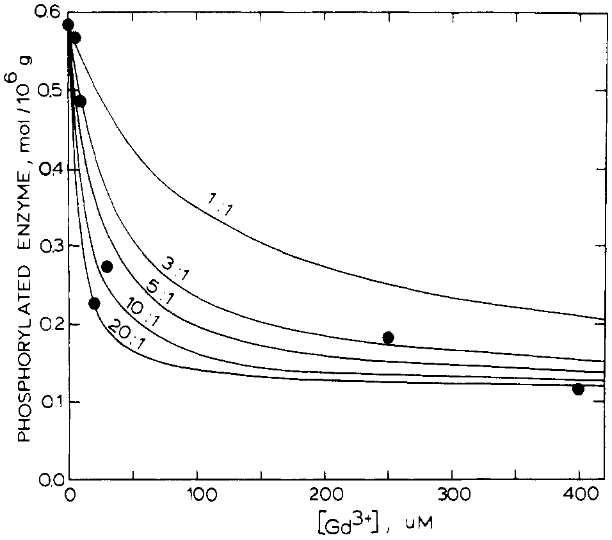

FIGURE 1: Steady-state levels of EP at 0 °C as a function of $GdCl_3$ concentration. Reaction mixture contained 50 mM Tris-HCl, pH 7.0, 100 mM KCl, 100 μM $CaCl_2$, 10 μM [γ-^{32}P]ATP, and 0.3 mg/mL Ca^{2+}-ATPase. A preincubation for 20 min at 25 °C was followed by an incubation of 30 min at 0 °C. The reaction was then initiated by addition of [γ-^{32}P]ATP and stopped by addition of 5% trichloroacetic acid. The precipitate was retained and washed on Millipore filters and counted for ^{32}P. The curves shown are calculated by assuming the ratios shown for $K_D^{Ca^{2+}}/K_D^{Gd^{3+}}$ as described in the text.

by assuming a variety of ratios for the dissociation constants of Ca^{2+} and Gd^{3+} from the calcium activator sites. For simplicity in the analysis, the activating calcium sites were assumed to be identical and independent. Superimposed on the data of Figure 1 are theoretical curves assuming $K_D^{Ca^{2+}}/K_D^{Gd^{3+}}$ ratios from 1:1 to 20:1. As can be seen, the best fit to the data lies between ratios of 3:1 and 20:1, with 10:1 providing a reasonable agreement with the data. These results indicate that Gd^{3+} binds much more tightly to the Ca^{2+} sites than Ca^{2+} itself. Our data, together with the previously determined K_m for Ca^{2+} of 0.35 μM (Yamada & Tonomura, 1972), would place the apparent dissociation constant for Gd^{3+} at $\sim 3.5 \times 10^{-8}$ M. At lower Ca^{2+} levels the decrease in EP occurs at proportionally lower Gd^{3+} concentrations, providing additional evidence supporting the competition of Gd^{3+} and Ca^{2+} at the Ca^{2+} sites.

$^7Li^+$ Nuclear Relaxation Studies. It has been previously demonstrated that the Ca^{2+}-ATPase from sarcoplasmic reticulum is activated by monovalent cations (Carvalho & Leo, 1967). The dissociation constants for K^+, Na^+, and Li^+, as determined by Duggan (1977), are 11 mM, 16 mM, and 50 mM, respectively. Since Li^+ provides 50% of the activation effected by K^+, $^7Li^+$ NMR studies should provide an effective means of characterizing the monovalent cation site or sites of the Ca^{2+}-ATPase. If, for example, the activating sites for Li^+ are near the binding sites for Ca^{2+} and if Gd^{3+} can bind to the Ca^{2+} sites, then the $^7Li^+$ nuclear relaxation rates should be increased by the presence of the paramagnetic Gd^{3+} ion because of the existence of electron–nuclear dipolar interactions (Mildvan & Cohn, 1970; Mildvan & Engle, 1972).

As shown in Figure 2, addition of Gd^{3+} to solutions of LiCl and Ca^{2+}-ATPase produces a dramatic increase in the longitudinal relaxation rate, $1/T_1$, of $^7Li^+$ which is proportional to the concentration of Gd^{3+} until the concentration of Gd^{3+} is equal to the enzyme concentration. Beyond this point, an additional, linear, but smaller increase in $1/T_1$ of $^7Li^+$ is observed. At $\sim$2.25 Gd^{3+} ions/100 000-dalton protein monomer the enzyme is saturated with Gd^{3+}, and no further increases in $1/T_1$ are observed beyond this point. The biphasic nature of this plot is consistent with the sequential binding of two Gd^{3+} ions to the ATPase, with the first of these Gd^{3+} sites exerting a larger paramagnetic effect on enzyme-bound Li^+. No such effects of Gd^{3+} on $1/T_1$ of $^7Li^+$ were observed in the absence of enzyme, although the enzyme itself exerts a minor[2] diamagnetic effect on $1/T_1$ of $^7Li^+$. Moreover, similar effects of Gd^{3+} on $1/T_1$ of Li^+ were observed at 6.9 °C as well as at 23–25 °C.

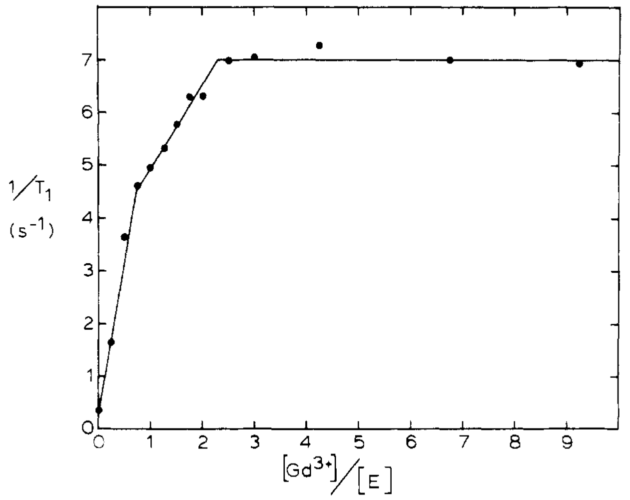

FIGURE 2: Effect of Gd^{3+} on $1/T_1$ of $^7Li^+$ in the presence of Ca^{2+}-ATPase. Solutions contained 0.1 M LiCl, 0.050 M NMe_4-Pipes, pH 7.0, and 1.62×10^{-4} M Ca^{2+}-ATPase.

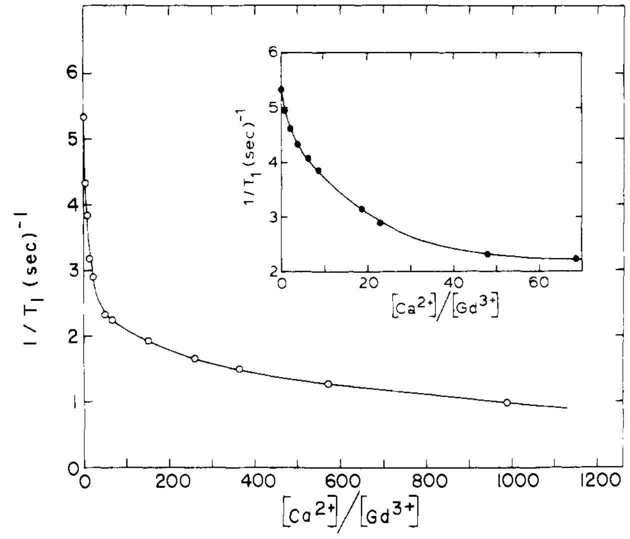

FIGURE 3: Displacement of Gd^{3+} from the ATPase–Gd^{3+} complex by Ca^{2+}. Solutions contained 1.62×10^{-4} M Ca^{2+}-ATPase, 4.80×10^{-4} M $GdCl_3$, and 0.050 M NMe_4-Pipes, pH 7.0.

