Phosphorus-31 Nuclear Magnetic Resonance Studies of the Conformation of an Adenosine 5′-Triphosphate Analogue at the Active Site of (Na^+ + K^+)-ATPase from Kidney Medulla†

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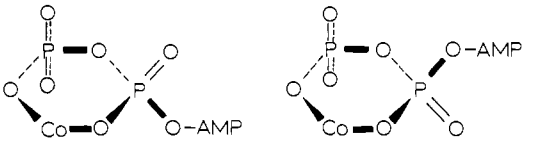
ABSTRACT: It has previously been shown that there are two sites for divalent metals at the active site of kidney (Na^+ + K^+)-ATPase, one bound directly to the enzyme and one coordinated to the ATP substrate [Grisham, C. (1981) *J. Inorg. Biochem. 14*, 45; O'Connor, S., & Grisham, C. (1980) *FEBS Lett. 118*, 303]. The conformation of the metal–nucleotide complex has been studied by using β,γ-bidentate Co(NH_3)$_4$ATP, a substitution-inert analogue of MgATP. Kinetic studies show that Co(NH_3)$_4$ATP is a competitive inhibitor with respect to MnATP for the (Na^+ + K^+)-ATPase. The K_i values under both high- and low-affinity conditions (K_i = 10 μM and K_i = 1.6 mM, respectively) are similar to the K_m values for MnATP under the same conditions (2.88 μM and 0.902 mM). From the paramagnetic effect of Mn^{2+} bound to the ATPase on the longitudinal relaxation rates of the phosphorus nuclei of Co(NH_3)$_4$ATP at the substrate site (at 40.5 and 145.75 MHz), Mn–P distances to all three phosphates are determined. The distances are consistent with the formation of a second sphere coordination complex on the enzyme between Mn^{2+} and the phosphates of Co(NH_3)$_4$ATP. In this respect, kidney (Na^+ + K^+)-ATPase appears to be similar to pyruvate kinase [Sloan, D., & Mildvan, A. (1976) *J. Biol. Chem. 251*, 2412] and phosphoribosylpyrophosphate synthetase [Granot, J., Gibson, K., Switzer, R., & Mildvan, A. (1980) *J. Biol. Chem. 255*, 10931]. Roles for both of the active site divalent cations are discussed.

Sodium and potassium ion activated adenosinetriphosphatase, or (Na^+ + K^+)-ATPase[1] (EC 3.6.1.3, ATP phosphohydrolase), is responsible for the active transport of sodium and potassium ions in animal cells. This transport is driven by the hydrolysis of ATP, and thus it is of interest to determine the mechanism of the energy coupling in this system. Our approach to this problem has been to attempt to determine the active site structure of the enzyme and then to evaluate possible energy coupling mechanisms within the context of an established structure. To this end, we have identified and characterized binding sites for sodium and potassium using $^{205}Tl^+$ and $^7Li^+$ NMR (Grisham et al., 1974; Grisham & Hutton, 1978) and a phosphate site by ^{31}P NMR (Grisham & Mildvan, 1975). We have characterized the existence and catalytic efficacy of a single divalent cation site on the enzyme in the absence of ATP and located this site with respect to the above-mentioned sodium, potassium, and phosphate sites (Grisham & Mildvan, 1974; O'Connor & Grisham, 1979).

Recently, we have shown that the β,γ-bidentate complexes of substitution inert Co(III) and Cr(III) and ATP can be effective analogues of Mg^{2+}–ATP with this and other ATPases (O'Connor & Grisham, 1980; Grisham, 1981; Gantzer et al., 1982). A unique consequence of the use of these analogues is the fact that the tight metal-coordinating site on the ATP substrate is completely occupied and, at the same time, any other metal binding sites on the enzyme are left empty and can then be filled selectively by the addition of another suitable metal. Taking advantage of this situation, we have recently demonstrated that (a) there are two divalent cation sites at the active site of the (Na^+ + K^+)-ATPase (Grisham, 1981) and (b) these two sites are 8.1 ± 0.5 Å apart, as measured by Mn^{2+} EPR (O'Connor & Grisham, 1980) and water ^1H NMR measurements (A. O'Neal and C. Grisham, unpublished data). In these studies, the Co(III) or Cr(III) of the metal–ATP analogues occupies the Mg^{2+} site on the ATP substrate, while Mn^{2+} is bound to a Mg^{2+} site on the enzyme that is involved in the activation of the ATPase (Grisham & Mildvan, 1974; O'Connor & Grisham, 1979; Robinson, 1981). This latter site binds Mn^{2+} with a very high affinity (K_D = 0.2–0.3 μM) and exists in the absence of the ATP substrate. We have previously characterized the locations of Na^+, K^+, and phosphate sites with respect to these two divalent metal sites (Grisham et al., 1974; Grisham & Mildvan, 1975; Grisham & Hutton, 1978).

