Stein, W. D., Lieb, W. R., Karlish, S. J. D., & Eilam, Y. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 275.
Tobin, T., Akera, T., & Brody, T. M. (1974) *Ann. N.Y. Acad. Sci. 242*, 120.
Uesugi, S., Dulak, N. C., Dixon, J. F., Hexum, T. D., Dahl, J. L., Perdue, J. F., & Hokin, L. E. (1971) *J. Biol. Chem. 246*, 531.
Yoda, A. (1974) *Ann. N.Y. Acad. Sci. 242*, 598.

Manganese Electron Paramagnetic Resonance Studies of Sheep Kidney $(Na^+ + K^+)$-ATPase. Interactions of Substrates and Activators at a Single Mn^{2+} Binding Site†

Sally E. O'Connor and Charles M. Grisham*

ABSTRACT: The interactions of Mn^{2+}, inorganic phosphate, and nucleotide substrate and substrate analogues with highly purified membrane-bound sodium plus potassium ion transport adenosine triphosphatase from sheep kidney medulla have been examined by using electron paramagnetic resonance techniques. EPR studies of both native and partially delipidated ATPase preparations indicate that the enzyme binds Mn^{2+} at one tight site with a K_D of 0.21×10^{-6} M. A second class of 24 ± 3 weaker binding sites for Mn^{2+} is also observed in the native enzyme, but these are removed upon removal of 50% of the essential phospholipids in the enzyme preparation, confirming that these are lipid binding sites for Mn^{2+} as had been previously suggested [Grisham, C., & Mildvan, A. (1974) *J. Biol. Chem. 249*, 3187]. The X-band EPR spectrum of the binary Mn^{2+}–ATPase complex exhibits a powder or quasi-solid state line shape consisting of a broad transition with partial resolution of the ^{55}Mn nuclear hyperfine structure, as well as a broad component to the low-field side of the main pattern. The spectrum of the delipidated enzyme is significantly broadened compared with the native enzyme, with a loss in resolution of the hyperfine lines. Low concentrations of phosphate produce a shift toward the center of the spectrum for the broad low-field component, with additional smaller effects on the hyperfine structure of the spectrum. ATP, ADP, AMP–PNP, and high concentrations of inorganic phosphate all broaden the hyperfine lines of the Mn^{2+} spectrum, consistent with a change in coordination geometry of the bound Mn^{2+}, a change in accessibility of the Mn^{2+} site to solvent, or both. These spectra strongly suggest that the true substrate for the enzyme is ATP and not Mn^{2+}–ATP. In contrast to the above effects, AMP causes a substantial narrowing of the Mn^{2+} spectrum, including the narrowing and eventual disappearance of the broad, low-field signal. These changes are consistent with a greatly reduced axial distortion of the Mn^{2+} ion geometry in the ternary ATPase–Mn^{2+}–AMP complex. Addition of phosphate to this complex provides a very slight broadening, while the further addition of Na^+ ion restores the broad, asymmetric spectrum observed in the binary ATPase–Mn^{2+} complex. By contrast, the addition of AMP to the delipidated enzyme–Mn^{2+} complex produces much different spectra, indicating unusual Mn^{2+} distortions and suggesting that lipid removal greatly alters the ability of the enzyme to interact with nucleotides. These results suggest that, while the divalent metal and substrate sites are preserved in the delipidated enzyme, their conformations are substantially altered.

Characterization of the structure and function of $(Na^+ + K^+)$-ATPase,[1] a plasma membrane bound enzyme responsible for Na^+ and K^+ transport in mammals, will depend on the development of specific spectroscopic probes. In this regard, use can be made of the monovalent and divalent cation requirements of the system as well as the substrate and inhibitor binding sites. Thus our previous studies have partially characterized a Mn^{2+} binding site on the enzyme which is responsible for the divalent cation activation of the enzyme and the transport system (Grisham & Mildvan, 1974, 1975; Grisham et al., 1974). Nuclear magnetic resonance studies which use the $^{205}Tl^+$, ^{31}P, and $^7Li^+$ nuclei have also shown that this Mn^{2+} site is very near (a) a Na^+ site which is probably involved in enzyme activation and ion transport (Grisham et al., 1974), (b) a K^+ site which has not previously been detected in kinetic studies (Grisham, 1978; Grisham & Hutton, 1978), and (c) a noncovalently binding phosphate site (Grisham & Mildvan, 1975) which had not been detected previously on this enzyme. Since the report of our NMR data on this phosphate site, kinetic (Froelich et al., 1976) and calorimetric (Kuriki et al., 1976) data have also been reported, which implicate a noncovalent enzyme–phosphate intermediate in the mechanism of the $(Na^+ + K^+)$-ATPase.

Electron paramagnetic resonance (EPR) spectra of the bound Mn^{2+} ion can provide additional insight into molecular motion and structure at the active site of enzyme–Mn^{2+} complexes. Spectra for macromolecular complexes of Mn^{2+} in solution typically resemble those for the ion in the solid state because of the slow rotational motion of the complex (Reed & Ray, 1971; Reed & Cohn, 1973). Magnetic anisotropies

† From the Department of Chemistry of the University of Virginia, Charlottesville, Virginia 22901. *Received December 20, 1978.* This work was supported by National Institutes of Health Grant AM19419, Grant 8757G-4 from the Petroleum Research Fund, administered by the American Chemical Society, and grants from the Research Corporation, the Muscular Dystrophy Association of America, and the University of Virginia as well as a grant from the National Science Foundation for the purchase of an EPR spectrometer and computer system.

[1] Abbreviations used: $(Na^+ + K^+)$-ATPase, sodium and potassium ion transport adenosinetriphosphatase; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; TMA, tetramethylammonium; EPR, electron paramagnetic resonance; PRR, proton relaxation rate; P_i, inorganic phosphate.

0006-2960/79/0418-2315$01.00/0

which arise from asymmetry in the electronic environment of the ion are apparent in the spectra, and changes in these parameters reflect changes in the ligand composition and geometry of the complex. Moreover, the electron spin relaxation time of the Mn^{2+} ion, which can be estimated from the widths of the EPR lines, is a sensitive indicator of molecular motion in the vicinity of the ion (Hudson & Luckhurst, 1969b; Reed et al., 1971). Correlation of parameters from the EPR spectra with data obtained from NMR studies of ligands, substrates, and metal-bound water molecules can provide unique information on the catalytic centers of enzymes.

Such EPR information would provide important insights into several aspects of the structure and function of the (Na^+ + K^+)-ATPase. For example, the divalent metal (Mg^{2+} or Mn^{2+}) and the nucleotide substrate (ATP) can both bind independently to the enzyme, and very little is known about the interaction of the metal and the ATP on the enzyme. Also our previous water proton relaxation studies (Grisham & Mildvan, 1974) have shown that the binding of inorganic phosphate to the enzyme–Mn^{2+} complex decreases the number of exchangeable water protons on the Mn^{2+} ion from 4 to 3. ^{31}P NMR studies are consistent with a Mn^{2+}–P separation in the ternary ATPase–Mn^{2+}–P_i complex of 6.9 Å. This would be too large for a Mn–O–P bond, which might result from a direct displacement of a Mn^{2+}-bound water molecule or hydroxide ion by the phosphate, and would suggest instead a second sphere complex, with a bridging water molecule between the Mn^{2+} and P_i on the enzyme. Mn^{2+} EPR spectra could be useful in distinguishing between these possibilities.

The effects of monovalent cations in facilitating formation of ATPase–Mn^{2+}–P_i complexes (Grisham & Mildvan, 1974) and the proximity of Na^+ (Grisham et al., 1974) and K^+ (Grisham, 1978; Grisham & Hutton, 1978) sites to the Mn^{2+} site on the ATPase raise questions about the effect of monovalent ions on the geometry of the Mn^{2+} site. Also in view of the absolute requirement of this enzyme for an ordered phospholipid matrix around the ATPase protein (Grisham & Barnett, 1972, 1973a,b), it seems reasonable to ask whether the various substrate and metal ion sites are preserved in the absence of some or all of the essential lipid.

