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Nuclear Magnetic Resonance Studies of the Conformation of Tetraamminecobalt(III)–ATP Bound at the Active Site of Bovine Heart Protein Kinase†

Joseph Granot, Hiroki Kondo, Richard N. Armstrong,‡ Albert S. Mildvan,* and E. T. Kaiser

ABSTRACT: The binding of metal–nucleotide substrates and substrate analogues to the catalytic subunit of cAMP-dependent protein kinase from bovine heart has previously been shown to induce the appearance of an additional tight, inhibitory, divalent cation binding site on the enzyme [R. N. Armstrong et al. (1979) *Biochemistry 18*, 1230]. The location of this inhibitory metal and the conformation of the metal–nucleotide substrate on the catalytic subunit have been studied by using β,γ-bidentate tetraammineCo(III)–$(NH_3)_4$ATP, a substitution inert analogue of MgATP, which is shown to be a linear competitive inhibitor ($K_I = 151 \pm 10$ μM) with respect to MgATP ($K_m = 2.8 \pm 0.3$ μM). From the paramagnetic effects of Mn^{2+} bound at the inhibitory site of the enzyme on the longitudinal relaxation rates of the protons (at 100 and 360 MHz) and phosphorus nuclei (at 40.5, 72.9, and 145.8 MHz) of $Co(NH_3)_4$ATP at the substrate site, nine distances from Mn^{2+} to $Co(NH_3)_4$ATP are determined. The distances indicate either bidentate α,γ or tridentate α,β,γ coordination of the triphosphate chain of both the Δ and Λ stereoisomers of $Co(NH_3)_4$ATP by the enzyme-bound Mn^{2+}. This location of Mn^{2+} is shown to be consistent with the known thermodynamic and kinetic properties of complexes of protein kinase with nucleotides and metal ions. In the absence of enzyme, Mn^{2+} binds weakly to the adenine ring of $Co(NH_3)_4$ATP.

Cyclic adenosine 3′,5′-monophosphate (cAMP)[1] dependent protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase) provides a major pathway, whereby cAMP can exert its effect on processes of cell regulation and metabolism (Krebs, 1972). This enzyme is composed of regulatory and catalytic subunits which form an inactive tetrameric holoenzyme. The activation of the enzyme by cAMP occurs through its dissociation into a dimeric regulatory subunit–cAMP complex and two active catalytic subunit monomers.

We have recently studied the interaction of Mn^{2+} and substrates with the catalytic subunit of protein kinase by magnetic resonance and kinetic methods (Armstrong et al., 1979a). While the enzyme alone was found to bind Mn^{2+} very weakly, enzyme complexes of nucleotides, such as ADP or the nonhydrolyzable β,γ-methylene analogue of ATP, bind 2 Mn^{2+} ions/mol of complex tightly. With the substitution-inert metal–nucleotide β,γ-bidentate complex $Co(NH_3)_4$ATP, only one tight Mn^{2+} binding site was detected. These findings

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[1] Abbreviations used: cAMP, adenosine 3′,5′-monophosphate; Co-$(NH_3)_4$ATP, tetraamminecobalt(III)-β,γ-phosphate–ATP.

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indicated that one of the two tight binding sites for Mn^{2+} induced by the presence of nucleotides was on the enzyme-bound nucleotide itself, while the other was on the enzyme. A kinetic analysis revealed the nucleotide-bound metal to be an essential activator, while the enzyme-bound metal was inhibitory.[2]

In the present work, ^1H and ^{31}P nuclear relaxation measurements of $Co(NH_3)_4ATP$ are made in the presence of Mn^{2+} and of the catalytic subunit of protein kinase. The distances from Mn^{2+} at the inhibitory site to $Co(NH_3)_4ATP$ at the substrate site are used to clarify the relative positions of the activating and inhibitory metal ions and the conformation of the bound nucleotide substrate. A preliminary report of this work has been published (Granot et al., 1979).

Experimental Section

Materials

The catalytic subunit of cAMP-dependent protein kinase from bovine heart muscle was prepared by the method of Demaille et al. (1977) with some modifications as previously described (Armstrong et al., 1979a). The β,γ-bidentate complex $Co(NH_3)_4ATP$ was prepared as described by Cornelius et al. (1977). [γ-^{32}P]ATP was purchased from Amersham/Searle. All other compounds were of the highest purity available commercially.

Methods

Miscellaneous Methods. The concentrations of the catalytic subunit of protein kinase and of ATP were determined spectrophotometrically by using $A_{280}^{1\%} = 14.9$ for the enzyme and by assuming a molecular weight of 40 000 (Armstrong & Kaiser, 1978) and ϵ_{260} 15.4×10^3 for the nucleotide. Solutions used in the NMR experiments contained 50 mM Tris-Cl buffer at pH 7.5, 150 mM KCl and 0.1 mM DTT. Trace metal impurities were removed from the solutions by passage through Chelex-100 (Bio-Rad). Purity of the $Co(NH_3)_4ATP$ complex was judged by thin-layer chromatography, and its structure was established by ^{31}P NMR (Cornelius et al., 1977).

Enzyme Assays. The catalytic activity of protein kinase was assayed by monitoring the phosphorylation of the synthetic peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly as previously described (Witt & Roskoski, 1975; Armstrong et al., 1979a).