Chart I



Δ-Co(NH_3)$_4$ATP Λ-Co(NH_3)$_4$ATP

In the present work, ^{31}P nuclear relaxation measurements of Co(NH_3)$_4$ATP (the diastereomeric structures of which are shown in Chart I) are made in the presence of Mn^{2+} and of the purified (Na^+ + K^+)-ATPase. The distances from Mn^{2+} at the activating metal site to Co(NH_3)$_4$ATP at the substrate site are used to clarify the relative positions of the two metal ions and the α-, β-, and γ-phosphorus atoms of the bound nucleotide substrate.

Experimental Procedures

Materials. The (Na^+ + K^+)-ATPase was purified from the outer medulla of sheep kidney as previously described (O'Connor & Grisham, 1979). The β,γ-bidentate complex of

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[1] Abbreviations: (Na^+ + K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase; CoATP, β,γ-bidentate Co(NH_3)$_4$ATP; TMA, tetramethylammonium; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

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$Co(NH_3)_4ATP$ was prepared as described by Cornelius et al. (1977).

Kinetic Studies. The ability of the substrate analogue $Co(NH_3)_4ATP$ to compete with Mn^{2+}–ATP at the ATP site of the $(Na^+ + K^+)$-ATPase was examined with a ^{32}P assay procedure described previously (Gantzer & Grisham, 1979). In these studies, Mn^{2+} was used as the divalent cation at a concentration equal to that of ATP + 0.5 mM, to assure that all of the ATP present was in the form of $Mn^{2+}ATP$. The analogue was added to the assay mixture immediately prior to the addition of enzyme. The CoATP studies were conducted in 20 mM Tes–TMA, pH 7.5, at 25 °C, with the addition of 100 mM NaCl and 10 mM KCl for the $(Na^+ + K^+)$-ATPase studies. In these kinetic studies, since the hydrolysis of MnATP is monitored by measuring the liberation of $^{32}P_i$ from Mn[γ-^{32}P]ATP, CoATP and CrATP will be observed to function as inhibitors. These analogues do, however, show substrate activity with these ATPases.

The effect of incubation of the ATPase with $Co(NH_3)_4ATP$ or the analogous $Cr(H_2O)_4ATP$ was examined with an assay that couples the hydrolysis of ATP to the oxidation of NADH (Grisham & Mildvan, 1974). Incubation solutions contained 83 mM imidazole–HCl, pH 7.25, 18% (w/w) sucrose, and 0.19 mM CrATP or 0.167 mM CoATP. At suitable times, aliquots were withdrawn and assayed.

NMR Measurements. Phosphorus-31 NMR spectra and longitudinal relaxation times (T_1) were obtained at 145.9 MHz on a Nicolet Magnetics Corp. NT-360/Oxford spectrometer equipped with a 1280/293B data system. The spectra were measured at 4 °C [to prevent breakdown of $Co(NH_3)_4ATP$] with a 10-mm broad-band probe with an internal ^2H lock (20% D_2O, total sample volume of 2.4 mL). Uninterrupted, incoherent proton decoupling at low levels (less than or equal to 1 W) was employed. The 90° pulse width at 145 MHz was 28–35 μs. Chemical shifts were referenced to 85% phosphoric acid. Spectra at 40.5 MHz were obtained on a JEOL PS-100 P/EC-100 spectrometer as previously described (Stephens & Grisham, 1979). T_1 values were determined with a 180–τ–90 pulse sequence (Carr & Purcell, 1954), with a delay between consecutive pulse sequences equal to at least 5 times T_1. After extended NMR measurements (4–5 h), the enzyme was found to retain more than half its activity, and no systematic changes were observed during the NMR measurements.

Theoretical Basis for Calculations. The calculations in this paper are based on the theory of Solomon and Bloembergen (Solomon, 1955; Solomon & Bloembergen, 1956; Bloembergen, 1957; Bernheim et al., 1959) for the paramagnetic effects on the nuclear spin relaxation rates of a magnetic nucleus that is bound near a paramagnetic species. The practical application of the theory to structural studies of biomolecules has been reviewed (Mildvan & Engle, 1972). In the limit of fast exchange, the equation that describes the Mn^{2+}–P dipolar interaction is

$$[CoATP]/([Mn^{2+}]T_{1_p}) = (601/r)^6 q f(\tau_c)$$

where

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

and where p is the number of P nuclei interacting with each paramagnet, r is the metal–nucleus distance, τ_c is the dipolar correlation time, and ω_I and ω_S are the nuclear and electronic Larmor frequencies, respectively.