The present paper provides a more thorough characterization of the single Mn^{2+}-binding site on the ATPase than was possible with the partially purified enzyme which was used in several of the earlier studies (Grisham & Mildvan 1974; Grisham et al., 1974) and describes EPR spectra obtained in the ATPase–Mn^{2+} system. Estimates of the relevant electron spin relaxation time and some features of the interaction between substrates and activators and the enzyme–Mn^{2+} complex are inferred from the EPR spectra.

Experimental Section

Materials and Assays. (Na^+ + K^+)-ATPase from sheep kidneys (Pel-Freeze Biological, Rogers, AR) was purified as previously described (Grisham & Mildvan, 1975; Jorgensen, 1974a), except that the second detergent treatment (Grisham & Mildvan, 1975) was often unnecessary. The final specific activities of these preparations were typically 30–38 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}. The (Na^+ + K^+)-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. Phosphate was determined by the method of Chen et al. (1956). Protein was determined by the method of Lowry et al. (1951) by using crystalline bovine serum albumin as a standard. Enzyme concentration was calculated by assuming a protein molecular weight of 250000 and using sodium dodecyl sulfate–polyacrylamide gel electrophoresis to determine the percentage of the total protein that was (Na^+ + K^+)-ATPase (these values were typically 80–95% pure). Inorganic phosphate solutions were prepared by neutralizing phosphoric acid with tetramethylammonium hydroxide. All sucrose solutions used in these studies were rigorously treated with Chelex-100 (Bio-Rad) to remove divalent metal contaminants.

The phospholipid content of the enzyme preparations was varied, when desired, by incubating the enzyme suspension for 15 min at room temperature in a mixture containing: 50 mM imidazole chloride, pH 7.2 at 20 °C, 1 mM TMA–EDTA, 1 mg/mL enzyme protein, and an appropriate concentration of sodium cholate. This mixture was then applied to a discontinuous sucrose gradient composed of layers of 29.4% (w/v) sucrose, 15% (w/v) sucrose, and 10% (w/v) sucrose, and the gradient was centrifuged 12–15 h at 50000 rpm in a Beckman 70 Ti rotor (18000g_{av}) and the pellet at the bottom of the tube was assayed for enzyme activity (Barnett, 1970), protein (Lowry et al., 1951), and phospholipid content (Chen et al., 1956; Bartlett, 1959).

EPR Measurements. A Varian E-109 EPR spectrometer was used for all EPR measurements. The spectrometer was equipped with a Varian E-257 variable temperature accessory and temperature was maintained within ±1 °C by heating a precooled stream of nitrogen gas which was passed through a Dewar assembly in the E-231-2 cavity. Temperature in the cavity was measured using a Texas Instruments thermistor placed in ethylene glycol in an EPR sample tube. The enzyme samples were centrifuged out of the appropriate solutions of buffer, salts, substrates, etc., and the enzyme pellet was mixed with a minimum of the same buffer solution and taken up in ultrapure quartz capillary tubes (Amersil). Sample volumes of up to 70 μL could be accommodated in the cavity in this way. Conditions for the EPR experiments included a modulation frequency of 100 kHz, modulation amplitude of 10 G, microwave power of 100 mW at 9.1 GHz, and field sweeps of 125–250 G/min. The spectra were taken at 22 °C unless otherwise indicated.

When appropriate, spectra were accumulated and stored on floppy disks by using a Digital Equipment Corp. PDP 11VO3 computer with 20K of core memory and video display. Among other options, this system permitted the spectra to be corrected for various base-line abnormalities, including the so-called "modulation offset" effect, which causes a systematic base-line drift at high modulation amplitudes.

Analysis of Titration Data. The concentration of free Mn^{2+} in solutions of the (Na^+ + K^+)-ATPase was determined from the intensity of the free Mn^{2+} EPR signal (Cohn & Townsend, 1954). The Mn^{2+}-binding data indicated two classes of binding sites. The data were analyzed with a Scatchard plot, from which initial estimates were obtained for the number of binding sites ($[E_1]_t$ and $[E_2]_t$) and the dissociation constants (K_1 and K_2). These parameters were refined by fitting the data to theoretical curves as follows. Assumed values of the dissociation constants and numbers of binding sites were used to compute the concentration of free Mn^{2+}, $[Mn]_f$, by means of the cubic equation (Miziorko, 1974) (eq 1) which was derived

$$[Mn]_f^3 + [Mn]_f^2\{[E_1]_t + [E_2]_t + K_1 + K_2 - [Mn]_t\} + [Mn]_f\{K_1([E_2]_t - [Mn]_t) + K_2([E_1]_t - [Mn]_t) + K_1K_2\} - K_1K_2[Mn]_t = 0 \quad (1)$$

by assuming two classes of noninteracting Mn^{2+}-binding sites. The concentration of bound Mn^{2+}, $[Mn]_b$, was calculated from the difference $[Mn]_t - [Mn]_f$, where $[Mn]_t$ is the total concentration of manganese. These values, together with the known concentration of total enzyme, were used to calculate

the theoretical Scatchard plots. This process was repeated until optimum fits were obtained.

Interpretation of EPR Spectra. The five unpaired 3d electrons and the relatively long electron spin relaxation time of the divalent manganese ion result in readily observable EPR spectra for Mn^{2+} solutions at room temperature. The Mn^{2+} ($S = 5/2$) ion exhibits six possible spin-energy levels when placed in an external magnetic field. These six levels correspond to the six values of the electron spin quantum number, M_s, which has the values $5/2$, $3/2$, $1/2$, $-1/2$, $-3/2$, and $-5/2$. The manganese nucleus has a nuclear spin quantum number of $5/2$, which splits each electronic fine structure transition into six components. Under these conditions, the selection rules for allowed EPR transitions are $\Delta M_s = \pm 1$, $\Delta m_I = 0$ (where M_s and m_I are the electron and nuclear spin quantum numbers) resulting in 30 allowed transitions. The spin Hamiltonian describing such a system is

$$\mathcal{H} = g\beta H \cdot S + hAS \cdot I + D[S_z^2 - (1/3)S(S+1)] + E[S_x^2 - S_y^2]$$

where S and I are the electron and nuclear spin operators, g and A are the Zeeman and hyperfine interaction constants, and D and E are the axial and rhombic distortion parameters of the zero-field splitting (ZFS) interactions (Abragam & Bleaney, 1970). The ZFS interactions are highly anisotropic, and the effect of these terms on the energy level is orientation dependent. In rapidly rotating complexes of manganese(II) ions, the terms involving D and E are effectively averaged to zero since $\Delta\omega\tau_r < 1$. Here τ_r is the rotational correlation time of the complex and $\Delta\omega$ is the ZFS in rad/s. Since ZFS terms make no net contribution to the spacing of energy levels, transition frequencies for all of the electronic fine structure components are equal, and the 30 allowed transitions thus give rise to six inhomogeneously broadened lines corresponding to the six values of m_I. Within each of the six resolved lines are the five unresolved fine structure components.

On the other hand, when rotational motion is slow, or when the symmetry of the complex is less than cubic, as in Mn^{2+} complexes with macromolecules, $\Delta\omega\tau_r$ is often greater than one, the anisotropic interactions are incompletely averaged, and EPR spectra similar to those for randomly oriented solid samples are observed. In these cases, the spectra depend upon the angular relationships between the magnetic field vector and the crystal field axis of the ion. Moreover, when the symmetry of the manganese ion complexes deviates greatly from cubic, the EPR spectra depend upon the sharing of spectral intensity between the normal and forbidden ($\Delta M_s = \pm 1$; $\Delta m_I = \pm 1$) transitions.