NMR Measurements. Longitudinal relaxation times (T_1) of the proton and phosphorus nuclei of ATP in the $Co(NH_3)_4ATP$ complex were measured at 100 and 360 MHz (^1H) and 40.5, 72.9, and 145.8 MHz (^{31}P). The measurements at 72.5, 145.8, and 360 MHz were carried out on a Bruker WH 180/360 spectrometer, and the T_1 values were determined using a 180°–τ–90° pulse sequence (Carr & Purcell, 1954). The measurements at 40.5 and 100 MHz were carried out on a Varian XL-100-FT spectrometer by using the 90° homogeneity spoil–τ–90° method (McDonald & Leigh, 1973). Transverse relaxation times (T_2) were calculated from linewidth measurements ($\Delta\nu$) by using the relation $T_2^{-1} = \pi\Delta\nu$. Multiplets are usually collapsed to singlets after the first addition of Mn^{2+}. In cases of partially overlapping resonances, the separate line widths were determined by manual simulation. Proton broad band decoupling was used to simplify the ^{31}P spectra, and selective proton irradiation was used to suppress the residual water signal in the ^1H spectra. Deuterated solutions of salts for the ^1H NMR measurements were made up by dissolving the solid materials in deuterium oxide (99.7%). Deuterated buffer solutions were prepared by repeated lyophilization in D_2O. The enzyme solutions were deuterated by repeated concentration by using vacuum dialysis and dilution with deuterated buffer and salt solutions. Since the deuterium signal was needed for field-frequency locking, 20% D_2O was added to the aqueous solutions used in the ^{31}P measurements. All measurements were carried out at 25 °C. After prolonged NMR studies (≥24 h), the enzyme was found to retain more than half of its activity, and no systematic changes were observed during the NMR measurements.

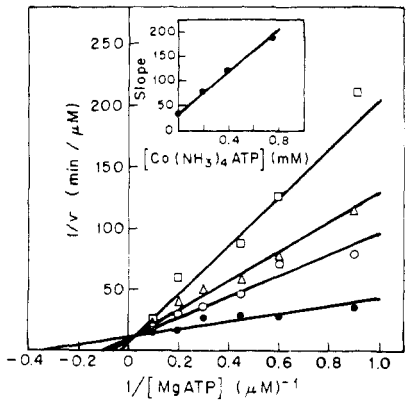


FIGURE 1: Double-reciprocal plot of the inhibition by $Co(NH_3)_4ATP$ of peptide phosphorylation by protein kinase. The assay mixture contained the synthetic heptapeptide (0.3–1.0 mg/mL), BSA (1 mg/mL), $MgCl_2$ (10 mM), Tris-Cl buffer (50 mM) at pH 7.5, KCl (150 mM), and the catalytic subunit of protein-kinase (30 nM).

Results

Inhibition of Peptide Phosphorylation by $Co(NH_3)_4ATP$. The effects of varying concentrations of $Co(NH_3)_4ATP$ on the rate of phosphorylation of the synthetic peptide are shown in the form of a double-reciprocal plot in Figure 1. The data when analyzed by a least-squares program (Cleland, 1967) indicate that $Co(NH_3)_4ATP$ is a linear competitive inhibitor with respect to MgATP, with a $K_I = 151 \pm 10$ μM, a value consistent with previous direct binding studies of $Co(NH_3)_4ATP$ to the catalytic subunit (Armstrong et al., 1979a). The K_m of MgATP (2.3 ± 0.3 μM) is in good agreement with dissociation constants previously determined by direct binding studies for various Mn^{2+}–nucleotide complexes (0.4–2.4 μM) and with the K_m of MgATP (4.2 μM) obtained in the enzyme catalyzed ATPase reaction (Armstrong et al., 1979b). This similarity with Mg^{2+} and Mn^{2+} supports the hypothesis that the binding of the metal–nucleotide substrate to the enzyme occurs through a nucleotide bridge (Armstrong et al., 1979a).

NMR Studies of the Binary Mn^{2+}–$Co(NH_3)_4ATP$ Complex. As a control for the enzyme studies, the interaction of Mn^{2+} with the $Co(NH_3)_4ATP$ complex was studied. Due to the chirality of the β-phosphorus, the bidentate $Co(NH_3)_4ATP$ complex has two stereoisomers denoted as Δ and Λ (Merritt et al., 1978). In the present study the racemic mixture was used. The different absolute configuration about the β-phosphorus gives rise to chemical shift differences between the phosphorus resonances of the two stereoisomers (Cornelius et al., 1977). At 145.8 MHz, this difference is 17, 8.5, and 2.0 Hz for P_α, P_β, and P_γ, respectively (Figure 2). Thus it was possible to obtain good differentiation between the two isomers in the high frequency ^{31}P spectra. In the proton spectra at 360 MHz all the protons of the adenosine moiety of ATP could be resolved (except for H_2' which was obscured by the residual

[2] The suggestion that the nucleotide-bound metal activates and that the second enzyme-bound metal inhibits is consistent with our recent observation that the Δ isomer of the stable complex β,γ-bidentate $Co(NH_3)_4ATP$ is a substrate for protein kinase in the absence of added divalent cations (Granot et al., 1979).