In order to use the above equations to calculate active site distances on an enzyme such as the $(Na^+ + K^+)$-ATPase, it is necessary to make an estimate of the correlation time, τ_c. If, as is the case for a number of Mn^{2+}–nucleus interactions on the $(Na^+ + K^+)$-ATPase (Grisham, 1979), the correlation time is dominated by the electron-spin relaxation time T_{1_e} of the Mn^{2+} ion, then it is given by (Bloembergen & Morgan, 1961)

$$\frac{1}{T_{1_e}} = B\left[\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right] \quad (1)$$

where B is a constant containing the electronic spin S and the zero-field splitting of Mn^{2+} and τ_v is a time constant for transient symmetry distortions of the Mn^{2+} complex. This correlation time has two limiting cases. For case 1

$$\omega_s^2\tau_v^2 \gg 1$$

In this case, eq 1 reduces to $\tau_s = \omega_s^2\tau_v/(2B)$, and the ratio of $1/(fT_{1_p})$ at two frequencies (ν_1 and ν_2) is given by

$$\frac{1/[fT_{1_p}(\nu_1)]}{1/[fT_{1_p}(\nu_2)]} = \frac{1 + 4\pi^2\nu_2^2(\nu_2/\nu_1)^4\tau_s^2}{(\nu_2/\nu_1)^2[1 + 4\pi^2\nu_1^2\tau_s^2]} \quad (2)$$

In this case, T_{1_e} displays maximum frequency dependence, and $\tau_s = T_{1_e}$ at ν_1.

For case 2

$$\omega_s^2\tau_v^2 \ll 1$$

As can be seen by consideration of eq 1, $T_{1_e} = 1/(5B\tau_v)$ for this case and is independent of frequency. For the ratio of $1/(fT_{1_p})$ at two frequencies, we obtain

$$\frac{1/[fT_{1_p}(\nu_1)]}{1/[fT_{1_p}(\nu_2)]} = \frac{1 + 4\pi^2\nu_2^2\tau_s^2}{1 + 4\pi^2\nu_1^2\tau_s^2} \quad (3)$$

Thus when T_{1_e} dominates τ_c, eq 2 and 3 can be used together with measurements of $1/(fT_{1_p})$ at two frequencies to estimate τ_c.

Results

Steady-State Kinetics of Competition between $Co(NH_3)_4ATP$ and MnATP. The kinetic studies characterizing the effects of $Co(NH_3)_4ATP$ on the hydrolysis of MnATP by the $(Na^+ + K^+)$-ATPase were plotted in the form of Dixon plots. This type of plot is useful for identifying the type of inhibition and for determining appropriate K_i values. In the case of linear competitive inhibition, the family of lines (where each line, in this case, is at a fixed MnATP concentration) in the Dixon plot intersects above the [I] axis in the second quadrant at [I] = $-K_i$. Replotting the y intercepts for each line in the form of a Lineweaver–Burk plot then allows determinations of the apparent K_m for MnATP under the same experimental conditions, where the inhibitor concentration is extrapolated to zero.

$Co(NH_3)_4ATP$ proved to be a linear competitive inhibitor with respect to MnATP in the presence of both high and low levels of MnATP.[2] At low MnATP, where the high-affinity form of the ATP site predominates, a K_i for CoATP of 10 μM was obtained (Figure 1A). From the secondary plot of the

[2] The $(Na^+ + K^+)$-ATPase typically exhibits biphasic response to ATP and ATP analogues. However, recent results from titrations of the ATPase with fluorescent ATP analogues have provided strong evidence that, although the affinity of the site for ATP changes remarkably under different circumstances, there is only one ATP site on each minimum asymmetric unit of the $(Na^+ + K^+)$-ATPase and this site is the active site (Moczydlowski & Fortes, 1981).

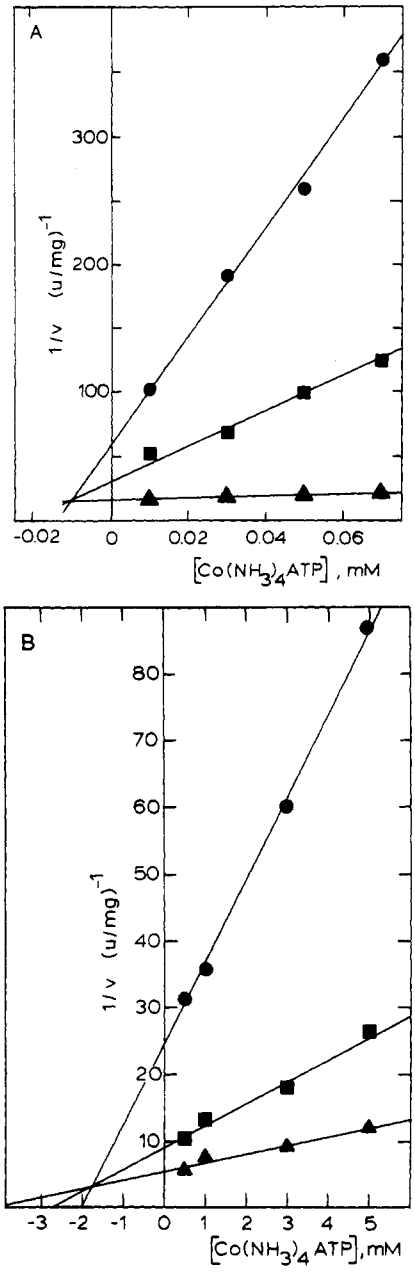

FIGURE 1: (A) Dixon plot of CoATP inhibition of MnATP hydrolysis at the high-affinity substrate site of the $(Na^+ + K^+)$-ATPase. Conditions and methods are as described in text. The ATP concentrations were as follows: (●) 1 μM; (■) 3 μM; (▲) 30 μM. The measured K_i was 10 μM. (B) Dixon plot of CoATP inhibition of MnATP hydrolysis at the low-affinity substrate site of the $(Na^+ + K^+)$-ATPase. Conditions and methods are as described in text. The ATP concentrations were as follows: (●) 0.2 mM; (■) 1.0 mM; (▲) 3.0 mM. The K_i was determined to be 1.6 mM.