The impact of solvent molecules and the resulting transient distortions of the Mn^{2+} complex determine the electron spin relaxation time of the system (Hudson & Luckhurst, 1969a). Thus efficient solvent collisions at the bound Mn^{2+} will yield broad EPR lines, while narrow lines should result when Mn^{2+} is inaccessible to this rapid, fluctuating motion.

Results

Manganese Binding Studies with Native and Delipidated $(Na^+ + K^+)$-ATPase. Previous Mn^{2+}-binding studies (Grisham & Mildvan, 1974) with the $(Na^+ + K^+)$-ATPase were complicated by the impurity of the enzyme and by the large number of lipid bilayer binding sites for Mn^{2+} in these preparations. Scatchard plots of the data obtained in these cases were consistent with 350 lipid membrane binding sites for Mn^{2+}/molecule of active enzyme. By contrast, the enzyme preparations used in the present studies (prepared according to Grisham & Mildvan, 1975) contained fewer phospholipids/enzyme molecule and fewer of the lipids appeared to bind Mn^{2+}. A Scatchard plot of Mn^{2+} binding to the purified enzyme is shown in Figure 1. The data were fitted by assuming two classes of noninteracting binding sites: 1 site with a K_D for Mn^{2+} of 0.21 μM and 24 sites with a K_D for Mn^{2+} of 185 μM. This single tight site is the same site previously characterized (Grisham & Mildvan, 1974) by EPR, water PRR, and kinetic studies as the divalent metal site involved in catalysis of ATP hydrolysis and, presumably, in ion transport. The dissociation constant obtained for the 24 weak binding sites is similar to that obtained for the lipid membrane binding sites for Mn^{2+} determined previously (Grisham & Mildvan, 1974), and the relatively small number of these sites ($n_2 = 24$) suggests that few of the lipids in these preparations can participate in Mn^{2+} binding. The membrane preparations used in these studies typically contained 230–300 phospholipids/active enzyme molecule.

In order to examine the effect of these phospholipids on the integrity of the single catalytic Mn^{2+} binding site and to confirm that the 24 weak Mn^{2+} sites/enzyme unit are in fact lipid sites, the enzyme was incubated with sodium cholate and centrifuged through sucrose gradients as described above. This treatment removes phospholipids in proportion to the con-

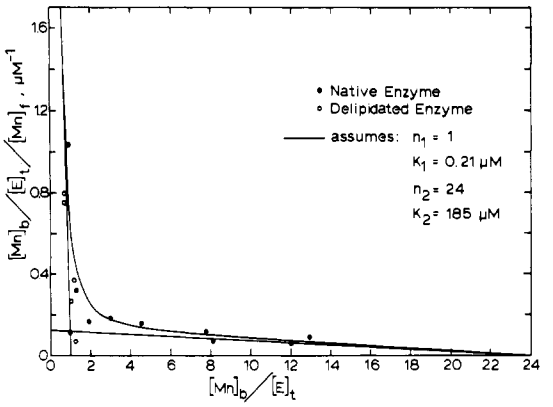

FIGURE 1: Scatchard plot of the binding of Mn^{2+} to native and partially delipidated $(Na^+ + K^+)$-ATPase. The free Mn^{2+} was determined by EPR in solutions containing $(Na^+ + K^+)$-ATPase (4.4 μM), $MnCl_2$ (0.01–0.20 mM), 25 mM Tes–TMA, pH 7.5, 100 mM NaCl, and 10 mM KCl in a total volume of 60 μL. The solid curve was computed as described in the text with the binding parameters shown. The solid lines show the Scatchard plots of the tight and weak sites separately. The delipidated enzyme was prepared as described in the text.

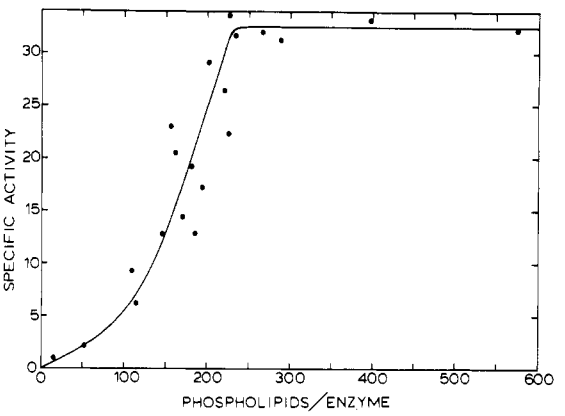

FIGURE 2: Dependence of $(Na^+ + K^+)$-ATPase activity on phospholipid content of the preparation. Native enzyme of greater than 90% purity was treated with sodium cholate as described in the text to remove lipids. As described in the text, the "native" enzyme used for the EPR studies contained 230 ± 20 phospholipids/enzyme molecule, while the "delipidated" preparation contained 120 phospholipids/enzyme.

centration of sodium cholate used, and the effect of lipid removal on the ATPase activity is shown in Figure 2. Below 230 phospholipids/enzyme, the activity decreases until, at approximately 100 phospholipids/enzyme molecule, the enzyme has lost 85% of its activity. These results are similar to those obtained with the Ca^{2+}–ATPase from sarcoplasmic reticulum (Warren et al., 1974; 1975). The results of a Mn^{2+}-binding study on such an enzyme preparation containing 120 phospholipids/enzyme and an activity of 6.6 u/mg (20% of the original activity) are shown in Figure 1. As can be seen, the 24 weak binding sites have been completely removed by this treatment, while the tight binding site has remained intact. (The binding studies on both the native and delipidated enzymes were carried to the limits of free and bound ligand suggested by a theoretical consideration of the Scatchard plot as a tool for ligand binding analysis (Deranleau, 1969), and thus the differences between the two preparations are not the result of artifacts in the analysis.) These results provide additional evidence that the 24 weak binding sites are lipid-binding sites for Mn^{2+} and clearly demonstrate that the single tight Mn^{2+} binding site is preserved upon delipidation.

X-Band EPR Spectra of $(Na^+ + K^+)$*-ATPase Complexes.* Successful Mn^{2+} EPR studies of Mn^{2+}–enzyme complexes require high concentrations of enzyme, not only for the sake of obtaining reasonably intense signals, but also to suppress the concentration of free Mn^{2+}, so as to minimize the spectral interference from the strong, isotropic signal for the free ion. For this reason, the $(Na^+ + K^+)$-ATPase, a plasma membrane bound enzyme, presents a potential problem. The particulate suspension state of this enzyme places a severe limit on obtainable enzyme concentrations, while the viscous nature of concentrated suspensions of the enzyme presents problems of sample handling and transfer. We have found, however, that enzyme concentrations of 0.15 mM or more can be obtained by careful resuspensions of centrifuge pellets of the ATPase; moreover, these suspensions can be drawn up into 1-mm i.d. quartz capillary tubing for EPR studies with a minimum sample loss due to handling.

The X-band EPR spectrum of Mn^{2+} bound to the $(Na^+ + K^+)$-ATPase and prepared as described above is shown in Figure 3. The spectrum observed for either the native or delipidated enzymes exhibits a "powder" line shape as would be expected for a macromolecular complex with Mn^{2+} (Reed & Cohn, 1970), and, therefore, the EPR transitions are spread over a considerable range of magnetic field. The central portion of the spectrum represents the $-1/2 \leftrightarrow 1/2$ fine structure transition in which partial resolution of the ^{55}Mn nuclear hyperfine structure is discernible. The spectrum also has a broad component to the low-field side of the main pattern. This low-field signal is part of the fine structure splitting which arises whenever there are asymmetries in the electronic environment of the bound Mn^{2+} (Reed & Ray, 1971). That the spectrum has a negligible contribution from free manganese is evidenced by the absence of noticeable temperature effects on the spectrum and by the width of the hyperfine lines. Free Mn^{2+} displays a marked temperature dependence (Reed & Cohn, 1973) as well as narrower hyperfine lines than are displayed here. Under the conditions of these experiments, it is possible to calculate that at least 93.3% of the manganese is bound to the single tight Mn^{2+}-binding site on the enzyme. Of the small amount of Mn^{2+} which remains, much is bound to the weaker, lipid bilayer sites and will exhibit a broad, but comparatively much weaker spectrum, which should not significantly affect the spectrum for the single, tight binding site.[2]