In order to determine whether the Gd^{3+} sites observed here are indeed Ca^{2+} sites, we titrated a solution of Li^+, ATPase, and Gd^{3+} with Ca^{2+}, as shown in Figure 3. As shown, the addition of Ca^{2+} produces a large decrease in $1/T_1$ of $^7Li^+$, presumably due to displacement of Gd^{3+} from the enzyme sites. At high levels of Ca^{2+}, essentially all of the paramagnetic effect of Gd^{3+} on $^7Li^+$ is removed, suggesting a complete displacement of Gd^{3+} by Ca^{2+}.

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[2] The normal $1/T_1$ for LiCl solutions in our experiments is 0.1–0.15 s^{-1}. Addition of 0.16 mM ATPase to such a solution increases $1/T_1$ to 0.3–0.35 s^{-1}. As can be seen in Figure 1, such changes are small compared to the effects of Gd^{3+}.

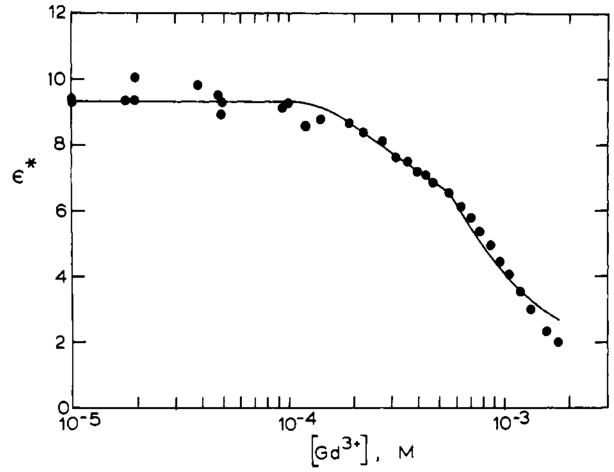

FIGURE 4: Effect of gadolinium on the enhancement of the longitudinal water proton relaxation rate in solutions containing Ca^{2+}-ATPase. The solutions contained 0.050 M NMe$_4$-Pipes buffer, pH 7.0, 0.24 mM Ca^{2+}-ATPase, and the noted concentration of gadolinium chloride. The temperature was 23 °C. The theoretical curve was fitted to the points by assuming that the sites were filled in the manner of the data of Figure 2 and assuming the enhancements (ϵ_B) described in the text.

Similarly, competition studies can be used to compare the relative affinities of monovalent cations for the sites observed in the NMR studies. Using the kinetically determined dissociation constants for Na$^+$ and Li$^+$ from the monovalent cation-activating site(s) of 16 and 50 mM, respectively (Duggan, 1977), we calculated that 33 ± 1% of the Li$^+$ should be displaced by 16 mM Na$^+$ under the conditions of the NMR experiments. In such experiments we observe 39 ± 5% displacement, in reasonable agreement with the predicted value.

PRR Studies of Gd^{3+} Binding to Ca^{2+}-ATPase. A study of the binding of Gd^{3+} to Ca^{2+}-ATPase was carried out by using the PRR technique. Figure 4 shows the behavior of the observed enhancement of the longitudinal water proton relaxation rate when Gd^{3+} is used to titrate a solution of the Ca^{2+}-ATPase. At the lower concentrations of Gd^{3+} the large observed enhancement of the water proton relaxation rate suggests the formation of a tight binary Gd^{3+}-ATPase complex. As the concentration of Gd^{3+} is increased, the enhancement decreases toward unity as the Gd^{3+} sites on the enzyme become saturated and the contribution of free Gd^{3+} becomes the predominant term in eq 1.

$$[\mathrm{Gd}]_T\epsilon^* = [\mathrm{Gd}]_F\epsilon_F + [\mathrm{Gd}]_{B1}\epsilon_{B1} + [\mathrm{Gd}]_{B2}\epsilon_{B2} \qquad (1)$$

In this equation the subscripts on the concentration and enhancement terms denote total Gd^{3+} (T), free Gd^{3+} (F), and Gd^{3+} bound at site 1 (B1) or Gd^{3+} bound at site 2 (B2). In this paper we will define site 1 as the site which binds Gd^{3+} more tightly. The data of Figure 4 are consistent with an ϵ_{B1} of 9.4 and an ϵ_{B2} of 5.4. When solutions containing 10 or 50 μM Gd^{3+} were titrated with Ca^{2+}-ATPase, the enhancement increased as the enzyme concentration increased. The reciprocal of the observed enhancement was plotted against the reciprocal of the total ATPase concentration (Figure 5) yielding a linear behavior, except at high levels of enzyme where a sharp increase in the observed enhancement is found. This behavior is consistent with two environments for bound Gd^{3+} ion on the Ca^{2+}-ATPase. At low concentrations of enzyme in Figure 5, the enzyme binds Gd^{3+} at both types of sites and the observed enhancements in the linear portion of the plot include weighted contributions from the bound enhancements at the two Gd^{3+} sites, as well as from the enhancement of free Gd^{3+} ion in the solution (ϵ_F = 1.0 by definition). The limiting enhancement of 7.4 at infinite enzyme, which is extrapolated from the linear portion of the plot, represents an average of the bound enhancements at the two Gd^{3+} sites. However, as the enzyme concentration is increased, the nature of the system changes from enzyme limited to metal limited and the first Gd^{3+} site, which is the tighter binding of the two Gd^{3+} sites and which also displays the larger enhancement, ϵ_{B1}, begins to pull Gd^{3+} away from site 2 (where the enhancement is lower). The net result is an increase in the observed enhancement and a downward deviation for $1/\epsilon^*$ at high enzyme in Figure 5.