y intercepts, an apparent K_m for MnATP of 2.88 μM was obtained under the same experimental conditions. At high levels of MnATP, where the low-affinity form of the ATP site predominates, the K_i for CoATP was found to be 1.6 mM (Figure 1B), while the apparent K_m for MnATP was 0.902 mM. Thus, under both conditions, the binding of Co$(NH_3)_4$ATP appears to be slightly weaker than that of MnATP. Similar results have been obtained with bidentate $Cr(H_2O)_4$ATP (Gantzer et al., 1982).

Slow Inactivation of $(Na^+ + K^+)$-ATPase by CrATP but Not CoATP. Recently, Pauls et al. (1980) demonstrated that CrATP causes a slow inactivation of the $(Na^+ + K^+)$-ATPase from beef brain and pig kidney. Their studies are complementary to the steady-state kinetic studies described in this

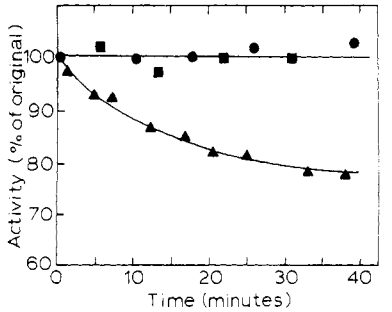

FIGURE 2: Effects of incubation with β,γ-bidentate $Cr(H_2O)_4$ATP and β,γ-bidentate Co$(NH_3)_4$ATP on sheep kidney $(Na^+ + K^+)$-ATPase. Incubation solutions contained 83 mM imidazole–HCl, pH 7.25, 18% (w/w) sucrose, and (▲) 0.19 mM CrATP, (■) 0.18 mM CoATP, or (●) no other additions. The enzyme concentration was 0.4 mg/mL in all incubations. At the times shown, aliquots were withdrawn and assayed as previously described (Grisham & Mildvan, 1974). The temperature was 37 °C in both incubation and assay.

paper and elsewhere (Gantzer et al., 1982). Thus the K_D for the enzyme–CrATP complex measured in the presence of 3 mM KCl by Pauls et al. (0.33 mM) agrees well with the K_i values reported for the $(Na^+ + K^+)$-ATPase (Gantzer et al., 1982). It should be noted here that at the temperature employed for the steady-state kinetics, and over the time required for the enzyme assays (0.5–10 min), no significant inactivation of the $(Na^+ + K^+)$-ATPase was observed. On the other hand, as shown in Figure 2, sheep kidney $(Na^+ + K^+)$-ATPase is inactivated by extended exposure to CrATP at 37 °C, in a manner similar to that described by Pauls et al. for the beef brain and pig kidney enzymes. For obvious reasons, it was of interest to us to see if Co$(NH_3)_4$ATP could likewise inactivate the $(Na^+ + K^+)$-ATPase. As shown in Figure 2, the Co(III) analogue causes no detectable inactivation after a 40-min incubation at 37 °C. Beyond this point, spontaneous breakdown of Co$(NH_3)_4$ATP releases significant amounts of Co(III), which causes some inactivation of the ATPase. This latter process has previously been characterized in our laboratory (McClaugherty, 1981).

NMR Studies of Ternary Mn^{2+}–ATPase–Co$(NH_3)_4$ATP Complex. ^{31}P relaxation rates of Co$(NH_3)_4$ATP were measured in solutions of Mn^{2+} (or Mg^{2+}) and $(Na^+ + K^+)$-ATPase at 145 and 40 MHz. The ^{31}P spectrum of Co$(NH_3)_4$ATP at these two frequencies is shown in Figure 3. At 40 MHz the α-P and β-P resonances overlap, whereas at 145 MHz, these two multiplets are resolved. The spectrum consists of superimposed resonances for both the Δ and Λ diastereomers of Co$(NH_3)_4$ATP. The correct assignments of the resonances of the diastereomers in Figure 3B were determined by using two-dimensional homonuclear chemical shift correlation maps obtained with a Jeener pulse sequence (Jeener, 1977; J. VanDivender and C. Grisham, unpublished results). The assignments of the β-P resonances are different from those first proposed by Granot et al. (1979), which were based on simulations by Cornelius et al. (1977). The $(Na^+ + K^+)$-ATPase has been shown to hydrolyze the Δ isomers of metal–nucleotide complexes and not the Λ isomers (Gantzer et al., 1982), although both isomers will bind to the enzyme.