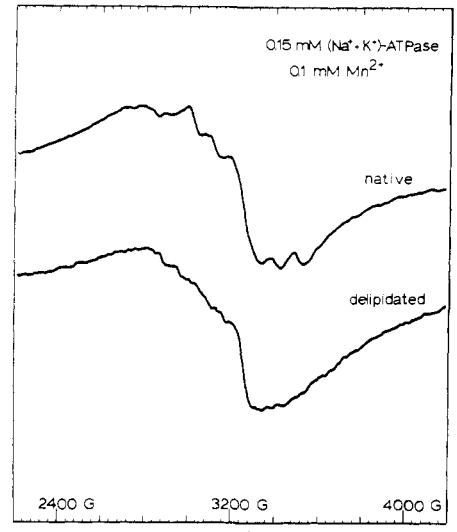

FIGURE 3: X-band EPR spectra of Mn^{2+} bound to a single site on $(Na^+ + K^+)$-ATPase. The native enzyme was prepared as described in the text. The partially delipidated enzyme (which contained 120 phospholipids/enzyme molecule) was prepared by incubating the enzyme preparation in a mix containing 21.5 mg/mL sodium cholate and following the procedures described in the text. All enzyme solutions were centrifuged out of 20 mM Tes–TMA, pH 7.5, and then combined with solutions of buffer and $MnCl_2$ so that the final concentrations were: 0.15 mM $(Na^+ + K^+)$-ATPase, 0.1 mM $MnCl_2$, 20 mM Tes–TMA, pH 7.5. EPR spectrometer settings were as described in the text.

Estimates of the electron spin relaxation time obtained from the EPR spectrum may be compared with the rotational correlation time and other relevant correlation times of this system. A lower limit on the transverse electron spin relaxation time, which can be measured from the line widths of the hyperfine transitions, is 1.7×10^{-9} s. By contrast, the rotational correlation time for the enzyme–Mn^{2+} complex can be estimated from a Stokes' law calculation to be at least 5×10^{-7} s (assuming a minimum mass for the enzyme–lipid complex of 420000 g/mol and a Stokes' radius of 81 Å). The electron spin relaxation time obtained previously (Grisham & Mildvan, 1974) for the ATPase–Mn complex from a frequency dependence of water proton relaxation was 1.68×10^{-9} s, in good agreement with the value obtained from the EPR line width for T_{2e}.

To examine further the effects of delipidation on the geometry of the single tight Mn^{2+}-binding site, EPR spectra of the native and delipidated enzyme preparations of the $(Na^+ + K^+)$-ATPase system were compared. The spectrum of Mn^{2+} in the delipidated enzyme is shown at the bottom of Figure 3. The removal of the essential lipids caused significant broadening of the manganese(II) EPR spectrum. This could result from a conformational change in the vicinity of the metal-binding site in the delipidated enzyme. If the line width of the EPR signal is determined primarily by the impact of outer sphere solvent molecules on the coordination sphere of the metal ion, then this broadening of the signal could be due to the greater accessibility of the metal site to solvent. It will

[2] We have observed low levels of paramagnetic impurities in the enzyme preparations used here. These impurities give rise to EPR spectra which are similar, but not identical, to the Mn^{2+} EPR spectra shown here. Treatment of the enzyme with 1 N HCl, followed by centrifugation, releases Mn^{2+} ion into the supernatant fluid (detected by its symmetrical six-line EPR signal). These impurities are ineffective in the relaxation of the protons of bulk water and their EPR spectra are insensitive to the addition of substrates and substrate analogues. Of the EPR spectra reported here, the spectra of the impurities are most like the delipidated enzyme–Mn^{2+} spectra (Figure 3).

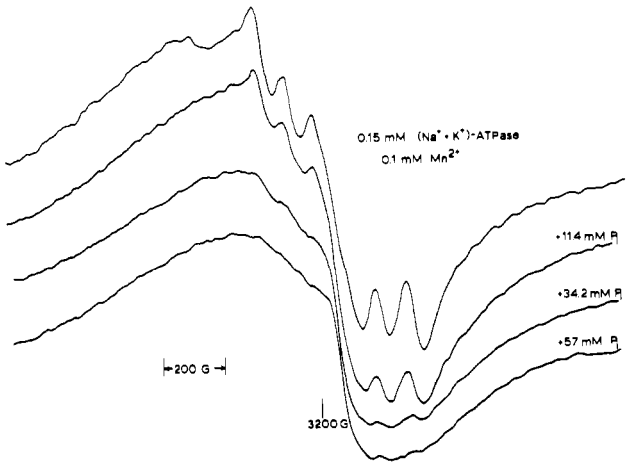

FIGURE 4: X-band EPR spectra for complexes of $(Na^+ + K^+)$-ATPase and inorganic phosphate. All solutions contained 20 mM Tes–TMA, pH 7.5, 0.15 mM ATPase, 0.1 mM $MnCl_2$, and the following concentrations of $(TMA)_2HPO_4$ (top to bottom): 0, 11.4, 34.2, and 57 mM. EPR spectrometer settings were as described in the text.

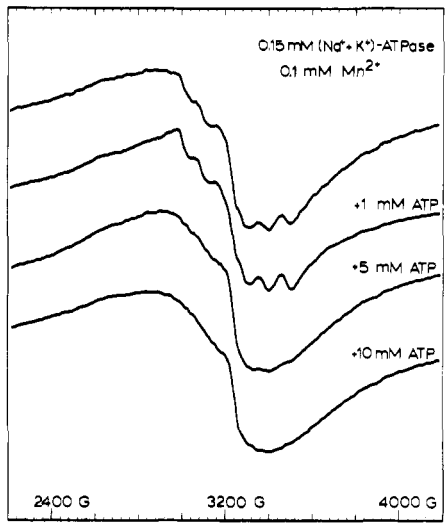

FIGURE 5: X-band EPR spectra for complexes of $(Na^+ + K^+)$-ATPase and ATP. The solutions and spectrometer settings were as described in Figure 4 and in the text. Solutions contained the indicated concentrations of ATP.

be seen later that the effects of AMP on the delipidated enzyme–metal complex are different from the effects produced on the native enzyme–metal complex. While the metal binding studies described above confirm that the single Mn^{2+} site is preserved on delipidation, the EPR spectra of the native and delipidated complexes are consistent with a conformational change of the enzyme upon delipidation. Such a change also accounts for the loss of enzyme activity upon lipid removal.

Manganese EPR Spectra of ATPase Complexes with Substrates, Activators, and Substrate Analogues. It has been demonstrated by a variety of techniques that inorganic phosphate binds tightly to the $(Na^+ + K^+)$-ATPase (Grisham & Mildvan, 1975; Froelich et al., 1976; Kuriki et al., 1976), but the interaction of phosphate with manganese on the enzyme is only partially understood. Mn^{2+} EPR spectra of the $(Na^+ + K^+)$-ATPase with several concentrations of added inorganic phosphate are shown in Figure 4. Low concentrations of phosphate, which would be sufficient to saturate the site observed in the NMR studies (K_D = 4.8 μM from Grisham & Mildvan, 1974), produce a shift in the broad, low-field component of the spectrum, with additional, smaller effects on the hyperfine structure of the spectrum. This can

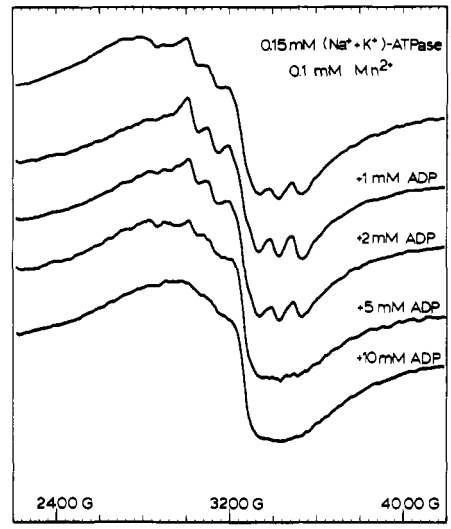

FIGURE 6: X-band EPR spectra for complexes of $(Na^+ + K^+)$-ATPase and ADP. The solutions and spectrometer settings were as described in Figure 4 and in the text. Solutions contained the indicated concentrations of ADP.

