Magnetic Resonance Measurements of Intersubstrate Distances at the Active Site of Protein Kinase Using Substitution-Inert Cobalt(III) and Chromium(III) Complexes of Adenosine 5′-(β,γ-Methylenetriphosphate)†

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ABSTRACT: Co^{3+} and Cr^{3+} complexes of β,γ-methylene-ATP (AMPPCP), which are substitution-inert substrate analogues inactive in phosphoryl transfer reactions, have been used in binding and structural studies of cAMP-dependent protein kinase. Dissociation constants of enzyme complexes with $Co(NH_3)_4$AMPPCP and CrAMPPCP and with Mn^{2+}, which binds at an inhibitory site, were determined by electron paramagnetic resonance and by proton relaxation rate enhancement techniques. Nuclear relaxation rate measurements at 100 and 360 MHz were used to determine the distance between Mn^{2+} and the β,γ-methylene protons of $Co(NH_3)_4$AMPPCP, yielding 7.4 ± 0.6 Å in the absence of enzyme and 5.0 ± 0.9 Å when both Mn^{2+} and $Co(NH_3)_4$AMPPCP were bound to the enzyme. The effect of the paramagnetic CrAMPPCP on the electron spin relaxation time of the enzyme-bound Mn^{2+} was used to calculate the distance between the two metal ions of 4.8 ± 0.4 Å. This distance and the Mn^{2+}–methylene distance are consistent with the previous finding that the inhibitory metal bridges the enzyme to the triphosphate chain of the enzyme-bound nucleotide [Granot, J., Kondo, H., Armstrong, R. N., Mildvan, A. S., & Kaiser, E. T. (1979) *Biochemistry 18*, 2339]. From the paramagnetic effects on the relaxation rates of the protons of the peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly, distances from Mn^{2+} and Cr^{3+} to the serine methylene protons of 9.1 ± 0.9 and 8.1 ± 0.8 Å, respectively, were calculated. These and previous measurements were used to estimate a distance of 5.3 ± 0.7 Å along the reaction coordinate between the γ-phosphorus of ATP and the serine hydroxyl oxygen. This distance is 2 Å greater than that required for molecular contact. The mechanistic implications of these findings are discussed.

Substitution-inert complexes of Co^{3+} and Cr^{3+} with nucleotides have been widely used in kinetic and structural studies of enzymes (Cleland & Mildvan, 1979). Recently we have used the β,γ-bidentate $Co(NH_3)_4$ATP[1] complex to elucidate the conformation of the nucleotide substrate bound at the active site of cAMP-dependent protein kinase from bovine heart (Granot et al., 1979a). Our finding that the Δ isomer of $Co(NH_3)_4$ATP is an active substrate for protein kinase, though a very slow one ($V_{max} \sim 0.5$ min^{-1}) (Granot et al., 1979b), rendered unfavorable, however, its use in further structural studies in which a phosphoryl acceptor substrate is also present. To avoid reaction in the presence of the enzyme and a substrate, such as the heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemp et al., 1977), the use of the inactive complexes of β,γ-methylene-ATP (AMPPCP) is to be preferred. In the present work we report magnetic resonance studies of the binding of Mn^{2+} and the exchange-inert complexes of Co^{3+} and Cr^{3+} with AMPPCP to protein kinase. ^{1}H nuclear relaxation studies are used to calculate the distance between Mn^{2+} bound at the inhibitory metal site of the catalytic subunit to the β,γ-methylene protons of $Co(NH_3)_4$AMPPCP. The effect of the paramagnetic CrAMPPCP on the electron spin relaxation time of the enzyme-bound Mn^{2+} is used to calculate the Mn^{2+}–Cr^{3+} distance, thus providing further structural information regarding the location of the inhibitory metal site with respect to the metal–nucleotide substrate on protein kinase. Intersubstrate distances from Mn^{2+} and Cr^{3+} to the methylene protons of the seryl residue of the peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly are measured on the enzyme. These distances are used to estimate the distance and detailed geometry along the reaction coordinate of the phosphoryl transfer process catalyzed by protein kinase.