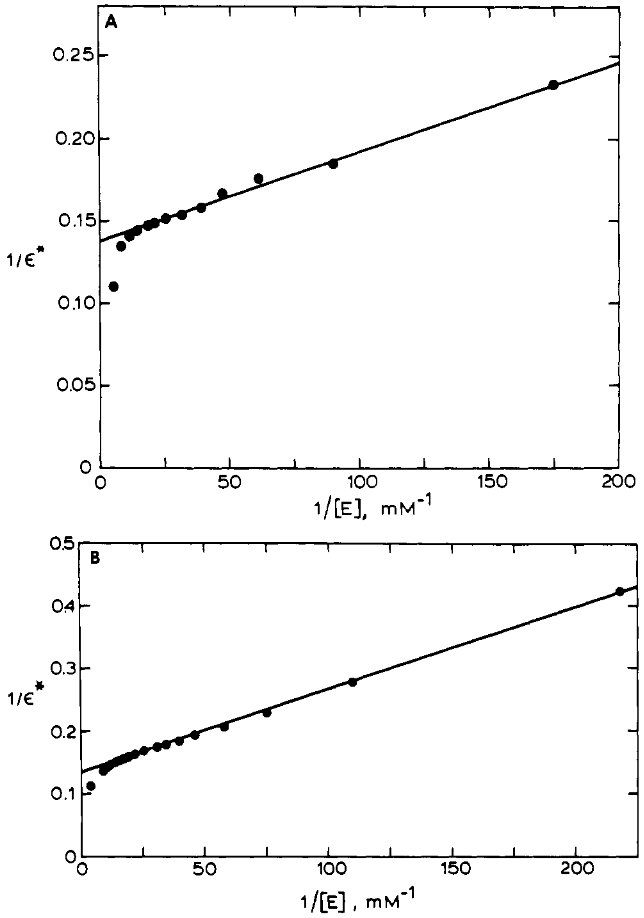

FIGURE 5: Titration of gadolinium chloride with Ca^{2+}-ATPase. The solutions contained 0.050 M NMe$_4$-Pipes buffer, pH 7.0, 24.4 μM GdCl$_3$ (A) or 49 μM GdCl$_3$ (B), and the noted concentrations of Ca^{2+}-ATPase. The value of ϵ^* obtained by extrapolation of the solid line to infinite protein concentration is the average of the enhancements at Gd^{3+} sites 1 and 2, denoted by ϵ_{B1} and ϵ_{B2}, respectively, in the text.

Formation of Ternary Complexes of Ca^{2+}-ATPase, Gd^{3+}, and Substrate Analogues. The interaction of two substrate analogues with the Gd^{3+}-ATPase complex was studied by PRR. β,γ-Imido-ATP (AMP-PNP) had previously been reported to bind to ATP-requiring enzymes, and it had also been found that this ATP analogue could not be hydrolyzed by a variety of ATPases (Yount et al., 1971). Kinetic studies indicate no detectable hydrolysis of AMP-PNP in solutions of the Ca^{2+}-ATPase and Gd^{3+} during the course of the PRR studies described below. The effect of AMP-PNP on the enhancement (ϵ^*) of the enzyme-Gd^{3+} complex (with Gd^{3+} at site 1 only) is shown in Figure 6A. The effect of the analogue on ϵ^* of the ATPase-Gd^{3+} complex is time dependent, with ϵ^* decreasing from 9 to 3 over a period of 20 min. While the time course of this change was subject to variation between preparations of the enzyme, the magnitude of the decrease in ϵ^* was reproducible between preparations.

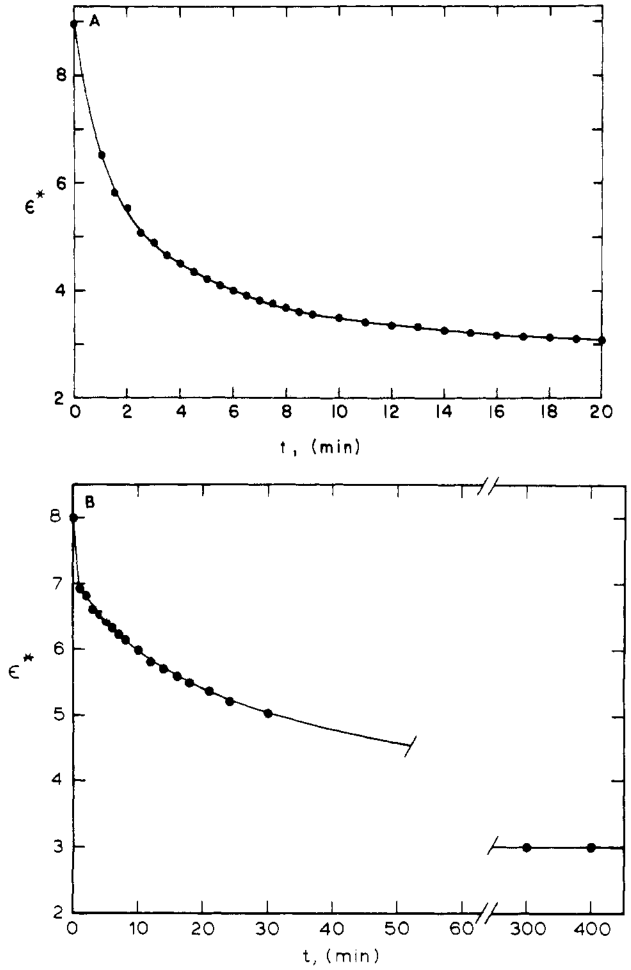

FIGURE 6: Effect of AMP-PNP (A) or CrATP (B) on the enhancement (ϵ^*) of water proton relaxation by ATPase-bound Gd^{3+}. Conditions were 0.06 mM Ca^{2+}–ATPase, 0.050 M NMe_4–Pipes buffer, pH 7.0, 0.05 mM $GdCl_3$, and either 0.1 mM AMP-PNP or 0.053 mM CrATP. T = 23 °C.

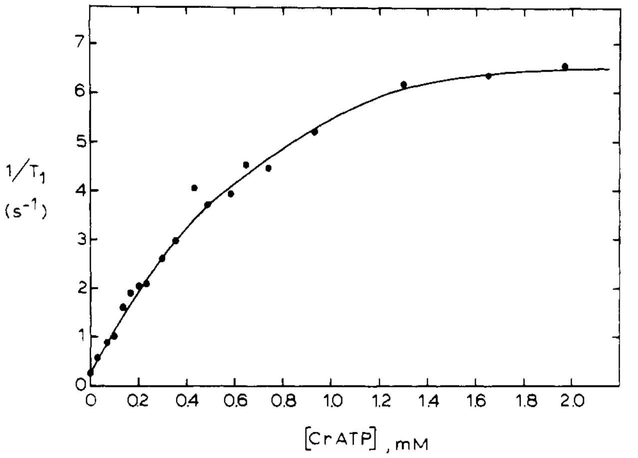

FIGURE 7: Effect of tridentate CrATP on $1/T_1$ of $^7Li^+$ in the presence of Ca^{2+}–ATPase. Solutions contained 0.1 M LiCl, 0.050 M NMe_4–Pipes, pH 7.0, and 0.2 mM Ca^{2+}–ATPase. T = 23 °C.

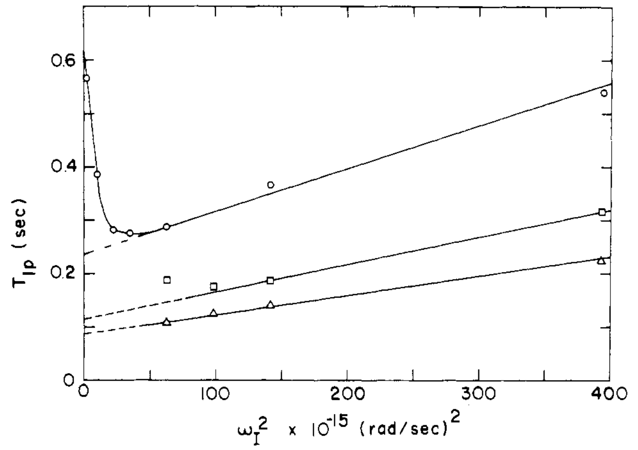

FIGURE 8: Paramagnetic contribution to the relaxation time (T_{1p}) of water protons as a function of the frequency squared (ω_I^2) for Ca^{2+}–ATPase–Gd^{3+} complexes. The solutions contained 50 mM NMe_4–Pipes, pH 7.0, and (○) 0.06 mM Ca^{2+}–ATPase and 0.05 mM $GdCl_3$, (△) 0.12 mM Ca^{2+}–ATPase and 0.05 mM $GdCl_3$, or (□) 0.12 mM Ca^{2+}–ATPase and 0.25 mM $GdCl_3$. The values of T_{1p} for the latter two cases (△, □) represent normalized values of T_{1p} at site 1 and site 2 for Gd^{3+}.