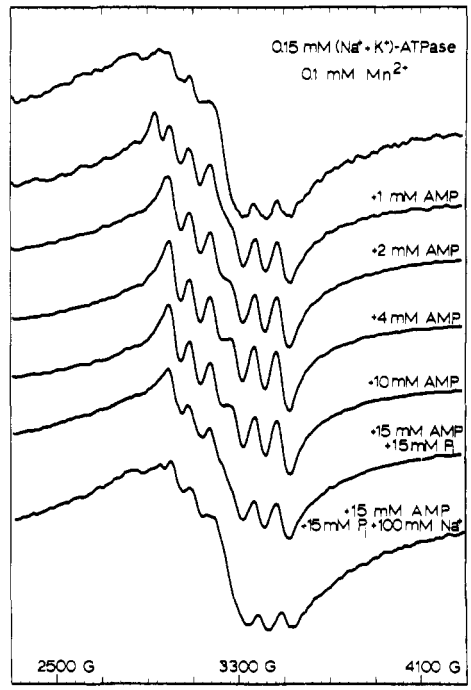

FIGURE 7: X-band EPR spectra for complexes of $(Na^+ + K^+)$-ATPase and AMP. The solutions and spectrometer settings were as described in Figure 4 and in the text. Solutions contained the indicated concentrations of AMP, inorganic phosphate, and sodium chloride.

be seen in the second spectrum in Figure 4, which was taken at 11.4 mM phosphate. The low-field signal is virtually eliminated, while approximately 50% of the resolution of the hyperfine lines is lost. The observed shift of the low-field signal toward the center of the spectrum could result from a significant reduction in the axial distortion of the enzyme-metal complex upon addition of phosphate substrate. The resolution of the hyperfine lines decreases with increasing concentrations of phosphate, until at 34 mM phosphate concentration, the hyperfine lines are barely discernible. Further addition of phosphate to the system (see bottom spectrum of Figure 4) seems to have little or no effect on the spectrum.

The effects of various nucleotide substrates and substrate analogues on the Mn^{2+} EPR spectrum are depicted in Figures 5–7. The changes in the Mn^{2+} EPR spectrum with increasing concentration of ATP and ADP substrates (Figures 5 and 6)

are similar to the effects produced by phosphate. As the concentration of ATP or ADP increases, the low-field signal shifts toward the center of the spectrum. Furthermore, the hyperfine lines broaden significantly as can be seen from the loss of resolution of the hyperfine peaks. However, the loss of resolution of the hyperfine signals occurs at a lower concentration of substrate for ATP and ADP (saturating at approximately 10 mM) than for phosphate, in which case saturation occurs at approximately 34 mM.

Under the conditions of these experiments, ATP is rapidly hydrolyzed by the high concentrations of enzyme employed, thus liberating inorganic phosphate, which itself would be expected to broaden the Mn^{2+} spectrum. However, there would appear to be an added effect which cannot be explained by liberation of inorganic phosphate since the broadening of the Mn^{2+} spectrum is complete at 10 mM ATP or ADP, while 34 mM phosphate is required to completely broaden the Mn^{2+} spectrum.

On the other hand, the effects of AMP on the Mn^{2+} EPR signal are drastically different from those produced by other phosphate substrates. Instead of the line broadening observed earlier, sharpening of the hyperfine lines is observed in the case of AMP. This can be seen in Figure 7. At 1 mM concentration of AMP, seven lines are observed. The six hyperfine lines corresponding to the central $1/2 \leftrightarrow -1/2$ fine transition narrow significantly, and the low-field line which is part of the fine structure splitting of the Mn^{2+} ion is very prominent. At concentrations of AMP greater than 1 mM, this low-field signal disappears, possibly as a result of a greatly reduced axial distortion of the enzyme–metal complex. The hyperfine lines narrow further at a concentration of AMP of 4 mM. Further addition of the substrate to the enzyme system (greater than 4 mM concentration of AMP) produces little or no further effect on the manganese ion spectrum.

The additional effects of inorganic phosphate and Na^+ ion on the enzyme–Mn^{2+}–AMP complex are also shown in Figure 7. Addition of 15 mM inorganic phosphate to the enzyme suspension at the end of the AMP titration causes a partial broadening of the hyperfine lines consistent with the effects of phosphate shown in Figure 4. However, the addition of sodium ion to this solution results in a further broadening of the hyperfine lines and, more significantly, the reappearance of the broad, low-field line, which is normally observed only in the spectrum of the binary enzyme–Mn^{2+} complex. Whereas phosphate or AMP, acting separately, both cause the disappearance of this line and alterations of the hyperfine lines, AMP plus phosphate plus sodium yield an ATPase–Mn^{2+} spectrum which is indistinguishable from that of the binary complex.

The effect of added AMP substrate on the EPR spectrum of the binary enzyme–metal complex is further evidence that the spectrum of the complex has a negligible contribution from free Mn^{2+} ion. If some free manganese ions were present in solution, addition of AMP would complex the free ions in the form of Mn^{2+}–AMP and broaden the EPR spectrum, resulting in a decrease in the apparent intensity of the hyperfine lines. However, the opposite effect, a narrowing and apparent increase in intensity of the hyperfine lines, is seen. This observation strongly suggests the absence of significant amounts of free Mn^{2+} in the enzyme solutions.

The examination of the interaction of the ATP site with the Mn^{2+} site on the $(Na^+ + K^+)$-ATPase is made difficult because most nucleoside triphosphates are hydrolyzed by high concentrations of the ATPase, even at trace levels of the monovalent cations. However, the methylene and amido

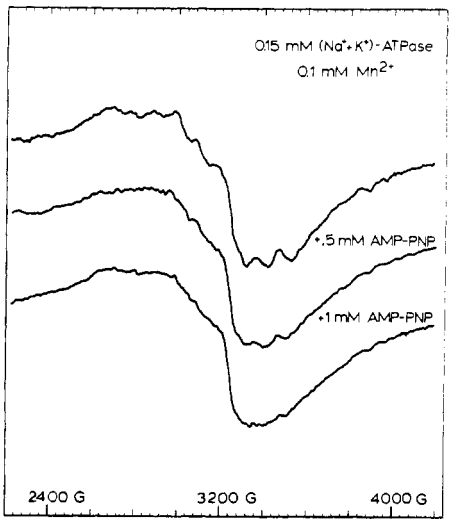

FIGURE 8: X-band EPR spectra for complexes of $(Na^+ + K^+)$-ATPase and AMP–PNP. The solutions and spectrometer settings were as described in Figure 4 and in the text. Solutions contained the indicated concentrations of AMP–PNP.