The presence of the $(Na^+ + K^+)$-ATPase causes a 6–10-fold enhancement of the effect of Mn^{2+} on the ^{31}P relaxation rates of Co$(NH_3)_4$ATP (Figure 4; J. VanDivender and C. Grisham, unpublished results), establishing the formation of a ternary Mn^{2+}–ATPase–Co$(NH_3)_4$ATP complex. The dissociation constant for Mn^{2+} ion from the $(Na^+ + K^+)$-ATPase, determined by Mn^{2+} EPR (O'Connor & Grisham, 1979) is 0.2 μM. Given a dissociation constant for the binary Mn^{2+}–Co-

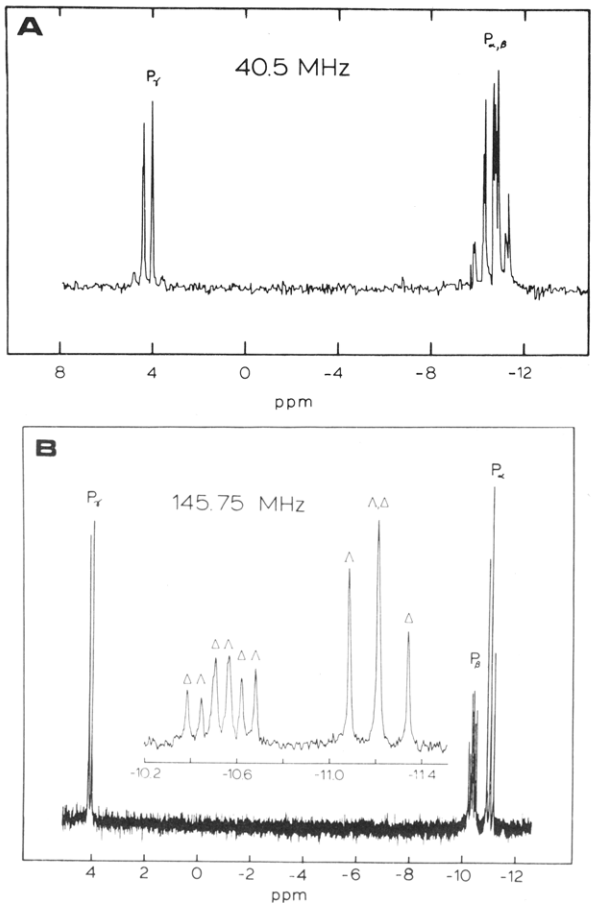

FIGURE 3: Phosphorus-31 nuclear magnetic resonance spectra of $Co(NH_3)_4ATP$ at 40.5 (A) and 145.75 (B) MHz at pH 7.5, 4 °C. The inset in (B) shows an expanded view of the α- and β-P resonances, with the assignments determined by J. VanDivender and C. Grisham (unpublished results).

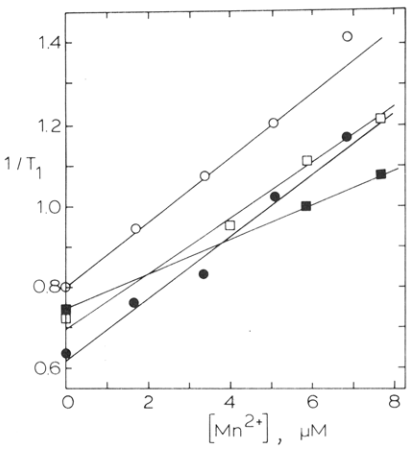

FIGURE 4: Effect of Mn^{2+} on longitudinal relaxation rates of phosphorus nuclei of $Co(NH_3)_4ATP$ in the presence of $(Na^+ + K^+)$-ATPase. The solutions contained either 20 or 25 mM β,γ-bidentate $Co(NH_3)_4ATP$, 82 mM Tris, pH 7.4, 100 mM NaCl, 10 mM KCl, and 8–10 μM enzyme. The solutions also contained 20% (v/v) D_2O for field/frequency locking. Data for the γ P (●), the β P (○), the α P (Δ) (□), and the α P (Λ) (■) are shown.

Table I: Paramagnetic Contributions to Relaxation Rates at 145.75 MHz for Phosphorus Nuclei of $Co(NH_3)_4ATP$ and Mn^{2+}–P Distances in the ATPase-Bound Mn^{2+}–$Co(NH_3)_4ATP$ Complex

P atom	$T_{1M}^{-1} \times 10^{-3}$ (s^{-1})	$1/(fT_{2p}) \times 10^{-4}$ (s^{-1})	r^a (Å)	r^b (Å)	r^c (Å)	r^d (Å)
P_α (Δ)	0.82 ± 0.06	5.0 ± 0.8	6.6	8.3	7.5	5.0
P_α (Λ)	0.50 ± 0.04	4.8 ± 0.7	7.2	9.0	8.1	5.5
P_β (Δ, Λ)	1.67 ± 0.10	23.4 ± 3.0	5.9	7.4	6.7	4.5
P_γ (Δ, Λ)	1.67 ± 0.08	7.0 ± 0.5	5.9	7.4	6.7	4.5