The final enhancement of 3 ± 0.5 is too large for a Gd^{3+}–AMP-PNP complex. Enhancements for a variety of Gd^{3+}–nucleotide complexes are in the range of 1.2 ± 0.3 (Valentine & Cottam, 1973). The observed effects, however, are consistent with the formation of a ternary complex of enzyme, Gd^{3+}, and AMP-PNP, in which the environment of bound Gd^{3+} has been altered. Further support for the formation of a complex which involves neither hydrolysis nor Gd^{3+} binding in solution to a nucleotide or substrate analogue is provided by the effects of CrATP on the Gd^{3+}–ATPase complex. CrATP is a substitution-inert complex of Cr^{3+} and ATP which is resistant to ATP hydrolysis by most ATP-requiring enzymes (DePamphilis & Cleland, 1973). The addition of CrATP to solutions of the ATPase–Gd^{3+} complex yields the data of Figure 6B. As in the case with AMP-PNP, the enhancement decreases upon addition of CrATP, reaching a final value of 3 after several hours. The CrATP complex does not readily form complexes involving an additional metal (Gupta et al., 1976b), and thus the decreased enhancement is not likely to be the result of the formation of a Gd^{3+}–CrATP complex in solution. If the sample containing the ATPase, Gd^{3+}, and analogue is centrifuged and the pellet is washed and resuspended in buffer, the relaxation rate of the water protons remains substantially unchanged, indicating that the decrease in enhancement upon substrate analogue addition does not result from the formation of a soluble metal–nucleotide complex, which would be removed upon centrifugation and washing. Rather the decreased enhancements are consistent with formation of ternary ATPase–Gd^{3+}–nucleotide complexes, with an altered environment for enzyme-bound Gd^{3+}.

Additional evidence for the formation of a complex between the Ca^{2+}–ATPase and CrATP is obtained from a $^7Li^+$ NMR study, in which solutions of Li^+ and the ATPase are titrated with CrATP, as shown in Figure 7. The longitudinal relaxation, $1/T_1$, of $^7Li^+$ is increased by addition of CrATP, until at ~1.6–2.0 mM the enzyme is saturated with CrATP. The apparent binding constant for CrATP from this titration is 0.4 mM, which is in reasonable agreement with previous estimates of the K_m for ATP itself of 0.5–1.0 mM from kinetic studies (Taylor & Hattan, 1979).[3]

Frequency Dependence of $1/T_{1p}$ of Water on Enzyme-Bound Gd^{3+}. Paramagnetic effects of metals such as those observed here with Gd^{3+} on the Ca^{2+}–ATPase can be used to determine distances, r, between the metal and pertinent nuclei

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[3] The kinetic studies of Taylor and Hattan provide evidence for both tight and weak ATP sites, with K_m values of 1–5 μM and 0.5–1.0 mM, respectively. This enzyme is thus similar to two other membrane-bound ATPases, the (Na^+ + K^+)-ATPase and the Mg^{2+}–ATPase from sheep kidney which both appear to utilize both tight and weak sites for ATP (Robinson, 1976; M. Gantzer and C. Grisham, unpublished experiments).

Table I: Frequency Dependence of $1/f(T_{1p})$ of Water Protons in the Presence of Binary and Ternary Ca^{2+}-ATPase Complexes

	$1/f(T_{1p})$ ($s^{-1} \times 10^{-6}$)		
frequency (MHz)	ATPase-$(Gd^{3+})_{site1}$	ATPase-$(Gd^{3+})_{site2}$[a]	ATPase-$(Gd^{3+})_{site1}$-AMP-PNP
8	4.30 ± 0.40		
16	6.31 ± 0.60		
24	8.61 ± 0.90		1.69 ± 0.2
30	8.74 ± 0.90		
40	8.45 ± 0.80	4.78 ± 0.50	1.97 ± 0.2
50	7.33 ± 0.70	5.13 ± 0.60	1.69 ± 0.2
55			1.61 ± 0.2
60	6.63 ± 0.70	4.87 ± 0.50	1.55 ± 0.2
100	4.15 ± 0.40	2.86 ± 0.30	

[a] The $1/f(T_{1p})$ of water protons at site 2 was determined by subtracting the $1/T_{1p}$ due to site 1 from the total paramagnetic effect on $1/T_1$ of water with both Gd^{3+} sites occupied.

or, if r is known, to determine q, the number of nuclei involved in the interaction with the metal. Such calculations, however, require a determination of τ_c, the correlation time for the metal–nucleus dipolar interaction. This is normally accomplished by a study of the frequency dependence of the metal-induced relaxation rate, $1/f(T_{1p})$ (Mildvan & Cohn, 1970). The results of such a study are shown in Figure 8 and Table III. As described elsewhere (Mildvan & Cohn, 1970; Peacocke et al., 1969), the frequency dependence of $1/f(T_{1p})$ may be used to determine the correlation time, τ_c, and the coordination number, q, for water protons on the ATPase-bound Gd^{3+}. It has been shown that in a plot of T_{1p} vs. ω_I^2, the correlation time τ_c can be determined from the slope of the linear portion of the curve (Peacocke et al., 1969). The values of τ_c determined from Figure 8 and the other experiments listed in Table I are summarized in Table II. The τ_c values for the Gd^{3+}–H_2O interaction are 1.91×10^{-9} and 2.12×10^{-9} s at Gd^{3+} sites 1 and 2, respectively. In the limit of fast exchange, the Solomon–Bloembergen equation which describes the Gd^{3+}–H_2O dipolar interaction (Solomon, 1955; Solomon & Bloembergen, 1956) is

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

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

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2\tau_c^2} \quad (3)$$

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 eq 2 and 3 to calculate q, the values of which are shown in Table II. These values of q are based on an assumed value of 3.1 Å for the Gd^{3+}–H_2O proton distance (Dwek, 1973). Although the absolute values of q are dependent on this distance, the relative magnitudes of q for the various complexes are accurately described by Table II. Thus, the value of q appears to be distinctly different at the two Gd^{3+} sites. Calculations using eq 2 yield three exchangeable water protons on Gd^{3+} at site 1 and two exchangeable protons at site 2. Moreover, at site 1, the addition to the enzyme of the nonhydrolyzing substrate analogue, AMP-PNP, results in a decrease from three to one in the number of fast-exchanging water protons on the enzyme-bound Gd^{3+}.

The existence of a minimum in the plot of T_{1p} vs. ω_I^2 (Figure 8) indicates that τ_c itself is frequency dependent below 30 MHz. This type of behavior indicates that τ_c is dominated by τ_s, the electron spin relaxation time of bound Gd^{3+}, as has been found for several enzyme–Gd^{3+} and enzyme–Mn^{2+} complexes (Nowak et al., 1973; Reuben & Cohn, 1970; Grisham & Mildvan, 1974). The values of τ_c shown in Table II are substantially longer than the values of τ_s of Gd^{3+} found in other systems.