analogues of ATP, AMP–PCP and AMP–PNP, which have been characterized by Yount (1977), will bind to the $(Na^+ + K^+)$-ATPase in place of ATP, are not hydrolyzed by the enzyme, and thus offer a means of characterizing the ATP site(s). The addition of the substrate analogue, adenylyl imidodiphosphate (AMP–PNP), to the ATPase–Mn^{2+} complex produces the spectra shown in Figure 8. As with ADP and ATP, AMP–PNP causes a loss of resolution of the hyperfine lines at a concentration much lower than that required for hyperfine broadening by phosphate. By assuming that the spectrum at the bottom of Figure 8 is due entirely to the ternary enzyme–metal–AMP–PNP complex, an estimate of the dissociation constant for AMP–PNP from the enzyme–metal complex can be made from the loss of resolution of the hyperfine lines. The titration behavior with AMP–PNP is consistent with a K_D for AMP–PNP from the enzyme–Mn^{2+} complex of 0.65 mM, which is similar to the K_m of 0.40–0.50 mM for AMP–PNP and ATP determined kinetically under similar conditions (Grisham & Mildvan, 1974; Robinson, 1976). Since the ATPase does not hydrolyze AMP–PNP, the observed effects on the Mn^{2+} EPR spectrum cannot be due to inorganic phosphate and must represent formation of an ATPase–Mn^{2+}–(AMP–PNP) complex. Moreover the titration with AMP–PNP absolutely rules out the possiblity that the hyperfine lines of the Mn^{2+} spectrum involve significant contributions from free Mn^{2+} ion. Under the conditions of this titration, 0.5 mM and 1 mM concentrations of AMP–PNP would be sufficient to complex at least 93 and 96% of any free Mn^{2+} present, respectively, whereas these concentrations of AMP–PNP decrease the hyperfine intensities of the Mn^{2+} spectrum by 29 and 58%. The relative affinities of Mn^{2+} for AMP–PNP (Yount et al., 1971) and the ATPase indicate that less than 8% of the total manganese is complexed by AMP–PNP under these conditions and this is corroborated by the lack of significant contributions from Mn^{2+}–AMP–PNP in the observed EPR spectra. The spectrum of Mn^{2+}–AMP–PNP (not shown here) is similar to the spectrum of Mn^{2+}–ATP (Reed et al., 1971).

The binding studies indicated the maintenance of the single, tight Mn^{2+} site upon removal of essential phospholipids in the enzyme. However, the delipidation of the enzyme was accompanied by loss of activity of the ATPase. A comparison of the EPR spectra of the native and delipidated enzyme–Mn^{2+}

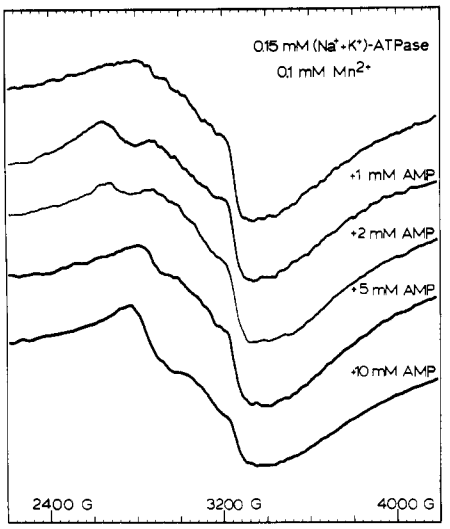

FIGURE 9: X-band EPR spectra for complexes of delipidated $(Na^+ + K^+)$-ATPase and nucleotides. The delipidated enzyme was prepared as described in the legend to Figure 3. All solutions contained 20 mM Tes–TMA, pH 7.5, 0.15 mM enzyme, 0.1 mM $MnCl_2$, and the indicated concentrations of AMP.

complexes and a comparison of the effects of substrate titration on these complexes might give additional information about the geometry of the metal site. The effects of titration of AMP on the delipidated enzyme–Mn^{2+} complex are shown in Figure 9. Whereas significant narrowing of the hyperfine lines is observed in the native enzyme–Mn^{2+} complex with increasing concentrations of AMP, the spectra in Figure 9 show virtually no change in the central portion upon addition of AMP. However, the low-field signal moves downfield at 1 mM and 2 mM AMP concentrations, while at concentrations greater than 2 mM AMP the signal moves upfield to almost the same position as the signal in the solution without added AMP. Clearly the effects of AMP on native vs. delipidated enzyme–Mn^{2+} complexes are different. Although these results reflect differences in the accessibility of the metal ion site to solvent (as can be expected from loss of some essential lipids), they also indicate different distortions of the complex in the native and delipidated enzymes. The results here indicate that, while the affinity of the enzyme for the Mn^{2+} is unchanged by removal of essential lipid, distortions of the active site of the enzyme do occur, as can be seen from a comparison of the EPR spectra.

The effect of monovalent cations on the Mn^{2+} spectrum of the ATPase system was also examined. No major changes in the central portion of the EPR spectrum occur for either Na^+, K^+, or Li^+, as compared with the effect produced by the other substrates mentioned above. Addition of sodium to the metal–enzyme system causes partial disappearance of the low-field signal, whereas addition of Li^+ or K^+ appears to have no effect on the low-field signal of the EPR spectrum. The behavior of the low-field component of the EPR spectrum is significant because this signal is sensitive to changes in the axial distortion of the complex; hence, the effect on this signal is indicative of changes in ligand geometry. The invariance of the appearance of the hyperfine lines in the central portion of the spectrum with different concentrations of monovalent cations possibly reflects little or no change in the ligand composition caused by these metal activators. The absence of large effects here is consistent with the failure to observe significant changes in the water proton relaxation due to Mn^{2+} upon addition of monovalent cations alone (Grisham & Mildvan, 1974). The change in the broad, low-field component of the spectrum by Na^+, but not by K^+ or Li^+, is also consistent with the observation (from NMR studies) that Na^+ binds closer to the Mn^{2+} site on the enzyme than K^+ or Li^+ (Grisham et al., 1974; Grisham & Hutton, 1978).

Discussion

The demonstration here that EPR spectra can be obtained for Mn^{2+} at the single, functional divalent metal binding site on the $(Na^+ + K^+)$-ATPase is significant for several reasons. First, it has not previously been demonstrated that membrane protein complexes, which typically consist of large, multimeric protein aggregates which require large amounts of lipid for maintenance of their native structure, can be prepared at concentrations sufficiently high to yield suitable EPR spectra for Mn^{2+} bound at a specific site. Indeed, the development of Mn^{2+} as an EPR probe of active sites of soluble enzymes (which can be prepared at very high concentrations with comparative ease) is itself a relatively recent advance (Reed & Cohn, 1973; Villafranca et al., 1976). Secondly, despite the characterization of a variety of amino acid residues at the $(Na^+ + K^+)$-ATPase active site, including sulfhydryl (Hokin & Dahl, 1972), aspartyl (Post & Kume, 1973), tyrosyl (Cantley et al., 1978), and arginyl (DePont et al., 1977) groups, and despite development of numerous substrate analogue probes (Yount, 1977), this enzyme has thus far resisted attempts to locate and observe a spectroscopic probe uniquely and specifically at the active site. As demonstrated by our previous NMR studies (Grisham & Mildvan, 1974, 1975; Grisham et al., 1974; Grisham & Hutton, 1978) and by the present EPR studies, the divalent manganese ion provides an unambiguous active site probe of the $(Na^+ + K^+)$-ATPase. Our kinetic studies provide compelling evidence that the site observed by NMR and EPR is indeed the catalytically important divalent metal binding site (Grisham & Mildvan, 1974).

The manganese binding studies presented here provide a more precise characterization of the single, catalytically involved manganese site on the $(Na^+ + K^+)$-ATPase than was possible previously (Grisham & Mildvan, 1974). The binding studies indicate 1 g-ion of tightly bound Mn^{2+}/250 000 g of protein. This combining weight for Mn^{2+} agrees with estimates of the molecular weight of the ATPase (Jorgensen, 1974b). The greatly reduced number of lipid membrane binding sites for Mn^{2+} in this system (a consequence of the differences in purification procedure) accounts for the improved resolution in the Scatchard analysis compared with previous data.

The data of Figures 1 and 2 suggest that approximately 230 ± 20 phospholipids are required per molecule of enzyme to maintain optimum enzyme activity, but that the stabilizing effects of the membrane bilayer are not related to the maintenance of the single Mn^{2+}-binding site on the enzyme. The Mn^{2+}-binding data for the delipidated preparation follows precisely the calculated Scatchard line for the single tight Mn^{2+}-binding site obtained with the native enzyme preparation.