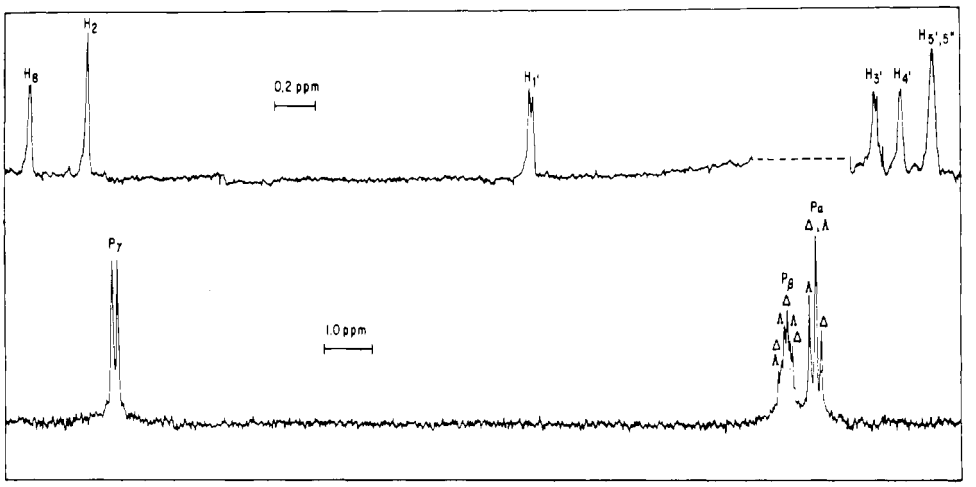


FIGURE 2: High-field proton (360 MHz) and $^{31}P[^{1}H]$ (145.8 MHz) magnetic resonance spectra and assignments of $Co(NH_3)_4ATP$ at pH 7.5, 25 °C.

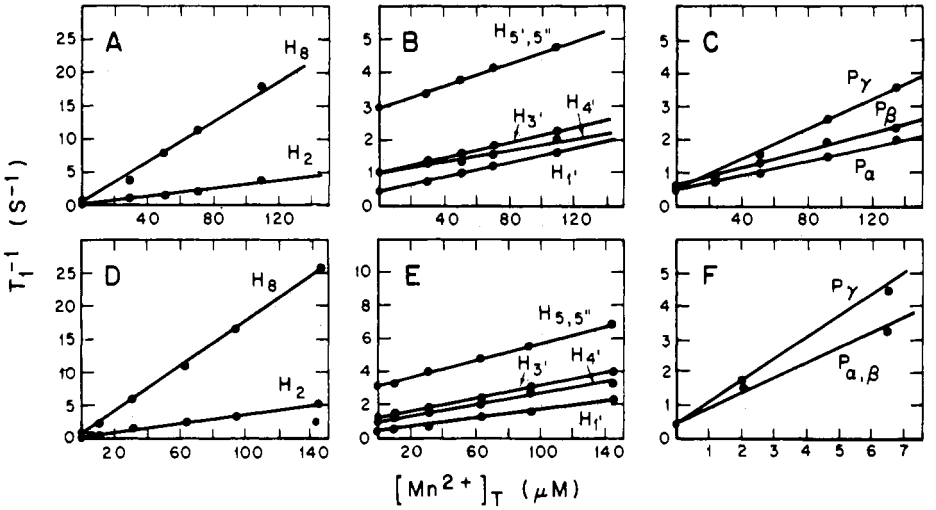


FIGURE 3: Effect of Mn^{2+} on the longitudinal relaxation rates of the proton and phosphorus nuclei of $Co(NH_3)_4ATP$ in the absence (A, B, C) and presence (D, E, F) of the catalytic subunit of protein kinase. The $Co(NH_3)_4ATP$ concentrations are 32.7 (A, B), 26.3 (C), 23.9 (D, E), and 6.6 mM (F). The enzyme concentrations are 51.5 μM (D, E) and 9.1 μM (F). As calculated by using the previously determined dissociation constants (Armstrong et al., 1979a), the fraction of Mn^{2+} which is bound to $Co(NH_3)_4ATP$ is 68.3% (A, B), 63.4% (C), 54.8% (D, E), and 29.1% (F). The fraction of Mn^{2+} in the ternary complex is 11.4% (D, E) and 4.4% (F).

HDO signal). Since the adenosine moiety is far from the chiral β-phosphorus the proton resonances of the two stereoisomers could not be resolved. Typical ^{1}H and ^{31}P spectra of $Co(NH_3)_4ATP$ are shown in Figure 2. Relaxation rates of the $Co(NH_3)_4ATP$ complex (14.5–33.5 mM), exemplified in Figure 3(A–C), were obtained as a function of Mn^{2+} concentration (1.5–108 μM) at 360 MHz (^{1}H) and 145.8 MHz (^{31}P). No differences were observed in the ^{31}P longitudinal relaxation rates of the resonances of the two stereoisomers. With the inversion–recovery method used, differences in T_1 beyond the estimated experimental error of 5% would have been detected, even for partially overlapping resonances. Bound state values, T_{1M}^{-1} and $1/fT_{2p}$[3] for the longitudinal and transverse relaxation rates, respectively, were calculated by using a value of 15 mM for the dissociation constant of the Mn^{2+}–$Co(NH_3)_4ATP$ complex (Armstrong et al., 1979a). The results are summarized in Table I. The largest value of the transverse relaxation rate in the binary complex (4.8×10^4 s^{-1}) sets a lower limit to $1/\tau_M$, the rate of dissociation of the binary complex, which is more than sixfold greater than the longitudinal relaxation rates. Hence, the latter values are not limited by chemical exchange and may be used for distance calculations.