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). β,γ-Methylene-ATP was purchased from Sigma Chemical Co. The β,γ-bidentate $Co(NH_3)_4$AMPPCP and CrAMPPCP complexes were prepared as described previously (Cornelius et al., 1977; Cleland & Mildvan, 1979). Tris, 95% deuterated (d11), was obtained from Stohler Isotope Chemicals. All other compounds were of the highest purity available commercially.

Methods

Miscellaneous Methods. The concentrations of the catalytic subunit of protein kinase and of the AMPPCP complexes were determined spectrophotometrically by using $A_{280}^{1\%} = 14.9$ for the enzyme, assuming a molecular weight of 40 000 (Armstrong & Kaiser, 1978) and $\epsilon_{260} = 15.4 \times 10^3$ for the nu-

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[1] Abbreviations used: $Co(NH_3)_4$ATP, β,γ-bidentate, Co^{3+}–tetraammine-ATP; $Co(NH_3)_4$AMPPCP, β,γ-bidentate, Co^{3+}–tetraammine–β,γ-methylene-ATP; CrAMPPCP, β,γ-bidentate, Cr^{3+}–β,γ-methylene-ATP; PRR, longitudinal proton relaxation rate ($1/T_1$) of water; EPR, electron paramagnetic resonance; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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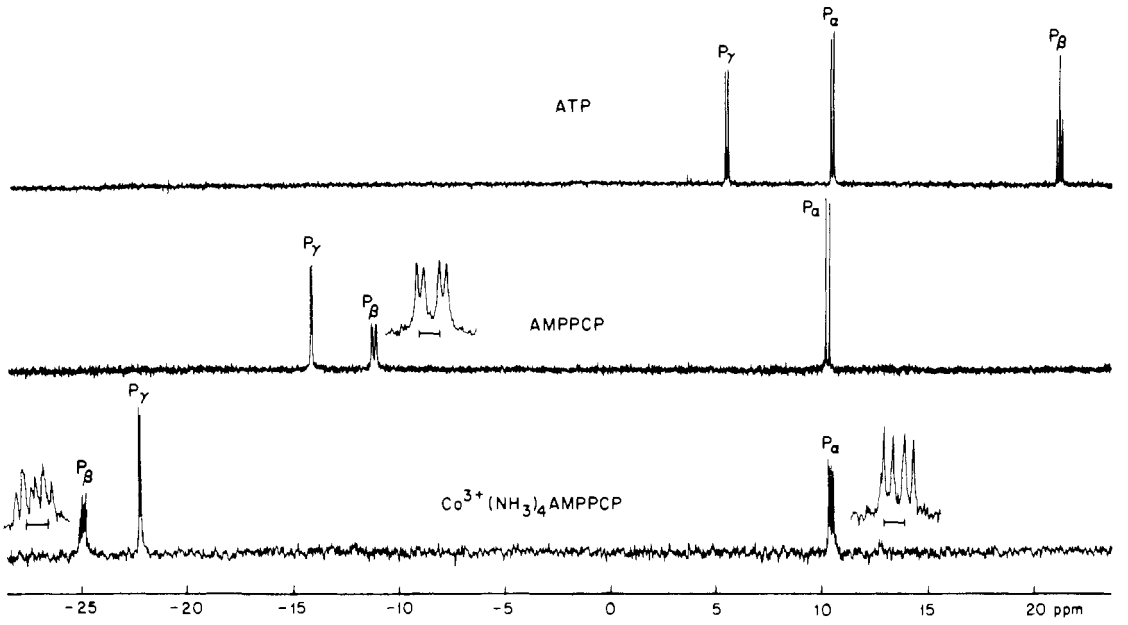


FIGURE 1: ^{31}P NMR spectra (at 145.8 MHz) of ATP, AMPPCP, and $Co(NH_3)_4$AMPPCP, pH 8.2, at 25 °C. The solutions contained 1 mM EDTA and 20% D_2O. Shifts are relative to 85% H_3PO_4. The inserts represent expanded multiplets. The bars correspond to 20 Hz.