Calculation of Gd^{3+}–Li^+ Distances on Ca^{2+}–ATPase. The paramagnetic effects of Gd^{3+} on $1/T_1$ of Li^+ can be used, under conditions of fast exchange, to calculate Gd^{3+}–Li^+ distances on the enzyme. The similar effects of Gd^{3+} at 6.9 and 23 °C are consistent with fast exchange (Mildvan & Cohn, 1970), and the Solomon–Bloembergen equation describing the Gd^{3+}–Li^+ dipolar interaction (Solomon, 1955; Solomon & Bloembergen, 1956) is

$$1/f(T_{1p}) = [Li^+]/([Gd^{3+}]T_{1p}) = (652/r)^6 q[f(\tau_c)] \quad (4)$$

where the quantities are as described above except that r now describes a Gd^{3+}–Li^+ separation. If, as described above, the correlation time τ_c for the Gd^{3+}–H_2O interaction is equal to the electron spin relaxation time τ_s of the bound Gd^{3+}, the same value of τ_c would modulate the Gd^{3+}–Li^+ interaction. Thus, we may presume that the appropriate correlation times for interpreting the data of Figure 2 are 1.91×10^{-9} s at Gd^{3+} site 1 and 2.12×10^{-9} s at Gd^{3+} site 2.

The simplest interpretation of Figure 2 would involve the sequential binding of two Gd^{3+} ions to the Ca^{2+}–ATPase. This is consistent with numerous reports identifying two Ca^{2+}-binding sites on the Ca^{2+}–ATPase (Hasselbach & Makinose, 1963; Martonosi & Feretos, 1964; Inesi, 1972; Baskin, 1977) and demonstrating that two Ca^{2+} ions are pumped across the SR membrane per molecule of ATP hydrolyzed. The smaller increase in $1/T_1$ caused by the second equivalent of Gd^{3+} suggests that the second Gd^{3+} binds to the enzyme at a greater distance from the Li^+ than the first Gd^{3+} since the correlation times for the 1:1 and 2:1 Gd^{3+}–ATPase complexes are very similar. If we assume a single Li^+-binding site near the Gd^{3+}-binding sites (i.e., $q = 1$), the above equation yields Gd^{3+}–Li^+ distances of 7.0 ± 0.7 and 9.1 ± 0.9 Å for the first and second Gd^{3+} sites, respectively (Table III).

X-Band Electron Paramagnetic Resonance Spectra of Gd^{3+} in the Ca^{2+}–ATPase System. The possibility of using the electron paramagnetic resonance properties of Gd^{3+} to probe its environment in and interactions with biological molecules has previously received little attention in the literature

Table II: Analysis of Frequency Dependence of T_{1p}

complex	τ_c[a] ($s \times 10^9$)	frequency (MHz)	$1/f(T_{1p})$ ($s^{-1} \times 10^{-6}$)	$f(\tau_c)$ ($s \times 10^9$)	q
ATPase-$(Gd^{3+})_{site1}$	1.91	40	8.45 ± 0.8	4.66	3.11 ± 0.1
		100	4.15 ± 0.4	2.35	3.03 ± 0.1
ATPase-$(Gd^{3+})_{site2}$	2.12	40	4.78 ± 0.4	4.95	1.66 ± 0.1
		100	2.86 ± 0.3	2.29	2.14 ± 0.2
ATPase-$(Gd^{3+})_{site1}$-AMP-PNP	2.14	40	1.97 ± 0.2	4.98	0.67 ± 0.03
		60	1.55 ± 0.2	3.89	0.68 ± 0.03

[a] Determined with the use of the linear portion of the curve in Figure 8.

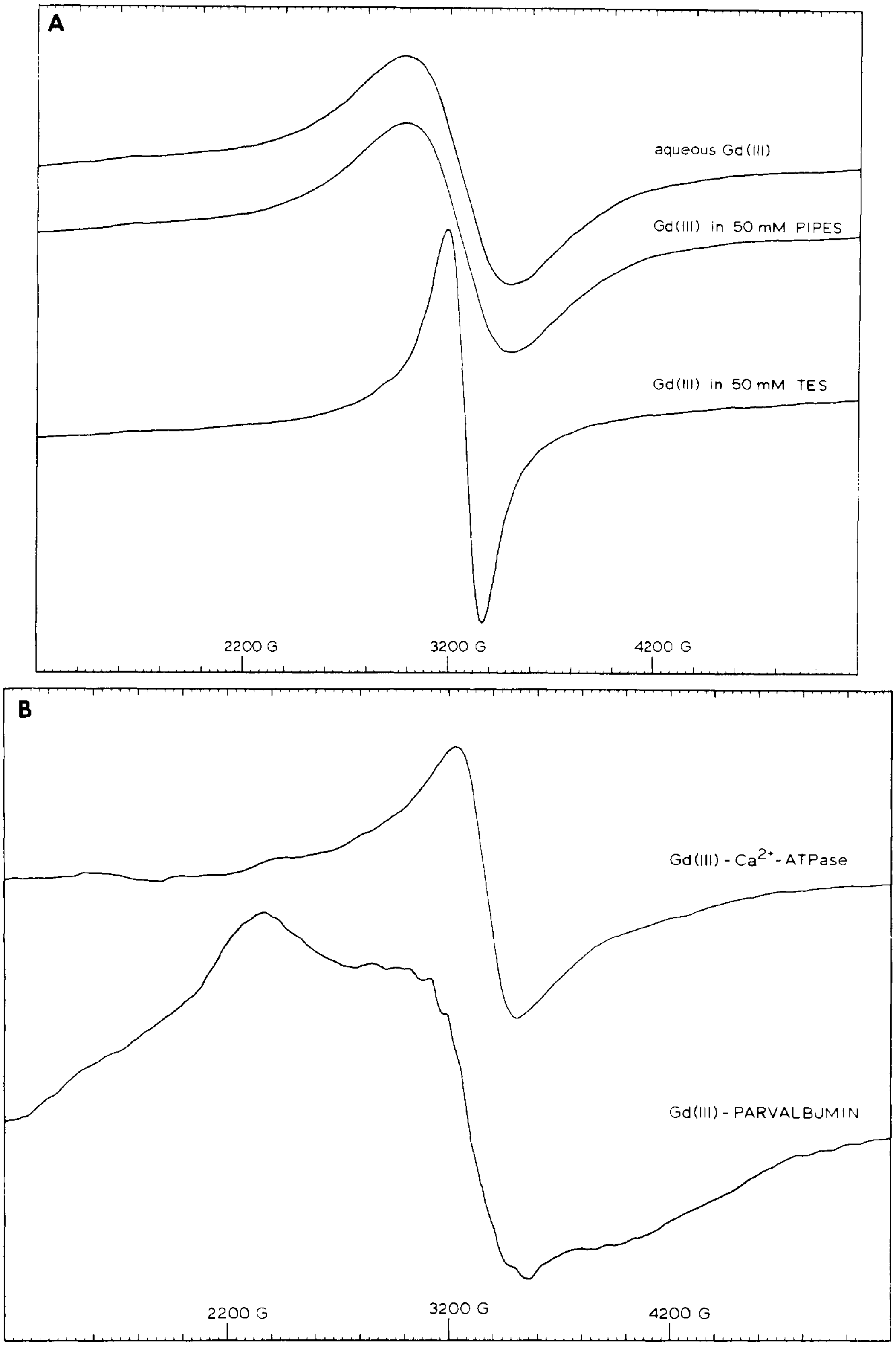

FIGURE 9: X-band EPR spectra of Gd^{3+} (A) in aqueous solution, in 50 mM NMe_4–Pipes, pH 7.0, and in 50 mM NMe_4–Tes, pH 7.0, 0.4 mM $GdCl_3$ in all cases, and (B) bound to Ca^{2+}–ATPase (0.4 mM Ca^{2+}–ATPase and 0.38 mM $GdCl_3$) or parvalbumin (20 mM parvalbumin and 14 mM $GdCl_3$). T = 23 °C.