Table I: Paramagnetic Contributions to the Relaxation Rates[a] of the ATP Nuclei and Mn^{2+}–Nuclei Distances in the Binary Mn^{2+}–$Co(NH_3)_4$ ATP Complex

atom	T_{1M}^{-1} (s^{-1})	$1/fT_{2p} \times 10^{-3}$ (s^{-1})	r (Å)
H_8	7300 ± 400	33.0 ± 2.0	5.4 ± 0.4
H_2	1300 ± 70	6.3 ± 0.5	7.2 ± 0.5
H_1'	520 ± 30	2.3 ± 0.3	8.4 ± 0.6
H_3'	530 ± 30	2.6 ± 0.3	8.4 ± 0.6
H_4'	470 ± 30	2.3 ± 0.3	8.6 ± 0.6
H_5', H_5''	820 ± 50	4.0 ± 0.3	7.8 ± 0.5
$P_\alpha(\Delta)$, $P_\alpha(\Lambda)$	920 ± 130	30.0 ± 3.0	6.3 ± 0.4
P_β	1010 ± 140	35.0 ± 3.0	6.4 ± 0.4
P_γ	1830 ± 150	48.0 ± 4.0	5.7 ± 0.4

[a] The proton and the phosphorus data were measured at 360 and 145.8 MHz, respectively. A correlation time $\tau_c = (5 \pm 2) \times 10^{-10}$ s, used in the distance calculations, was determined as described in the text.

Determination of Correlation Times and Distances in the Binary Complex. In order to calculate the distances between

[3] In the case of the transverse relaxation, $1/fT_{2p}$ rather than T_{2M}^{-1} is used in order to avoid ambiguity due to a possible significant exchange contribution to this relaxation rate. f is the ratio of the concentration of Mn^{2+}-bound ligand to that of the total concentration of the ligand.

Mn^{2+} and the proton and phosphorus nuclei of ATP, the correlation time (τ_c) for the relaxation in the binary complex had to be determined. This has been done in several ways. (a) From the average ratio $T_{1M}/fT_{2P} = 4.7 \pm 0.4$ for the proton resonances, at 360 MHz, of ATP in the binary complex a value of $\tau_c = 1.0 \times 10^{-9}$ s was obtained. This may be considered as an upper limit to τ_c due to a possible scalar contribution to $1/fT_{2p}$. (b) T_1 values for H_8, H_2, and H_1' of ATP in the binary complex were measured at 100 MHz. The average ratio $T_{1M}^{-1}(100\ MHz)/T_{1M}^{-1}(360\ MHz) = 2.0 \pm 0.2$ yielded $\tau_c = 4.8 \times 10^{-10}$ s. (c) The effect of Mn^{2+} on the longitudinal relaxation rate of water protons was measured at 24.3[4] and 360 MHz. A ratio $T_{1M}^{-1}(24.3\ MHz)/T_{1M}^{-1}(360\ MHz) = 2.4 \pm 0.2$ yielded $\tau_c = 5.2 \times 10^{-10}$ s. Consistent with these short τ_c values, the paramagnetic effects of Mn^{2+} on $1/T_1$ of the phosphorus resonances of $Co(NH_3)_4ATP$ were found to be independent of frequency between 40.5 and 145.8 MHz.

The distances (r) in Table I were calculated with $\tau_c = 5 \times 10^{-10}$ s, by using eq 1 (Solomon, 1955; Bloembergen & Morgan, 1961) where ω_I and ω_S are the nuclear and electron

$$r = C[T_{1M}f(\tau_c)]^{1/6} \quad (1)$$

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

Larmor frequencies and C is a constant equal to 812 or 601 for the Mn^{2+}–^1H or Mn^{2+}–^{31}P interactions, respectively.

NMR Studies of the Ternary Mn^{2+}–Enzyme–$Co(NH_3)_4ATP$ Complex. ^{31}P relaxation rates of $Co(NH_3)_4ATP$ (6.6–22.2 mM), exemplified in Figure 3F, were obtained as a function of Mn^{2+} concentration (1.3–6.5 μM) in the presence of enzyme (9.1–9.5 μM), at 145.8 MHz. The two isomers of $Co(NH_3)_4ATP$ which previously were shown to bind with the same affinity to protein kinase (Armstrong et al., 1979a), were also observed to have indistinguishable relaxation rates in the ternary complex, as judged from the ^{31}P NMR spectra which are partially resolved at this frequency (Figure 2).

The presence of the enzyme causes an order of magnitude enhancement of the effects of Mn^{2+} on the ^{31}P relaxation rates of $Co(NH_3)_4ATP$ (Figure 3) establishing the formation of a ternary Mn^{2+}–enzyme–$Co(NH_3)_4ATP$ complex. Since under the experimental conditions $Co(NH_3)_4ATP$ was present both in binary and ternary Mn^{2+} complexes, the small contribution of the binary complex to the measured paramagnetic relaxation rates (T_{1p}^{-1}) had to be subtracted. The data were thus analyzed with eq 2 where [SM], [MES], and $[S]_T$ denote the

$$T_{1p}^{-1} = \frac{[SM]}{[S]_T}(T_{1M}^{-1})_{binary} + \frac{[MES]}{[S]_T}(T_{1M}^{-1})_{ternary} \quad (2)$$

concentrations of the binary and ternary complexes and of the total $Co(NH_3)_4ATP$, respectively, and $(T_{1M}^{-1})_{binary}$ and $(T_{1M}^{-1})_{ternary}$ are the respective bound-state relaxation rates of ATP in the binary and the ternary complexes. An analogous equation was used for the paramagnetic transverse relaxation rate with $(1/fT_{2p})_{binary}$ and $(1/fT_{2p})_{ternary}$ as the bound-state values. In the analyses [SM] and [MES] were calculated with the appropriate dissociation constants previously determined (Armstrong et al., 1979a), and $(T_{1M}^{-1})_{binary}$ values were taken from Table I. The results are summarized in Table II.