cleotide. Unless otherwise specified, solutions used in the experiments contained 50 mM Tris-HCl buffer, pH 7.5, 150 mM KCl, and 0.1 or 1.0 mM dithiothreitol. Solutions used in the ^1H NMR experiments were made up by using deuterium oxide (99.7%) and contained 10 mM deuterated Tris-HCl buffer, pD 7.5. Trace metal impurities were removed from the solutions by passage through Chelex-100 (Bio-Rad). Enzyme solutions were deuterated by repeated concentration and dilution by vacuum filtration using Millipore immersible ultrafiltration units. 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). After prolonged NMR experiments, the enzyme was found to retain 70–90% of its activity.

Magnetic Resonance Measurements. Binding studies were carried out by the PRR and EPR methods as previously described (Armstrong et al., 1979a). PRR measurements were done at 24.3 MHz using a spectrometer described elsewhere (Mildvan & Engle, 1972). EPR spectra were recorded at 9.15 GHz on a Varian E-4 spectrometer. ^1H NMR spectra were recorded on a Bruker WH 180/360 spectrometer operating at 360 MHz and on a Varian XL-100-FT spectrometer operating at 100 MHz. Longitudinal relaxation times (T_1) were determined by using a 180°–τ–90° pulse sequence. Transverse relaxation times (T_2) were calculated from line width ($\Delta\nu$) measurements using the relation $T_2^{-1} = \pi\Delta\nu$. ^{31}P NMR spectra were recorded at 145.8 MHz on a Bruker WH 180/360 spectrometer. The EPR and PRR measurements were carried out at 22 °C and the NMR measurements were carried out at 25 °C.

Results and Discussion

^{31}P NMR Characterization of the $Co(NH_3)_4$AMPPCP Complex. The proton-decoupled ^{31}P NMR spectra at 145.8 MHz, pH 8.2, of ATP, AMPPCP, and $Co(NH_3)_4$AMPPCP are shown in Figure 1. Chemical-shift and spin coupling data are given in Table I. Introduction of the methylene group between the β- and γ-phosphates of ATP causes a marked change in the chemical shifts of P_β and P_γ (i.e., 32.3- and 19.6-ppm downfield shifts, respectively) and in their spin coupling constants (i.e. $J_{\alpha\beta}$ increases by 6.3 Hz, while $J_{\beta\gamma}$ decreases by 11.8 Hz). Upon formation of the $Co(NH_3)_4$AMPPCP complex, P_β and P_γ are further shifted downfield (coordination shifts of 13.8 and 8.1 ppm, respectively), accompanied by small changes in the coupling constants. As with $Co(NH_3)_4$ATP (Cornelius et al., 1977; Granot et al., 1979a), the different absolute configuration about the β-phosphorus gives rise to chemical shift differences between the two stereoisomers of $Co(NH_3)_4$AMPPCP, which are 12, 9, and <2 Hz for P_α, P_β, and P_γ, respectively.

Table I: ^{31}P Chemical Shifts and Coupling Constants at 145.8 MHz, pH 8.2, and 25 °C of ATP, AMPPCP, and $Co(NH_3)_4$AMPPCP[a]

	chemical shift (ppm)[b]			coupling constants (Hz)			
	P_α	P_β	P_γ	$J_{\alpha\beta}$	$J_{\beta\gamma}$	$J_{\beta H_2}$	$J_{\gamma H_2}$
ATP	10.54	21.26	5.56	19.8	19.8		
AMPPCP	10.30	−11.07	−14.05	26.1	8.0	20.9	19.5
$Co(NH_3)_4$AMPPCP (Δ)[c]	10.54	−24.79	−22.11	24.7	10.3		
$Co(NH_3)_4$AMPPCP (Λ)[c]	10.46	−24.85	−22.11	24.7	10.3		

[a] The uncertainty in the chemical shifts is 0.01 ppm and in the coupling constants is 0.3 Hz. [b] Relative to 85% H_3PO_4 external reference. Positive values are upfield. [c] This assignment of Δ and Λ was made by analogy to the $Co(NH_3)_4$ATP complex (Cornelius et al., 1977; Granot et al., 1979a).