[a] Calculated using a correlation time ($\tau_c = 1.9 \times 10^{-9}$ s) obtained from the frequency dependence of H_2O proton relaxation (Grisham & Mildvan, 1974). As stated in the text, the absolute errors in r in this table may be ±20%, but the relative errors within a given experiment may be considered to be less than 1–2%. [b] Calculated using a correlation time ($\tau_c = 7.19 \times 10^{-9}$ s) obtained from comparisons of $^6Li^+$ and $^7Li^+$ relaxation data (S. Bainbridge and C. Grisham, unpublished results). [c] Calculated under assumption of maximum frequency dependence of the correlation time with $\tau_c = 4.0 \times 10^{-9}$ s. [d] Calculated under assumption of no frequency dependence of the correlation time with $\tau_c = 4 \times 10^{-10}$ s.

$(NH_3)_4ATP$ complex of 15 mM (Armstrong et al., 1979), it is easy to show that, under the conditions of these experiments [8–10 μM $(Na^+ + K^+)$-ATPase[3] and 20–30 mM $Co(NH_3)_4ATP$], at least 97% of the added Mn^{2+} was enzyme bound. Thus no corrections must be made for the small contribution of the binary complex to the measured paramagnetic relaxation rates ($T_{1_p}^{-1}$). Also, addition of saturating levels of diamagnetic Mg^{2+} ion (8.33 mM) produced no measurable increase in the relaxation rates of the phosphorus nuclei of $Co(NH_3)_4ATP$, indicating that the effects of added Mn^{2+} are due entirely to electron–nuclear dipolar interactions.

Determination of Correlation Times and Distances in the Ternary Complex. In order to use the data of Figure 4 to calculate the distances between Mn^{2+} and the phosphorus nuclei of ATP in the ternary Mn^{2+}–ATPase–$Co(NH_3)_4ATP$ complex, it was necessary to determine the correlation time (τ_c) for the dipolar effects on the ^{31}P relaxation. This has been done for the case at hand in several ways. (a) From the frequency dependence of water proton relaxation in the presence of the enzyme–Mn^{2+} complex (both in the presence and absence of ATP analogues), dipolar correlation times of $(1.9 \pm 0.1) \times 10^{-9}$ s have been measured (Grisham & Mildvan, 1974; S. McClaugherty and C. Grisham, unpublished data). In these studies it was determined that τ_c is dominated by τ_s, the electron-spin relaxation time of Mn^{2+}. If this is the case for the water protons of the Mn^{2+}–enzyme and Mn^{2+}–enzyme–$Co(NH_3)_4ATP$ complexes, it must also be the case that τ_s is the dominant term in the determination of τ_c for the ^{31}P–Mn^{2+} interaction of interest here. (b) We have measured values for T_{1_p} (T_{1_M}) for $^6Li^+$ and $^7Li^+$ complexes with the $(Na^+ + K^+)$-ATPase and Mn^{2+} (S. Bainbridge and C. Grisham, unpublished results) at the same magnetic field strength as that used in the 145.8-MHz ^{31}P studies described here. As Raushel & Villafranca (1980a,b) have shown, such measurements can be used to calculate a value of τ_c for the interaction of interest. A τ_c for the Li^+–Mn^{2+} pair on the ATPase of 7.19×10^{-9} s was calculated in this way. Using the same argument as in (a) above, we can use this value as an appropriate estimate of τ_c in the ^{31}P–Mn^{2+} interaction. (c) The paramagnetic effect of Mn^{2+} on the longitudinal relaxation rates of ^{31}P of $Co(NH_3)_4ATP$ was measured at two frequencies: 40.5 and 145.8 MHz. Within the experimental error, no frequency dependence of the relaxation rate was observed.

[3] The molar concentration of the ATPase was calculated by (a) determining the protein concentrations of the enzyme samples by the method of Lowry et al. (1951) with bovine serum albumin as a standard, (b) applying the correction to this method determined by Craig & Kyte (1980), (c) assuming that the molecular weights of the ATPase polypeptides are 121 000 (α) and 49 000 (β) (Craig & Kyte, 1980; Peters et al., 1981), and (d) correcting for the presence in our preparations of a 30 000-dalton peptide that accounts for 7–9% of the total protein (Grisham & Mildvan, 1975).

Given the assumption of a 10% error in the measurement of the ratio of $1/(fT_{1_p})$ at two frequencies, we may assume that a ratio of $(1/[fT_{1_p}(\nu_1)])/(1/[fT_{1_p}(\nu_2)])$ of 0.9 could have gone undetected. If this is the case, the assumption that τ_c is frequency independent gives a value for τ_c of 4×10^{-10} s (eq 3). On the other hand, the assumption of a maximum frequency dependence of τ_c (eq 2) gives $\tau_c = 4.0 \times 10^{-9}$ s at 145.75 MHz.