Proton relaxation rates of $Co(NH_3)_4ATP$ (11.1–23.7 mM) were determined as a function of Mn^{2+} concentration (5.3–144 μM) in the presence of enzyme (30.1–51.5 μM) (Figure 3).

[4] The measurements at 24.3 MHz were carried out on a PRR spectrometer described elsewhere (Mildvan & Engle, 1972).

Table II: Paramagnetic Contributions to the Relaxation Rates[a] of the ATP Nuclei and Mn^{2+}–Nuclei Distances in the Enzyme-Bound Mn^{2+}–$Co(NH_3)_4ATP$ Complex

atom	$T_{1M}^{-1} \times 10^{-3}$ (s^{-1})	$1/fT_{2p} \times 10^{-6}$ (s^{-1})	r^c (Å)	r^d (Å)	r_{av} (Å)
H_8	1.2 ± 0.8	[b]	6.1	7.3	6.7 ± 1.0
H_2	0.41 ± 0.25	[b]	7.4	8.8	8.1 ± 1.1
H_1'	0.74 ± 0.22	[b]	6.6	8.0	7.3 ± 0.8
H_3'	1.55 ± 0.38	[b]	5.8	7.0	6.4 ± 0.7
H_4'	1.23 ± 0.32	[b]	6.1	7.3	6.7 ± 0.7
H_5', H_5''	1.46 ± 0.42	[b]	5.8	7.0	6.4 ± 0.7
$P_\alpha(\Delta)$, $P_\alpha(\Lambda)$	81 ± 6	2.0 ± 0.3	3.0	3.0	3.0 ± 0.2
P_β	78 ± 6	1.9 ± 0.3	3.0	3.0	3.0 ± 0.2
P_γ	113 ± 8	2.7 ± 0.4	2.8	2.9	2.9 ± 0.2

[a] The proton and the phosphorus data were measured at 360 and 145.8 MHz, respectively. [b] Due to the relatively small effect and the relatively higher uncertainty in T_2^{-1} values obtained from line-width data, these values could not be accurately determined. [c] Calculated under assumption of maximum frequency dependence of the correlation time, with $\tau_c = 2.7 \times 10^{-9}$ s. [d] Calculated under assumption of no frequency dependence of the correlation time with $\tau_c = 7 \times 10^{-10}$ s.

The presence of the enzyme slightly decreased the observed paramagnetic effects of Mn^{2+} on the relaxation rates of the protons of $Co(NH_3)_4ATP$. Since a ternary complex was established by the ^{31}P data, eq 2 was used to calculate the proton relaxation rates ($1/T_{1M}$) in the ternary complex (Table II). Because of the relatively small paramagnetic effects on the proton relaxation rates in the ternary complex, the corresponding T_{1M}^{-1} ternary values have larger estimated errors of 30–70% (Table II).

Determination of Correlation Times and Distances in the Ternary Complex. In order to determine the correlation time, the paramagnetic contributions to the longitudinal relaxation rates of ATP in the ternary complex were measured at several frequencies. ^{31}P relaxation rates were measured at 40.5, 72.9, and 145.8 MHz. Within the experimental error, no frequency dependence of the relaxation rate was observed, indicating that $\tau_c \lesssim 1.1 \times 10^{-9}$ s at 145.8 MHz. Longitudinal ^1H relaxation rates were measured at 100 and 360 MHz, yielding an average $T_{1M}^{-1}(100\ MHz)/T_{1M}^{-1}(360\ MHz) = 3.0 \pm 0.7$. The correlation time was calculated with eq 9 and 11 from Bean et al. (1977). By using the assumption that the correlation time is frequency independent, a value of 7.0×10^{-10} s was obtained. By assuming that the correlation time is dominated by the electron spin relaxation time and that it has maximum frequency dependence, a τ_c value of 2.7×10^{-9} s at 360 MHz was obtained. This value applies also to the ^{31}P relaxation data (145.8 MHz) measured at the same magnetic field. Distances calculated with these two limiting assumptions are given in Table II. As with the binary complex, the largest value of the transverse relaxation rate (2.7×10^6 s^{-1}, Table II) sets a lower limit on $1/\tau_M$ which is much greater than the longitudinal relaxation rates. This fact and the minimal (inner sphere) distances from Mn^{2+} to phosphorus justify the use of the $1/T_{1M}$ values for distance calculations.

Molecular Model of the Binary Mn^{2+}–$Co(NH_3)_4ATP$ Complex. Skeletal and space-filling molecular models for the Mn^{2+}–$Co(NH_3)_4ATP$ complex were constructed on the basis of the Mn^{2+}–nuclei distances, determined by NMR (Table I). In the absence of enzyme, the experimental distances (Table I) indicate close proximity of Mn^{2+} to the adenine ring of ATP but not to the polyphosphate chain. Since the addition of $Co(NH_3)_4ATP$ to Mn^{2+} solutions caused marked effects on the EPR spectrum of Mn^{2+},[5] it follows that Mn^{2+} must be