However, EPR spectra of the enzyme–metal complexes suggest that the removal of some essential phospholipids in the enzyme occurs with conformational change in the vicinity of the metal binding site. Since this metal binding site is located at the active site of the ATPase enzyme, it is to be expected that the conformational change upon delipidation would result in loss of the ATPase activity. In this case, a loss of more than 90% of the activity takes place upon removal of 80% of the essential phospholipids.

In Mn^{2+} EPR spectra of the type observed here, it is important to consider at least two of the possible factors which

Table I: Nuclear and Electron Spin Relaxation Parameters for Mn^{2+}-($Na^+ + K^+$)-ATPase Complexes with Substrates and Activators

complex	nucleus obsd	ϵ^*	$1/fT_{1p} \times 10^{-3}$ (s^{-1})	$\tau_c \times 10^9$ (s)	K_D[g] (μM)	q	r	ref
ATPase-[Mn^{2+}]	^1H (H_2O)	7.8		1.68	0.21	4		*a*
ATPase-Mn^{2+}-[P_i]	^1H (H_2O)	6.0		1.96	4.8	3		*a*
ATPase-Mn^{2+}-[CH_3PO_3]	^1H (H_2O)	6.0		1.88	7.3	3		*a*
ATPase-Mn^{2+}-Tl^+	$^{205}Tl^+$		115	1.68[f]			4.0	*b*
ATPase-Mn^{2+}-Tl^+-P_i	$^{205}Tl^+$		20	1.96[f]			5.4	*b*
ATPase-Mn^{2+}-CH_3PO_3	^{31}P		2.0	1.88[f]			6.9	*c*
ATPase-Mn^{2+}-Li^+	$^7Li^+$		1.2	1.68[f]			7.2	*d, e*
ATPase-Mn^{2+}-Li^+-P_i	$^7Li^+$		1.2	1.96[f]			7.2	*d, e*

[a] Grisham & Mildvan, 1974. [b] Grisham et al., 1974. [c] Grisham & Mildvan, 1975. [d] Grisham, 1978. [e] Grisham & Hutton, 1978. [f] Assumed value from water proton studies. [g] K_D of [ligand] from indicated complex.

contribute to EPR spectral line widths. One is the static distortion of the Mn^{2+} ion geometry induced by ligands which results in inhomogeneous broadening. The other is the transient distortion of the Mn^{2+} complex caused by impact of outer sphere water molecules, which provides the principal mechanism for electron spin relaxation of Mn^{2+} in solution and which is manifested and reflected by the spectral line width. Thus the changes in the Mn^{2+} EPR spectrum on addition of inorganic phosphate could have been anticipated on the basis of the previous PRR studies (Grisham & Mildvan, 1974) which indicate that P_i and methylphosphonate decrease the number of fast exchanging water protons on enzyme-bound Mn^{2+} from 4 to 3. Such an effect could involve a direct ligand substitution of P_i on the Mn^{2+}, or could, on the other hand, involve coordination of P_i to the manganese via an OH^- or *HOH* bridge to form an Mn–O–H–O–P complex. Such an interaction could "freeze" the previously exchangeable water proton on the NMR time scale and manifest itself in a decrease in q, as shown in Table I. ^{31}P NMR studies on the same system (Grisham & Mildvan, 1975) yield a Mn–P distance in the ATPase–Mn^{2+}–$CH_3PO_3^-$ complex of 6.9 Å, which is too large for a direct ligand substitution and favors instead the water bridge coordination mentioned above.

If indeed the coordination of P_i to the Mn^{2+} on the ATPase is through a second sphere complex and if the phosphate is acting mainly to freeze a water proton (or molecule) on the Mn^{2+}, then the effect of P_i on the line width of the Mn^{2+} EPR spectrum in this case might be expected to be relatively minor. This is what is observed in Figure 4. At concentrations which should saturate the tight site for P_i (K_D measured by PRR, Table I), there is virtually no change in the line width of the EPR signal. However, a shift in the position of the low-field signal is observed, which seems to suggest an increase in symmetry of the coordination environment of the bound Mn^{2+} ion. These results show the importance and value of the EPR technique as a tool for detecting changes in the environment around the metal ion, which could not be detected by other techniques. Although the precise manner in which distortions occur cannot be deduced from the EPR spectra, the fact that the presence and possibly the magnitude of such distortions could be inferred from the EPR spectra makes this technique invaluable in characterizing the structure and function of metals in enzymes.

Higher concentrations of P_i cause a significant loss of hyperfine structure for the Mn^{2+} EPR signal. This could reflect binding of P_i to additional sites on the enzyme, perhaps those phosphate sites involved in nucleotide binding. This possibility is consistent with the similar effects on the Mn^{2+} spectrum of low concentrations of ATP, ADP, and AMP–PNP or of high concentrations of phosphate. The substantial differences between the broadening effects of all these phosphate compounds and the narrowing effects of AMP suggest that the β- and/or γ-phosphate liganding portions of the ATP site on the enzyme may bind substrates in a manner which produces transient distortions of the Mn^{2+} coordination geometry and broadens the EPR spectrum. On the other hand, the sharpening of the hyperfine lines of the spectrum and the sharpening and disappearance of the broad low-field component of the spectrum induced by AMP indicate an opposite change in ligand geometry around the Mn^{2+} compared with those induced by ATP, ADP, AMP–PNP, and phosphate. The narrowing of the spectral lines resulting from the interaction of AMP with the binary ATPase–Mn^{2+} complex may reflect an increase in the electron spin relaxation time of Mn^{2+}. Since the principal mechanism for electron spin relaxation has been ascribed to distortion of the Mn^{2+} octahedral complex by outer sphere solvent molecules (Bloembergen & Morgan, 1961; Reed et al., 1971), it is possible that the collision frequency of solvent water molecules with the bound Mn^{2+} coordination complex is reduced for the enzyme–Mn^{2+}–AMP complex.

The presence of significant distortions of the complex is suggested by preliminary 35-GHz EPR spectra of the binary enzyme–Mn^{2+} complex. Forbidden nuclear transitions of considerable amplitude are seen in the spectrum. A value of D (in the spin Hamiltonian) of approximately 800 G is obtained from the spectrum by assuming a purely axial distortion of the complex. This value is not consistent with the position of the low-field signal seen in the X-band spectrum. These results lead us to believe that significant rhombic distortions, instead of purely axial distortions, exist in the binary enzyme–Mn^{2+} complex.

The Mn^{2+} EPR spectrum of the binary Mn^{2+}–ATPase complex is more asymmetric than that obtained for many other protein systems (Reed & Cohn, 1973; Villafranca et al., 1976). On the other hand, the Mn^{2+} spectra of the ($Na^+ + K^+$)-ATPase are similar to the asymmetric spectra obtained for pyruvate carboxylase (Reed & Scrutton, 1974), phosphoglucomutase (Reed & Ray, 1971), and the inorganic complexes of manganese bis(iminodiacetic acid) and manganese–EDTA. These latter, model complexes display low-field asymmetries not unlike that of the ATPase.