(Geraldes & Williams, 1977). However, the possibility exists that Gd^{3+} will be a sensitive EPR probe for characterizing macromolecular biological systems such as the Ca^{2+}–ATPase. The EPR spectra of Gd^{3+}, which has $S = {}^7/_2$, in neutral water and in two different buffers are shown in Figure 9A. The line widths were found to be independent of pH over the usable range of these buffers and independent of temperature between 4 and 30 °C. The spectrum of Gd^{3+} in neutral water is centered around 3248 G, with a line width of 530 G. As shown, Gd^{3+} in Pipes buffer, but not in Tes buffer, yielded a spectrum similar to that of the aqueous Gd^{3+} solution. On this basis, all of our Gd^{3+} EPR and NMR studies were performed in Pipes buffer. As shown in Figure 9B, the formation of the Gd^{3+}–ATPase complex results in a shift of the resonance to 3315 G and a decrease in the line width to 285 G. Such a decrease in the line width may result from a

Table III: ^{7}Li Relaxation Rates, Correlation Times, and Gd^{3+}-Li^+ Distances on Ca^{2+}-ATPase

complex	$1/f(T_{1p})$ ($s^{-1} \times 10^{-3}$)	τ_c[a] ($s \times 10^9$)	$f(\tau_c)$[a] ($s \times 10^9$)	r (Å)
ATPase–$(Gd^{3+})_{site1}$–Li^+	3.55 ± 0.4[b]	1.91	5.254	7.0 ± 0.7
ATPase–$(Gd^{3+})_{site2}$–Li^+	0.75 ± 0.05	2.12	5.718	9.1 ± 0.9

[a] From the frequency dependence of $1/T_{1p}$ of water protons. [b] The value of $1/f(T_{1p})$ for site 1 was calculated from the initial slope of the line in Figure 2. The $1/f(T_{1p})$ at site 2 was obtained by subtracting the $1/T_{1p}$ due to site 1 from the total change of $1/T_1$ in Figure 2.

decrease in the Gd^{3+} coordination number upon formation of the macromolecular complex, which could result in greater symmetry and a lower zero-field splitting for the Gd^{3+} ion. This spectrum is independent of temperature between 4 and 25 °C and is independent of the Gd^{3+}/ATPase ratio up to 2 Gd^{3+} ions/ATPase molecule. The peak-to-peak line width of 285 G sets a lower limit of 2.3×10^{-10} s on the electron spin relaxation time of enzyme-bound Gd^{3+}. This symmetric, narrow EPR spectrum for the Gd^{3+}–ATPase complex is compared in Figure 9B to that of Gd^{3+} bound to parvalbumin, a Ca^{2+}-binding protein from carp. In this case, the spectrum is extremely broad and suggests a greatly distorted Gd^{3+} coordination geometry compared to the Ca^{2+}–ATPase.

Discussion

Considerable evidence has accumulated to indicate that the active transport of Ca^{2+} ions across the sarcoplasmic reticulum membrane involves the binding and subsequent transport of 2 Ca^{2+} ions/molecule of ATP hydrolyzed (Baskin, 1977). Thus, in studies of Ca^{2+} uptake in fragmented sarcoplasmic reticulum vesicles, Meissner found that 2 Ca^{2+} ions are accumulated per ATP hydrolyzed (Meissner, 1973). Using a purified Ca^{2+}–ATPase preparation, Ikemoto has measured 2 Ca^{2+}-binding sites/100 000-dalton protein monomer (Ikemoto, 1975). Ikemoto has found that, while two sites are observed at 25 °C, only one of these sites is maintained at 2–5 °C. With this exception, no other studies have been able to distinguish between the two Ca^{2+}-binding sites.

In the present study of the Ca^{2+}–ATPase, gadolinium ion has been shown to bind to 2 sites/100 000-dalton protein monomer. These two sites for Gd^{3+} have been characterized by using $^7Li^+$ NMR, water proton NMR, and Gd^{3+} EPR. The Gd^{3+} sites on this enzyme are the activating Ca^{2+}-binding sites, as demonstrated by the displacement of Gd^{3+} by Ca^{2+} in the $^7Li^+$ NMR studies (Figure 3) and by the comparable effect of Gd^{3+} on the Ca^{2+}-activated phosphorylation (Figure 1). Furthermore, these two sites can be distinguished on the basis of the effects of Gd^{3+} ion on the relaxation rate of enzyme-bound Li^+ and by the enhancement of water proton relaxation by Gd^{3+}. In these experiments, the first additions of Gd^{3+} to the enzyme produce a sharp increase in the $1/T_1$ of Li^+ and effect a high enhancement of water proton relaxation. At levels of Gd^{3+} ion greater than 0.8 Gd^{3+}/enzyme monomer, smaller increases in $1/T_1$ of Li^+, as well as a smaller enhancement of water proton relaxation, are observed. The water proton relaxation enhancement data of Figure 4 are best fit by assuming 1 Gd^{3+}-binding site/protein monomer with an enhancement factor, ϵ_{B1}, of 9.4 and 1 Gd^{3+}-binding site/protein monomer with an enhancement of 5.4. The value of ϵ^* obtained in Figure 5 from an extrapolation to high enzyme levels of 7.4 thus represents an average of the enhancements at the two Gd^{3+}-binding sites. The downward bend at high enzyme in the data of Figure 5 is consistent with the existence of two Gd^{3+} sites, which differ in their affinities for Gd^{3+} and also in their abilities to enhance water proton relaxation. Thus, at low levels of enzyme both of the Gd^{3+} sites are saturated with Gd^{3+} and the extrapolation of this portion of the curve to high enzyme yields a value for ϵ^* of 7.4. This graphically determined value is then an average of the enhancement factors for Gd^{3+} at the two Ca^{2+}-binding sites. At high levels of enzyme in Figure 5, the ATPase preferentially binds Gd^{3+} at site 1 and the observed enhancement increases sharply. Thus, both the $^7Li^+$ NMR and water PRR measurements are consistent with the binding of 2 g ions of Gd^{3+} per 100 000 g of protein. The first of these, which we have called site 1, binds Gd^{3+} more tightly than the second site (site 2), exerts a larger paramagnetic effect on enzyme-bound Li^+, and exhibits a larger enhancement of water proton relaxation.