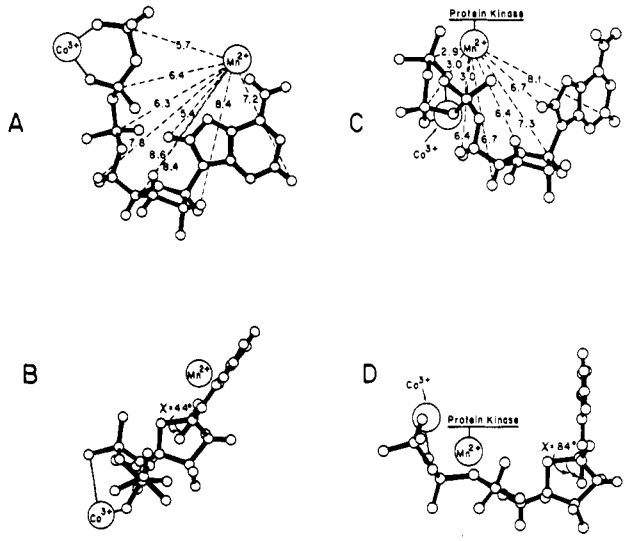


FIGURE 4: Conformations of $Co(NH_3)_4ATP$ in its binary complex with Mn^{2+} (A, B) and in its ternary complex with Mn^{2+} and the catalytic subunit of protein kinase (C, D). Distances are expressed in Å. Views A and C have the same ribose orientation. Views B and D show the glycosidic torsion angle (χ) and manifest the differences in the relative orientations of the polyphosphate chain and the adenine ring in the binary and ternary complexes.

directly coordinated to the adenine ring, most probably at N_7 and/or C_6–NH_2. From Dreiding molecular model studies, it was determined that Mn^{2+} binding to the adenine ring in a bidentate chelate between N_7 and C_6–NH_2 would yield a distance ratio $r(Mn^{2+}$–$H_2)/r(Mn^{2+}$–$H_8)$ of 2.0 ± 0.1. The corresponding distance ratios expected for the monodentate complexes at C_6–NH_2 or at N_7 are 1.1 ± 0.4 and 2.5 ± 0.1, respectively. The experimental distances (Table I) yield a value of 1.3 ± 0.1 for this ratio, which may indicate binding of Mn^{2+} to the adenine at the C_6–NH_2 group. However, in view of the low basicity of the amino nitrogen, and other observations which do not favor monodentate binding at this site (Izatt et al., 1971; Marzilli, 1977), the present results may reflect a mixture of species including multimeric complexes, in which the coordination of Mn^{2+} to the adenine ring is through N_7 or N_7 and C_6–NH_2 or even N_1 and N_3. On the basis of these arguments, use was made of a computer program which searches among all the possible conformations that yield Mn^{2+}–nuclei distances within their experimental values and their errors (Mildvan & Gupta, 1978; Mildvan et al., 1979) in order to determine the optimum model and its uniqueness. The initial structure of $Co(NH_3)_4ATP$ was constructed using the X-ray coordinates of Kennard et al. (1971) for the adenosine moiety and those of Merritt et al. (1978) for the tetraamino-Co^{3+}–triphosphate part. These two residues were combined and protons were placed on the adenosine moiety by using other computer programs (Rao & Rossmann, 1973; Mildvan et al., 1979). Rotations were generated about the six flexible single bonds (N_9–C_1'; C_4'–C_5'; C_5'–O_5'; O_5'–P_α; P_α–O_6'; O_6'–P_β), and Mn^{2+} distances to the protons and phosphorus nuclei were calculated for each solution allowing ≤10% van der Waals overlap at each atom. For the binary Mn^{2+}–$Co(NH_3)_4ATP$ complex, one solution was obtained which was within the van der Waals and distances tolerance. A perspective model of this solution being unique to 5–10° of rotation about each bond is depicted in Figures 4A and 4B. This model shows a folded conformation of the ATP molecule, although the phosphate chain itself appears to be in an extended conformation. The Co^{3+}–tetraamino group is located far from the Mn^{2+}, as may be expected in view of the electrostatic repulsion between the two metal ions. The glycosidic torsion angle (χ) is found to be 44 ± 10° which is in the range usually obtained for various nucleosides and nucleotides in the crystalline state (Sundaralingam, 1969; Kennard et al., 1971) and in solution (Dhingra et al., 1978; Imoto et al., 1977).

Molecular Models of Enzyme-Bound Mn^{2+}–Co-$(NH_3)_4ATP$. In the presence of the enzyme, in marked contrast with the binary complex, Mn^{2+} binds to the polyphosphate chain of $Co(NH_3)_4ATP$ (Table II). By using space-filling and skeletal models, it was found that coordination of a second metal ion by the β,γ-bidentate–Co^{3+}–triphosphate is possible either with α,γ-bidentate binding or with α,β,γ-tridentate chelation. Both types of Mn^{2+} chelation yielded very similar conformations of the adenosine portion of the enzyme-bound $Co(NH_3)_4ATP$ in the molecular model studies. By using the computer search program, again with the initial coordinates of Kennard et al. (1971) and Merritt et al. (1978), solutions within the van der Waals tolerances and measured distances were obtained only for the α,γ-bidentate chelation of Mn^{2+}. Tridentate coordination required changes in the given X-ray conformation of the β,γ-Co^{3+}–pyrophosphate ring, which, while possible in solution, could not be generated by the computer program used in the present study. It is noteworthy that α,γ-bidentate chelation would better explain the similar dissociation constants of Mn^{2+} from enzyme-bound ADP, ATP, and AMP-PCP (Armstrong et al., 1979a).