Distances from the bound Mn^{2+} to the three phosphorus atoms of $Co(NH_3)_4ATP$ were calculated with the four values of τ_c discussed above. As shown in Table I, the Mn^{2+}–P distances are the same for the γ and β phosphorus atoms of $Co(NH_3)_4ATP$ on the enzyme. While the spectra of the β P for the Δ and Λ diastereomers of $Co(NH_3)_4ATP$ are not resolved and while the β-P resonances broaden upon the addition of Mn^{2+} and thus prevent the quantitative analysis of the β-P resonances, it should be noted that no systematic qualitative differences in the β-P resonances of the two diastereomers were detected. On the other hand, the Mn^{2+}–(α-P) distance for the Λ isomer is significantly longer than that of the Δ isomer. (It should perhaps be pointed out that while the absolute errors in the average distances, as given in Table I, may be ±20%, the relative errors within a given experiment may be considered to be less than 1–2%.) Thus the difference in the α-P distances for the two isomers should be considered significant. Equal affinities by the enzyme for the two diastereomers of $Co(NH_3)_4ATP$ were assumed in these calculations. The difference in $1/T_{1_M}$ at the α P could also be the result of different affinities of the enzyme for the two diastereomers of $Co(NH_3)_4ATP$.

Also compared in Table I are the paramagnetic contributions to the longitudinal and transverse relaxation rates of the phosphorus nuclei. The largest value of the transverse relaxation rate in Table I (23.4×10^4 s^{-1}) sets a lower limit on $1/\tau_M$ that is much greater than the longitudinal relaxation rates. Thus the fast-exchange assumption and the calculation of distances from the values of $1/T_{1_M}$ are justified.

Discussion

The data presented in this paper establish that the phosphorus nuclei of an ATP analogue are bound to the enzyme near the high-affinity divalent metal ion site that we have previously characterized (Grisham & Mildvan, 1974; O'Connor & Grisham, 1979, 1980). We have established the existence of binding sites for sodium, potassium, phosphate, and $Cr(H_2O)_4ATP$ near this site, using Mn^{2+} as a paramagnetic analogue of Mg^{2+} (Grisham, 1979). Thus, a Na^+ transport site has been identified by Tl^+ NMR 5.4 Å from the Mn^{2+} site (Grisham et al., 1974), and a K^+ site of undetermined function has been observed 7.2 Å from the Mn^{2+} site by $^7Li^+$ NMR (Grisham & Hutton, 1978). Using the dipolar interaction between Cr^{3+} and Mn^{2+}, as reflected in Mn^{2+} EPR spectra, we had measured a Mn^{2+}–Cr^{3+} distance in the ATPase–Mn^{2+}–$Cr(H_2O)_4ATP$ complex of 8.1 Å (O'Connor & Grisham, 1980). The structural similarities between paramagnetic $Cr(H_2O)_4ATP$ and diamagnetic $Co(NH_3)_4ATP$, as well as the similar interactions of these two analogues with the $(Na^+ + K^+)$-ATPase (Gantzer et al., 1982), permit a comparison between the Mn^{2+} EPR studies with $Cr(H_2O)_4ATP$ and the present ^{31}P NMR results with $Co(NH_3)_4ATP$. As shown in Figure 5, the present results place the phosphorus atoms of $Co(NH_3)_4ATP$ approximately between the enzyme-bound Mn^{2+} ion and the ATP-bound Co^{3+}. The Co–P distance of 3.4 Å has been established crystallographically by Merritt et al. (1978). This value, taken together with the Mn^{2+}–Cr^{3+} distance of 8.1 Å, confines the β and γ phosphorus atoms of ATP to a range of Mn^{2+}–P distances

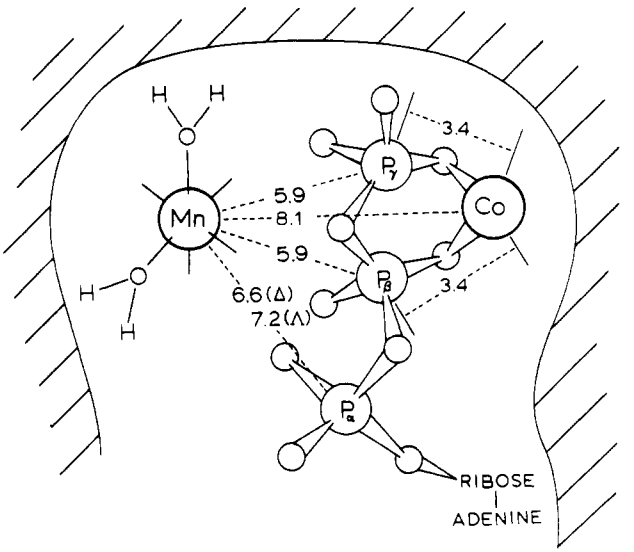

FIGURE 5: Conformation of triphosphate chain of $Co(NH_3)_4ATP$ with respect to bound Mn^{2+} ion on sheep kidney $(Na^+ + K^+)$-ATPase. Distances are expressed in angstroms. The Δ diastereomer of $Co(NH_3)_4ATP$ is depicted in this drawing. The Mn^{2+}–Cr^{3+} distance of 8.1 Å was determined by Mn^{2+} EPR as previously described (O'Connor & Grisham, 1980). The Mn^{2+}–P distances shown were calculated by using a correlation time, τ_c, of 1.9×10^{-9} s (see Table I).

from 4.7 to 11.5 Å.[4] Clearly, the values obtained here are in the low end of this range.