Binding Studies. (1) Mn^{2+}–E–$Co(NH_3)_4$AMPPCP. The binding of Mn^{2+} to the enzyme–$Co(NH_3)_4$AMPPCP complex was studied by PRR and EPR. A mixture of enzyme (126 μM) and $Co(NH_3)_4$AMPPCP (324 μM) was titrated with $MnCl_2$ (67–402 μM). The data shown in Figure 2A in the form of a Scatchard plot yield 1.25 ± 0.20 tight Mn^{2+} binding sites with K_D = 172 ± 20 μM. The stoichiometry and affinity for Mn^{2+} binding to the enzyme–$Co(NH_3)_4$AMPPCP complex are very similar to those found with the active enzyme–$Co(NH_3)_4$ATP complex (Table II). Next, a solution containing the enzyme (59 μM) and $MnCl_2$ (101 μM) was titrated with an otherwise identical solution which also contained $Co(NH_3)_4$AMPPCP, and the PRR of water was measured. The best fit to the data (Figure 2B) was obtained by assuming a

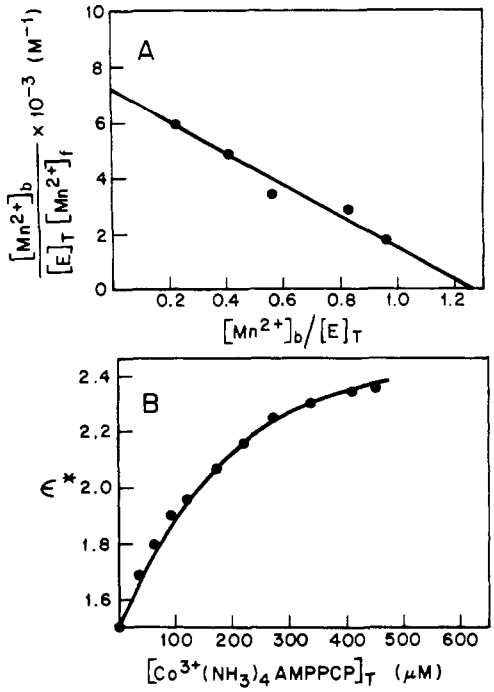


FIGURE 2: (A) Scatchard plot of the binding of Mn^{2+} to the E–$Co(NH_3)_4$AMPPCP complex. The concentrations of the enzyme and of $Co(NH_3)_4$AMPPCP are 125.7 and 324 μM, respectively. (B) Effect of $Co(NH_3)_4$AMPPCP binding to protein kinase (59.4 μM) in the presence of Mn^{2+} (101 μM) on the enhancement of PRR of water. The solutions also contained 50 mM Tris-HCl buffer, pH 7.5, 150 mM KCl, and 1 mM dithiothreitol. T = 22 °C.

Table II: Dissociation Constants and Enhancement Factors of Mn^{2+}-Protein Kinase-Nucleotide-Metal Systems[a]

SM	K_D (μM)[b] E-SM	Mn^{2+}E-SM	Mn^{2+}-ESM	ϵ_{Mn}[c]	ref
$Co(NH_3)_4$-AMPPCP	281	48	172	10.4 ± 1.0	*e*
$Co(NH_3)_4$ATP	301	39	130	9.6 ± 1.0	*d*
CrAMPPCP	25	1.4	55	4.2 ± 0.5	*e*
MnAMPPCP	30	1.8	60	*f*	*d*