For the α,γ-bidentate Mn^{2+}–$Co(NH_3)_4ATP$ on the enzyme, two solutions were obtained, each unique to 5–15° about the six rotated bonds. The two solutions show indistinguishable conformations within their accuracies, except for the torsion angle at the glycosidic bond. Thus one solution yielded χ = 84 ± 10°, an anti conformational angle, while the other yielded χ = 284 ± 10°, a syn conformational angle. This ambiguity, which arises from the planarity of the adenine ring and its remoteness from the Mn^{2+} ion, cannot be resolved on the basis of the present data or by further NMR experiments at sensitivities currently available. However, the anti conformation is to be preferred over the syn since it is much more generally found in purine nucleotides and their complexes in the crystalline state and in solution. The overall conformation of enzyme-bound $Co(NH_3)_4ATP$ (Figure 4C and 4D) is folded, with the polyphosphate chain almost perpendicular to the plane of the adenine ring (Figure 4D). This conformation differs considerably from that of the binary Mn^{2+}–$Co(NH_3)_4ATP$ complex in which the polyphosphate chain lies essentially in the plane defined by the adenine ring (Figure 4B).

Kinetic Properties of the Mn^{2+}–Enzyme–$Co(NH_3)_4ATP$ System. The largest transverse relaxation rate ($1/fT_{2p}$) sets a lower limit to the pseudo-first-order rate constant for the exchange of $Co(NH_3)_4ATP$ into the paramagnetic environment ($1/\tau_M \geq 2.7 \times 10^6$ s^{-1}, Table II) (eq 3). The simplest

$$Co(NH_3)_4ATP^* + Mn^{2+}\text{–E–}Co(NH_3)_4ATP \xrightarrow{1/\tau_M} Co(NH_3)_4ATP + Mn^{2+}\text{–E–}Co(NH_3)_4ATP^* \quad (3)$$

kinetic scheme consistent with this value, and with previously measured dissociation constants (Armstrong et al., 1979a), is given by eq 4 and 5. Defining $1/\tau_A$ as the rate constant

$$Mn^{2+}\text{–E–}Co(NH_3)_4ATP \underset{k_{on}(Mn^{2+})}{\overset{1/\tau_A}{\rightleftharpoons}} Mn^{2+} + E\text{–}Co(NH_3)_4ATP \quad (4)$$

[5] The intensities of the EPR resonances of Mn^{2+} were found to decrease upon the addition of $Co(NH_3)_4ATP$, indicating inner sphere complexation. Detailed titrations of 3.4 mM and 11.9 mM $Co(NH_3)_4ATP$ with Mn^{2+} measuring the free Mn^{2+} by EPR yielded in both cases a 1:1 stoichiometry and a dissociation constant of 15 ± 5 mM.

$$E\text{–}Co(NH_3)_4ATP \underset{k_{on}(Co(NH_3)_4ATP)}{\overset{1/\tau_B}{\rightleftharpoons}} E + Co(NH_3)_4ATP \quad (5)$$

for dissociation of Mn^{2+} from the ternary Mn^{2+}–E–Co-$(NH_3)_4ATP$ complex, and $1/\tau_B$ as the rate constant for dissociation of the E–$Co(NH_3)_4ATP$ complex, it can be shown that the observed paramagnetic contribution to the transverse relaxation rate is given by eq 6 where $1/T_{2M}' = f_2/(T_{2M} +$

$$1/T_{2p} = f_1/(T_{2M}' + \tau_B) \quad (6)$$

$\tau_A)$, $f_1 = [ES]/[S]_T$, and $f_2 = [MES]/[ES]$. The value of τ_A sets a lower limit to T_{2M}', i.e., $T_{2M}' \geq \tau_A/f_2$, leading to the relationship

$$1/T_{2p} \leq f_1/(\tau_A/f_2 + \tau_B) \quad (7)$$

If it is assumed that $\tau_B \gtrsim \tau_A$, eq 7 together with the present data yields $1/\tau_A \geq 1/\tau_B = 1/\tau_M \geq 2.7 \times 10^6\ s^{-1}$. The limit value of $1/\tau_A$ together with the dissociation constant of Mn^{2+} from the Mn^{2+}–E–$Co(NH_3)_4ATP$ complex (130 μM, Armstrong et al., 1979a) yields $k_{on}(Mn^{2+}) \geq 2 \times 10^{10}\ M^{-1}\ s^{-1}$. Similarly the limit value of $1/\tau_B$ and the dissociation constant of the binary E–$Co(NH_3)_4ATP$ complex (301 μM, Armstrong et al., 1979a) yields $k_{on}(Co(NH_3)_4ATP) \geq 9 \times 10^9\ M^{-1}\ s^{-1}$. Assuming the other limiting case, i.e., $\tau_B \ll \tau_A$, yields k_{on} values ($\gg 10^{10}\ M^{-1}\ s^{-1}$) which exceed the diffusion-controlled limit and this case is, therefore, considered unlikely. Hence, in this system the residence time of Mn^{2+} in the ternary complex (τ_A) is of the same order or shorter than the residence time of the nucleotide in the binary complex (τ_B).

Discussion

Previous studies of the interaction of enzyme-bound Mn^{2+} with nucleoside or deoxynucleoside triphosphates have been made with pyruvate kinase (Sloan & Mildvan, 1976), DNA polymerase (Sloan et al., 1975), and RNA polymerase (Bean et al., 1977). A major advantage of $Co(NH_3)_4ATP$, as used here, over ATP is the 1000-fold lower affinity of the Co-$(NH_3)_4ATP$ complex for Mn^{2+} (Armstrong et al., 1979a). Thus the amount of Mn^{2+} in the binary complex in the present system is relatively small requiring smaller corrections in calculating the paramagnetic effects of Mn^{2+} on Co-$(NH_3)_4ATP$ in the ternary enzyme complex. In addition, in the present system, only one Mn^{2+} binding site exists on the enzyme–$Co(NH_3)_4ATP$ complex, rather than two as found with ATP and with other nucleotides (Armstrong et al., 1979a).