Given the estimates of the Mn^{2+}–P distances obtained here, it is of interest to determine the exact nature of the interaction between the enzyme-bound Mn^{2+} and the phosphates of bound ATP. The distances in Table I are too large for an inner sphere complex. From the results of small-molecule crystallography and molecular model studies (Mildvan & Grisham, 1974), the Mn^{2+} to phosphorus distance is 2.8–3.0 Å for an inner sphere complex of tetrahedral phosphate. On the other hand, a second sphere complex in which an inner sphere water or a ligand of comparable size intervenes between the Mn^{2+} and phosphate typically gives Mn^{2+}–P distances of 6.1 ± 0.5 Å. The distances obtained in the present studies are thus consistent with a second sphere complex between Mn^{2+} and the γ- and β-phosphates of $Co(NH_3)_4ATP$. In support of this model, we have previously shown that Mn^{2+} at the high-affinity site of this enzyme coordinates four rapidly exchanging water protons (i.e., two water molecules), one of which is lost (either displaced or "frozen" in place) upon addition of phosphate (Grisham & Mildvan, 1974). ^{31}P NMR studies subsequently established the existence of a phosphate site 6.9 Å from the enzyme-bound Mn^{2+} (Grisham & Mildvan, 1975). These results were interpreted in terms of a second sphere complex of Mn^{2+} and phosphate with an intervening water molecule. The previously characterized phosphate site is quite possibly distinct from the sites involved in coordination of $Co(NH_3)_4ATP$, but this point has yet to be established with certainty. [It should be noted, however, that phosphate and ATP can bind simultaneously to the ATPase (Grisham, 1979).]

This study adds additional evidence to that obtained previously (O'Connor & Grisham, 1980; Grisham, 1981), all of which suggests that the active complex of ATP with the $(Na^+ + K^+)$-ATPase involves two divalent cations. The predominant form of ATP as it is available to the ATPase in vivo is the

[4] It should be noted here that the lower limit distance of 4.7 Å, on the basis of EPR data, argues against an occult inner sphere CoATP complex that is dissociating at a rate significantly slower than $1/T_{1_M}$.

Mg^{2+}–ATP complex. In the present study, $Co(NH_3)_4ATP$ provides a nonhydrolyzable analogue of Mg^{2+}–ATP. On the other hand, we (Grisham & Mildvan, 1974; O'Connor & Grisham, 1979) and others (Robinson, 1981) have established the activating role of the high-affinity Mn^{2+} site on the ATPase that exists even in the absence of ATP. The notion of the two divalent metal ions interacting in catalytic roles at the active site of an enzyme is not new. Such a model was proposed by Foster et al. (1967) for phosphoenolpyruvate carboxylase, on the basis of the synergistic kinetic effects of Mn^{2+} and Mg^{2+}. Likewise, evidence for a dual divalent cation requirement has been obtained for pyruvate kinase (Gupta et al., 1976a,b) and for phosphoribosylpyrophosphate synthetase (Granot et al., 1980). In all these cases, one metal ion is directly coordinated to the nucleotide substrate, while the other is bound directly to the enzyme. The present work and other recent data (O'Connor & Grisham, 1980; Grisham, 1981) would appear to place the $(Na^+ + K^+)$-ATPase in this same class of enzymes. It is particularly interesting that the distances measured from the enzyme-bound cation to the phosphorus atoms of the nucleotide substrate on the $(Na^+ + K^+)$-ATPase are second coordination sphere distances, since this is also the case for two of the other members of the dual divalent cation club, namely, pyruvate kinase (Sloan & Mildvan, 1976) and phosphoribosylpyrophosphate synthetase (Granot et al., 1980).

The roles of the two divalent metals in effecting the hydrolysis of ATP are not entirely clear in the case of the $(Na^+ + K^+)$-ATPase, but certain inferences may be made. The nucleotide-bound metal is probably not involved in adjusting the protein conformation, since its role can be filled by Cr^{3+}, which is substitution inert and therefore cannot acquire ligands from the enzyme. [It should be noted here that, despite its substitution-inert nature, $Cr(H_2O)_4ATP$ is a substrate for the $(Na^+ + K^+)$-ATPase (Gantzer, 1980; Gantzer et al., 1982).] A more likely role for the nucleotide-bound metal would be to adjust the conformation of the polyphosphate backbone and to polarize and thereby increase the electrophilicity of the γ-phosphoryl group of ATP. The role of the enzyme-bound metal may be to adjust the protein conformation and/or to orient water ligands near the phosphoryl groups of ATP. Such interactions could serve to stabilize the phosphorane transition state in the S_N2 displacements occurring during the formation and breakdown of the aspartyl phosphate intermediate, which is a requisite intermediate in the hydrolytic pathway of this enzyme.

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