Information on the structure of these Gd^{3+}-binding sites, including the hydration state of the metal, accessibility of solvent molecules, and distances to other metal- and substrate-binding sites, can be obtained under appropriate conditions if the dipolar correlation time for Gd^{3+}–nucleus interactions is known. From the frequency dependence of the effect of the enzyme-bound Gd^{3+} on $1/T_1$ of H_2O (Figure 8 and Tables I and II), the correlation times for sites 1 and 2 are found to be 1.9×10^{-9} and 2.1×10^{-9} s, respectively.[4] These values are too short to be influenced significantly by τ_r, the rotational correlation time of the enzyme–Gd^{3+} complex, or τ_m, the mean residence time of water molecules in the first coordination sphere of the metal. Moreover, the minima in the plots of T_{1p} vs. ω_I^2 indicate that τ_c must be dominated by τ_s, the electron spin relaxation time. The τ_s values determined here are longer than most of those determined previously for Gd^{3+}. The electron spin relaxation time for aqueous Gd^{3+} is $(4–7) \times 10^{-10}$ s at 30 MHz (Bloembergen & Morgan, 1961), while values for τ_s of $(2–7) \times 10^{-10}$ s have been reported for complexes of Gd^{3+} with pyruvate kinase (Cottam et al., 1974) and a value of 2.2×10^{-10} s has been found for a Gd^{3+}–lysozyme complex (Dwek & Richards, 1971). Moreover, we have estimated a τ_c of 6.8×10^{-10} s for Gd^{3+} bound to parvalbumin.[5] The long Gd^{3+} correlation times found in the present study are consistent with a poor accessibility of these Gd^{3+} sites to solvent water molecules. The electron spin relaxation time, τ_s, is given by eq 5 (Bloembergen & Morgan, 1961)

$$\frac{1}{\tau_s} = B\left[\frac{\tau_v}{1 + \omega_S^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_S^2\tau_v^2}\right] \qquad (5)$$

where τ_v is a correlation time which is related to the rate at which the zero-field splitting is modulated by impact of the solvent molecules on the complex and B is a constant containing the value of the electronic spin and the zero-field splitting parameters. This theory assumes that $\tau_v \ll \tau_s$ and also that the electron spin–lattice relaxation time is much larger than the electron spin–spin relaxation time. The latter assumption will be considered further below. The values of τ_c (i.e., τ_s) obtained for Gd^{3+} bound to the Ca^{2+}–ATPase are 3–10 times longer than those observed with the Gd^{3+} aquo cation and with the protein complexes cited. Since the impact of outer sphere solvent molecules on the Gd^{3+} complex provides the predominant mechanism for electron spin–lattice relax-

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[4] In analyzing the frequency-dependence data to determine τ_c at site 2 for Gd^{3+}, it was assumed that no dipole–dipole interactions occur between Gd^{3+} at sites 1 and 2. Such interactions may in fact occur, so that τ_c for Gd^{3+} at site 1 would change when site 2 is filled. Experiments to determine the nature and extent of these interactions are in progress.

[5] E. M. Stephens and C. M. Grisham, unpublished experiments.

ation, the long values for τ_s found here are thus consistent with a reduced accessibility of solvent water to the Gd^{3+} ion in the ATPase complex.

The fully hydrated Gd^{3+} ion is thought to be nine- or tenfold coordinated (Dwek, 1973; Valentine & Cottam, 1973). In complexes with proteins, where the coordination number could be six or eight, the symmetry of the Gd^{3+} could be increased over the aquo complex, resulting in a reduced zero-field splitting and a narrower EPR line width. This is found to be the case in the EPR spectra of Figure 9. The Gd^{3+} aquo cation exhibits an EPR line width of 530 G. The EPR spectrum of the Ca^{2+}–ATPase–Gd^{3+} complex, on the other hand, consists of a single transition with a line width of 285 G and is consistent with an increased symmetry for Gd^{3+} upon binding to the ATPase. These line widths set lower limits for τ_s of Gd^{3+} of 1.2×10^{-10} and 2.3×10^{-10} s for the aquo cation and ATPase complexes, respectively. The latter value is significantly shorter than the values of τ_s obtained from the frequency dependence of water proton relaxation, suggesting that the assumption made for eq 5, that $\tau_s \gg T_{2e}$, is valid.

The values of q, the number of exchangeable water molecules, calculated for the two Gd^{3+} sites ($q_1 = 3.0 \pm 0.5$ and $q_2 = 2.0 \pm 0.5$) indicate that these metal sites are relatively unhydrated or perhaps that most of the first coordination sphere water at these sites is "frozen" on the NMR time scale and does not exchange rapidly with the bulk solvent. Poorly hydrated lanthanide-binding sites on proteins have been observed in other systems (Epstein et al., 1974; Moews & Kretsinger, 1975a,b). For example, the EF Ca^{2+} site in parvalbumin, a Ca^{2+}-binding protein from carp, binds Ca^{2+} or Tb^{3+} with only one water of hydration (Moews & Kretsinger, 1975a,b). It is worth considering the significance of τ_s and q for the Gd^{3+} sites (i.e., Ca^{2+} sites) on the Ca^{2+}–ATPase. The long electron spin relaxation time for Gd^{3+}, which almost certainly reflects a limited accessibility of solvent water, and the small number of exchangeable water molecules at these metal sites are consistent with Ca^{2+} sites which are "buried" within a pocket or cleft in the ATPase molecule. Neither the long τ_s nor the small values of q would be consistent with surface sites for Ca^{2+} or Gd^{3+} which were in intimate contact with the solvent. While verification of this possibility will require additional structural information, the notion of occluded Ca^{2+} sites could be anticipated for a membrane enzyme involved in Ca^{2+} transport.

A fundamental question regarding the structure and function of the Ca^{2+}–ATPase (and other transport enzymes) concerns the location of the catalytic site with respect to the transport site and the locations of the transport sites with respect to each other. In the present study we have demonstrated the existence of a binding site or sites near both of the Ca^{2+}-binding sites on the enzyme. Under conditions of fast exchange the effects of Gd^{3+} on $1/T_1$ of $^7Li^+$ can be used to calculate Gd^{3+}–Li^+ distances to both Gd^{3+} sites. It is unlikely that $1/T_{1p}$ of $^7Li^+$ in the presence of Gd^{3+} is exchange limited in these studies. If exchange were limiting the value of $1/f(T_{1p})$, one would expect a positive temperature dependence and an activation energy greater than 3 cal/mol (Mildvan & Cohn, 1970), contrary to observation.

As can be seen from eq 2, errors in r in this type of calculation go as the sixth root of errors in q. If more than one Li^+ were bound near the Gd^{3+}, i.e., if $q > 1$, the absolute Gd^{3+}–Li^+ distance would increase with $q^{1/6}$. For example, if two Li^+ ions were bound at equal distances from the two Gd^{3+} sites, the Gd^{3+}–Li^+ distances would increase by 12% to 7.84 and 10.2 Å instead of 7.0 and 9.1 Å, respectively. The possibility of more than one Li^+ ion bound near the Gd^{3+} sites cannot be ruled out in the present study. Li^+ ions bound at greater distances would contribute little to $1/f(T_{1p})$ because of the inverse sixth power relationship in eq 4.