This is the first evidence of any kind relating the Mn^{2+} and ATP sites on the enzyme, and the suggestion from the data is that ATP and its analogues are not strongly perturbing the electronic environment of the Mn^{2+} under the conditions of the experiment. It should be mentioned, however, that this does not strictly rule out close approach of ATP or its analogues to the Mn^{2+} site. Questions of this type will probably be best answered by NMR studies of the nuclei of substrate analogues in solutions of the ATPase and Mn^{2+}, or by EPR studies of Mn^{2+}–ATPase complexes with paramagnetic substrates such as CrATP. Experiments in both these areas are underway in our laboratory. In any case these spectra of ternary enzyme–Mn^{2+}–nucleotide complexes provide strong

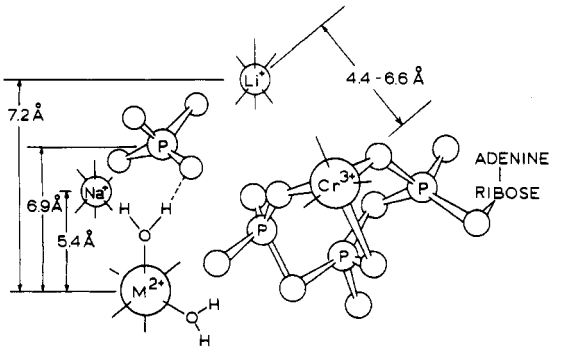

FIGURE 10: Active site structure of $(Na^+ + K^+)$-ATPase as determined by ^1H, $^{205}Tl^+$, ^{31}P, and $^7Li^+$ NMR, Mn^{2+} EPR, and kinetic studies.

evidence that the true substrate for the ATPase is ATP and not M^{2+}–ATP, since the spectra of these ternary complexes bear no resemblance to that of MnATP and MnADP. For enzymes which utilize M^{2+}–ATP as the substrate and which form enzyme–ATP–M^{2+} bridge complexes, the EPR spectrum of the ternary complex is usually identical (or nearly identical) with that of the binary Mn^{2+}–nucleotide complex (Buttlaire & Cohn, 1974; Reed & Cohn, 1972). Our EPR results alone would not rule out the possibility that the enzyme-bound Mn^{2+} interacts directly with the nucleotides or that a second divalent cation may interact with the nucleotide. However, we have shown that binding of Mn^{2+} to the single tight site on the enzyme is sufficient for full ATPase activity (Grisham & Mildvan, 1974), eliminating any need for a second divalent cation. This, together with the EPR spectra shown here for Mn^{2+} at the tight site, would indicate that ATP alone is a sufficient substrate for the $(Na^+ + K^+)$-ATPase.

The data presented here can be combined with our previous NMR studies to form a picture of the active site of the $(Na^+ + K^+)$-ATPase as it is currently understood. In addition to the water proton and ^{31}P NMR studies discussed above, $^{205}Tl^+$ and $^7Li^+$ NMR experiments have demonstrated the existence of both Na^+ and K^+ sites near the Mn^{2+} site on the enzyme (Grisham et al., 1974; Grisham & Hutton, 1978). Interactions between Li^+ at the K^+ site and enzyme-bound, paramagnetic CrATP have also been observed by $^7Li^+$ NMR (Grisham & Hutton, 1978). Figure 10 shows the structural information currently available on the active site of the enzyme. Molecular models constructed with typical Mn–OH_2, P–O, O–H, and Na^+–O bond lengths indicate that the Mn^{2+}–P and Mn^{2+}–Na^+ distances from NMR are consistent with the complex shown here. In a survey of several phosphoryl- and nucleotidyl-transferring enzymes, including phosphoglucomutase, pyruvate kinase, and DNA polymerase I, Mildvan (1977) has suggested Mn^{2+}–P second sphere complexes (such as that shown in Figure 10) as a general mode of activating phosphoryl groups for nucleophilic attack in the phosphoryl transfer reaction. To distinguish between this and other possible mechanisms, it will be important to determine the structure and location of ATP itself on the ATPase. Toward this end, we have explored the substitution-inert Cr^{3+} and Co^{3+} complexes of ATP as substrate analogue probes of the $(Na^+ + K^+)$-ATPase and have already established that CrATP and Mn^{2+} can bind simultaneously and proximally at the active site of this enzyme. This work will be the subject of future communications.

Acknowledgments

We wish to thank Sherry Sutherland and Jannequin Bennett for excellent technical assistance with the enzyme preparations and Susan Pilch and Patricia Radloff for assistance with the Mn^{2+}-binding and phospholipid activation studies.

References

Abragam, A., & Bleaney, B. (1970) *Electron Paramagnetic Resonance of Transition Ions*, p 491, Oxford University Press, London.
Barnett, R. E. (1970) *Biochemistry 9*, 4644.
Bartlett, G. (1959) *J. Biol. Chem. 234*, 466.
Bloembergen, N., & Morgan, L. (1961) *J. Chem. Phys. 34*, 842.
Buttlaire, D., & Cohn, M. (1974) *J. Biol. Chem. 249*, 5741.
Cantley, L., Gelles, J., & Josephson, L. (1978) *Biochemistry 17*, 418.
Chen, P., Toribara, T., & Warner, H. (1956) *Anal. Chem. 28*, 1756.
Cohn, M., & Townsend, J. (1954) *Nature (London) 173*, 1090.
DePont, J., Schoot, B., Van Prooijen-Van Eeden, A., & Bonting, S. (1977) *Biochim. Biophys. Acta 482*, 213.
Deranleau, D. (1969) *J. Am. Chem. Soc. 91*, 4044, 4050.
Froelich, J., Albers, R., Koval, G., Goebel, R., & Berman, M. (1976) *J. Biol. Chem. 251*, 2186.
Grisham, C. M. (1978) in *Biomolecular Structure and Function*, p 385, Academic Press, New York.
Grisham, C. M., & Barnett, R. E. (1972) *Biochim. Biophys. Acta 266*, 613.
Grisham, C. M., & Barnett, R. E. (1973a) *Biochim. Biophys. Acta 311*, 417.
Grisham, C. M., & Barnett, R. E. (1973b) *Biochemistry 12*, 2635.
Grisham, C. M., & Mildvan, A. S. (1974) *J. Biol. Chem. 249*, 3187.
Grisham, C. M., & Mildvan, A. S. (1975) *J. Supramol. Struct. 3*, 304.
Grisham, C. M., & Hutton, W. C. (1978) *Biochem. Biophys. Res. Commun. 81*, 1406.
Grisham, C. M., Gupta, R. K., Barnett, R. E., & Mildvan, A. S. (1974) *J. Biol. Chem. 249*, 6738.
Hokin, L., & Dahl, J. (1972) in *Metabolic Pathways, VI Metabolic Transport* (Hokin, L. E., Ed.) p 269, Academic Press, New York.
Hudson, A., & Luckhurst, G. (1969a) *Chem. Rev. 69*, 191.
Hudson, A., & Luckhurst, G. (1969b) *Mol. Phys. 16*, 395.
Jorgensen, P. (1974a) *Biochim. Biophys. Acta 356*, 36.
Jorgensen, P. (1974b) *Methods Enzymol. 32B*, 277.
Kuriki, Y., Halsey, J., Biltonen, R., & Racker, E. (1976) *Biochemistry 15*, 4956.
Lowry, O., Rosebrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193*, 265.
Mildvan, A. (1977) *Acc. Chem. Res. 10*, 246.
Miziorko, H. (1974) Ph.D. Thesis, University of Pennsylvania.
Post, R., & Kume, S. (1973) *J. Biol. Chem. 248*, 6993.
Reed, G. H., & Cohn, M. (1970) *J. Biol. Chem. 245*, 662.
Reed, G. H., & Ray, W. J., Jr. (1971) *Biochemistry 10*, 3190.
Reed, G. H., & Cohn, M. (1972) *J. Biol. Chem. 247*, 3073.
Reed, G. H., & Cohn, M. (1973) *J. Biol. Chem. 248*, 6430.
Reed, G., & Scrutton, M. (1974) *J. Biol. Chem. 249*, 6156.
Reed, G. H., Leigh, J. S., Jr., & Pearson, J. E. (1971) *J. Chem. Phys. 55*, 3311.
Villafranca, J., Ash, D., & Wedler, F. (1976) *Biochemistry 15*, 544.
Warren, G., Toon, P., Birdsall, N., Lee, A., & Metcalfe, J. (1974) *Biochemistry 13*, 5501.
Warren, G., Houslay, M., Metcalfe, J., & Birdsall, N. (1975) *Nature (London) 255*, 684.
Yount, R. (1977) *Adv. Enzymol. 37*, 1.
Yount, R., Babcock, D., Ballantyne, W., & Ojala, D. (1971) *Biochemistry 10*, 2484.