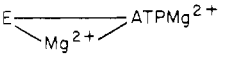
The binding of $Co(NH_3)_4ATP$ to the enzyme is an order of magnitude weaker than that of Mn^{2+}–nucleotide complexes (Armstrong et al., 1979a). However, linear competitive inhibition by $Co(NH_3)_4ATP$ with respect to the substrate MgATP indicates that $Co(NH_3)_4ATP$ occupies the nucleotide-binding site of the enzyme and, therefore, can be used as a substrate analogue in structural studies. Control studies of the binary Mn^{2+}–$Co(NH_3)_4ATP$ complex show that the Mn^{2+} binds directly to the adenine ring of ATP. Although the phosphate chain carries a net negative charge and the α-phosphate is not coordinated in the β,γ-bidentate Co(III) complex, Mn^{2+} is not directly coordinated to the phosphate chain.

An entirely different geometry is found in the presence of the enzyme (Figure 4). The enzyme-bound Mn^{2+} is directly coordinated to the polyphosphate chain of $Co(NH_3)_4ATP$, either α,γ-bidentate or α,β,γ-tridentate ($r(Mn^{2+}\text{–}P) \sim 3$ Å), while being further removed from the adenine ring (~ 8 Å) (Table II).

The location of the Mn^{2+}-binding site on the enzyme–$Co(NH_3)_4ATP$ complex is in accord with several properties previously observed in the Mn^{2+}–enzyme–substrate system (Armstrong et al., 1979a). While protein kinase binds Mn^{2+} very weakly in the absence of a nucleotide, two tight binding sites appear when a nucleotide is present. In addition, binding of Mn^{2+} at the inhibitory site produces significant increases in the affinity of the enzyme for nucleotides or metal–nucleotides (typically 10–50-fold). Furthermore, the data for the water proton relaxation enhancement suggest the presence of site–site interaction between the two Mn^{2+}-binding sites (Armstrong et al., 1979a). All these findings can be accounted for by considering the coordination of both metal ions by the polyphosphate chain of the enzyme-bound nucleotide. The k_{on} value for the binding of Mn^{2+} to the enzyme–Co-$(NH_3)_4ATP$ complex ($\geq 2 \times 10^{10}\ M^{-1}\ s^{-1}$) is comparable to the rate constant under similar conditions for formation of the binary Mn^{2+}–ATP complex ($1.1 \times 10^{10}\ M^{-1}\ s^{-1}$, Sloan & Mildvan, 1976) in which polyphosphate chelation also occurs.

The change in the glycosidic torsional angle (χ) of Co-$(NH_3)_4ATP$ from 44 ± 10° in the binary complex to the energetically less favored value of 84 ± 10° in the ternary complex indicates that the enzyme interacts strongly with the purine ring of the bound substrate analogue. This view is consistent with the high preference of protein kinase for ATP as a substrate over other purine and pyrimidine nucleotides (Walsh & Krebs, 1973), and with the recent finding that ATP analogues chemically modified on the adenine ring have considerably lower affinity for the catalytic subunit of rabbit muscle protein kinase (Hoppe et al., 1978).

The strong (98.6%) inhibitory effect of the enzyme-bound Mn^{2+} on the catalytic rate (Armstrong et al., 1979a) can be ascribed to the bridging by this metal of the enzyme to the polyphosphate chain of the Mn–ATP substrate, and possibly to the MnADP and phosphopeptide products of the protein kinase reaction. The weaker (80%) inhibition found with the enzyme-bound Mg^{2+}, together with the competition between Mn^{2+} and Mg^{2+} at the inhibitory site (Armstrong et al., 1979a), suggests a similar structure and mechanism of inhibition in the



complex. Space-filling models indicate that the γ-phosphorus of enzyme-bound ATP (Figure 4B) remains accessible to nucleophiles even when both metals together with their inner sphere ligands are coordinated to the polyphosphate chain. This observation is in accordance with the incomplete inhibition by both Mn^{2+} and Mg^{2+} and suggests that steric effects are not the major source of the inhibition by Mg^{2+} or Mn^{2+}. The finding that both stereoisomers of $Co(NH_3)_4ATP$ bind to the enzyme with similar affinity (Armstrong et al., 1979a) and geometry suggests little steric hindrance at the active site as might be expected on an enzyme with large protein substrates.

The role of a high affinity site for the binding of inhibitory divalent cations to the catalytic subunit of protein kinase is not clear. Numerous other enzymes which utilize ATP as a substrate do not have such a site, although they may show multiple weak and nonspecific inhibitory sites for Mn^{2+} (Mildvan & Loeb, 1979). It seems clear that the inhibitory metal ion does not compete directly with the peptide at the active site (Armstrong et al., 1979a). It is tempting to speculate that the inhibitory Mn^{2+} site on protein kinase may constitute a portion of the binding site for the regulatory subunit. Indeed, the regulatory subunit is in close proximity to the triphosphate chain of ATP in the holoenzyme–ATP

complex. This is demonstrated by the fact that the regulatory subunit is a substrate for the catalytic subunit in an intracomplex autophosphorylation reaction (Rangel-Aldao & Rosen, 1976; Todhunter & Purich, 1977). Recent binding and kinetic studies with ATP analogues suggest that the regulatory subunit of the type I isoenzyme interacts with the triphosphate chain of ATP (Hoppe et al., 1978) analogous to the present findings with Mn^{2+}. The inhibition by the regulatory subunit may manifest itself in two domains, one of which is the peptide binding site, while the other is the triphosphate chain of bound metal–ATP.

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