Another central question in the elucidation of the mechanism for calcium transport by the Ca^{2+}–ATPase is the matter of coupling between ATP hydrolysis and the movement of calcium ions across the membrane. It is interesting in this regard to note the decrease in ϵ^* for the ATPase–Gd^{3+} complex upon addition of the nonhydrolyzable substrate analogues AMP–PNP and tridentate CrATP. Such decreases could be due to any one of several different processes on the enzyme. The substrate analogues could function to (1) displace the Gd^{3+} from its sites on the enzyme, (2) complex the Gd^{3+} and remove it from the enzyme, (3) alter the environment of the Gd^{3+} site on the enzyme, or (4) promote the movement of Gd^{3+} to a new site on the enzyme. Our data eliminate the first two processes from consideration. The paramagnetic effect of Gd^{3+} on $1/T_1$ of water protons is not removed by centrifugation and washing of the enzyme. The correlation time for the enzyme–Gd^{3+}–AMP–PNP complex is unchanged from the value of the binary ATPase–Gd^{3+} complex and is much too long for either aqueous Gd^{3+} ion or for Gd^{3+}–AMP–PNP complexes. Moreover, CrATP does not readily form complexes with additional metals (Gupta et al., 1976a) and would not be able to pull Gd^{3+} off of its site on the ATPase. The decrease in q for fast-exchanging water protons on the enzyme-bound Gd^{3+}, on the other hand, is consistent with either of the third or fourth processes above. Whatever the nature of the change in the Gd^{3+} environment, it occurs without hydrolysis of the nucleotide analogue.

There is an additional structural consideration arising from the $^7Li^+$ NMR studies. If only one Li^+ ion site can interact with the Gd^{3+}-binding sites in the NMR experiments, the distances measured can set an upper limit on the distance between the two Ca^{2+} ion sites. The Li^+–Gd^{3+} distances of 7.0 and 9.1 Å thus set an upper limit on the Ca^{2+}–Ca^{2+} distance of 16.1 Å. If there are instead two Li^+ sites which can both interact with the Ca^{2+} (i.e., Gd^{3+}) sites, then the Ca^{2+}–Ca^{2+} distance limit increases to 18 Å. In any case, if the Li^+ sites we observe can interact with both Gd^{3+} sites, these two sites must be close together on the Ca^{2+}–ATPase. If this was indeed the case, one might anticipate that Gd^{3+}–Gd^{3+} dipolar interactions could be observed on the Ca^{2+}–ATPase. Our EPR studies (Stephens & Grisham, 1978) of the Ca^{2+}–ATPase have already detected such Gd^{3+}–Gd^{3+} interactions upon addition of ATP to the enzyme–Gd^{3+} complex. This latter work will be the subject of future communications.

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References

Balakrishnan, M., & Villafranca, J. (1978) *Biochemistry 17*, 3531.

Barnett, R. E. (1970) *Biochemistry 9*, 4644.

Baskin, R. A. (1977) in *Membrane Proteins and Their Interactions with Lipids* (Capaldi, R., Ed.) p 151, Marcel Dekker, New York.

Bloembergen, N., & Morgan, L. (1961) *J. Chem. Phys. 34*, 842.

Carvalho, A., & Leo, B. (1967) *J. Gen. Physiol. 50*, 1327.

Chen, P. S., Toribara, T., & Warner, H. (1956) *Anal. Chem. 28*, 1756.

Cottam, G. L., Valentine, K. M., Thompson, B. C., & Sherry, A. D. (1974) *Biochemistry 13*, 3532.

DePamphilis, M. L., & Cleland, W. W. (1973) *Biochemistry 12*, 3714.

Duggan, F. (1977) *J. Biol. Chem. 252*, 1620.

Dwek, R. A. (1973) *Nuclear Magnetic Resonance in Biochemistry*, Clarendon Press, Oxford.

Dwek, R., & Richards, R. (1971) *Eur. J. Biochem. 21*, 204.

Ebashi, S., & Lipmann, F. (1962) *J. Biol. Chem. 214*, 389.

Eisinger, J., Shulman, R., & Szymanski, B. (1962) *J. Chem. Phys. 36*, 1721.

Epstein, M., Levitzki, A., & Reuben, J. (1974) *Biochemistry 13*, 1777.

Froelich, J. P., & Taylor, E. (1975) *J. Biol. Chem. 250*, 2013.

Geraldes, E., & Williams, R. (1977) *J. Chem. Soc., Dalton Trans. 18*, 1721.

Goa, J. (1953) *Scand. J. Clin. Lab. Invest. 5*, 218.

Grisham, C. M. (1978) in *Biomolecular Structure and Function* (Agris, P., Ed.) p 385, Academic Press, New York.

Grisham, C., & Mildvan, A. (1974) *J. Biol. Chem. 249*, 3187.

Grisham, C. M., & Hutton, W. C. (1978) *Biochem. Biophys. Res. Commun. 81*, 1406.

Gupta, R., Oesterling, R., & Mildvan, A. (1976a) *Biochemistry 15*, 2881.

Gupta, R., Fung, C., & Mildvan, A. (1976b) *J. Biol. Chem. 251*, 2421.

Hasselbach, W., & Makinose, M. (1963) *Biochem. Z. 339*, 94.

Hutton, W. C., Stephens, E. M., & Grisham, C. M. (1977) *Arch. Biochem. Biophys. 184*, 166.

Ikemoto, N. (1975) in *Calcium Transport in Contraction and Secretion* (Carafoli, E., Ed.) p 289, North-Holland Publishing Co., Amsterdam.

Inesi, G. (1972) *Annu. Rev. Biophys. Bioeng. 1*, 191.

Makinose, M. (1969) *Eur. J. Biochem. 10*, 74.

Martonosi, A., & Feretos, R. (1964) *J. Biol. Chem. 239*, 648.

Meissner, G. (1973) *Biochim. Biophys. Acta 298*, 246.

Mildvan, A. S., & Cohn, M. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol. 33*, 1.

Mildvan, A., & Engle, J. (1972) *Methods Enzymol. 26C*, 654.

Moews, P., & Kretsinger, R. (1975a) *J. Mol. Biol. 91*, 201.

Moews, P., & Kretsinger, R. (1975b) *J. Mol. Biol. 91*, 229.

Nowak, T., Mildvan, A., & Kenyon, G. (1973) *Biochemistry 12*, 1690.

Peacocke, A., Richards, R., & Sheard, B. (1969) *Mol. Phys. 16*, 177.

Reuben, J., & Cohn, M. (1970) *J. Biol. Chem. 245*, 6539.

Robinson, J. (1976) *Biochim. Biophys. Acta 429*, 1006.

Solomon, I. (1955) *Phys. Rev. 99*, 559.

Solomon, I., & Bloembergen, N. (1956) *J. Chem. Phys. 25*, 261.

Stephens, E., & Grisham, C. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1483.

Taylor, J. S., & Hattan, D. (1979) *J. Biol. Chem. 254*, 4402.

Valentine, K., & Cottam, G. (1973) *Arch. Biochem. Biophys. 158*, 346.

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 622.

Yamada, S., & Tonomura, Y. (1972) *J. Biochem. (Tokyo) 74*, 417.

Yamamoto, T., & Tonomura, Y. (1968) *J. Biochem. (Tokyo) 64*, 137.

Yount, R., Babcock, D., Ballantyne, W., & Ojala, D. (1971) *Biochemistry 10*, 2484.