[a] Conditions are as given in Figure 2. [b] K_D(E-SM) = [E][SM]/[ESM]; K_D(Mn^{2+}E-SM) = [Mn^{2+}E][SM]/[Mn^{2+}ESM]; K_D(Mn^{2+}-ESM) = [Mn^{2+}][ESM]/[Mn^{2+}ESM]. Estimated errors in these parameters are ±30%. [c] ϵ_{Mn} is the enhancement due to Mn^{2+} binding at the inhibitory site of the enzyme. [d] Armstrong et al. (1979a). [e] This work. [f] An average ϵ of 7.0 ± was found for Mn^{2+} bound at both the inhibitory and activating sites of the Mn^{2+}-E-AMPPCP-Mn^{2+}. This value of ϵ could not be resolved into the separate ϵ_{Mn} values for each bound Mn^{2+}.

single nucleotide binding site and a single Mn^{2+} binding site by using the dissociation constants of Table II. The stoichiometry and the dissociation constants and the enhancement factor of the Mn^{2+}–E–$Co(NH_3)_4$AMPPCP system (Table II), are indistinguishable, within experimental error, from those previously found with the substrate $Co(NH_3)_4$ATP (Armstrong et al., 1979a).

(*2*) *Mn^{2+}–E–CrAMPPCP.* The binding of Mn^{2+} to the enzyme–CrAMPPCP complex was studied by PRR and EPR by titrating a mixture of enzyme (54 μM) and CrAMPPCP (126 μM) with $MnCl_2$ (23–233 μM).[2] The data shown in

[2] The solutions containing enzyme and CrAMPPCP at pH 7.5 were not used for periods exceeding 5 min because of the slow decomposition of Cr–nucleotide complexes at pH values greater than 6.8 (Cleland & Mildvan, 1979).

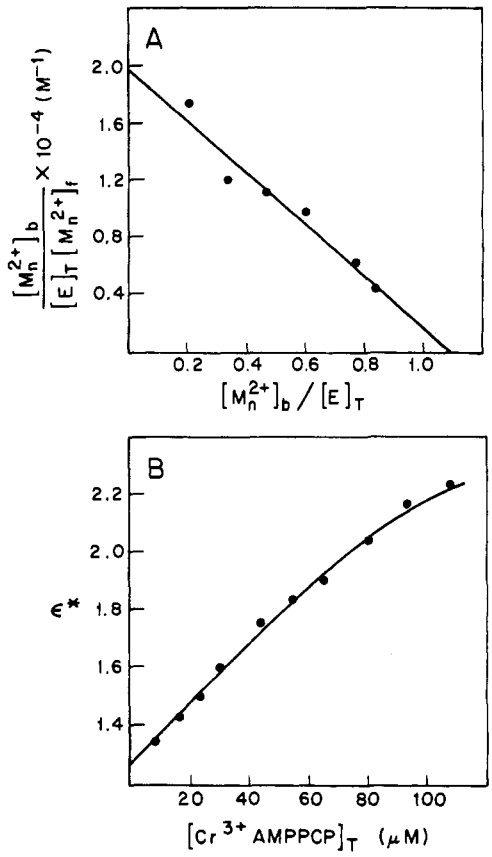


FIGURE 3: (A) Scatchard plot of the binding of Mn^{2+} to the E–CrAMPPCP complex. The concentrations of the enzyme and CrAMPPCP are 53 and 126 μM, respectively. (B) Effect of CrAMPPCP binding to protein kinase (64.6 μM) in the presence of Mn^{2+} (83.3 μM) on the enhancement of PRR of water due to the binding of Mn^{2+} to the enzyme. Other components and conditions are as given in Figure 2.

Figure 3A yield 1.1 ± 0.1 tight Mn^{2+} binding sites with K_D = 55 ± 10 μM. Since CrAMPPCP is paramagnetic [by contrast with the diamagnetic $Co(NH_3)_4$AMPPCP], its binding to protein kinase could be monitored by PRR even in the absence of Mn^{2+}. Analysis of the enhancement data obtained by titrating an enzyme solution (65 μM) with CrAMPPCP (8.2–320 μM) yielded a 1:1 enzyme–nucleotide stoichiometry, a dissociation constant for the enzyme–CrAMPPCP complex of 25 ± 10 μM, and an enhancement factor of 1.4 ± 0.2. In the presence of 5.1 mM $MgCl_2$ a similar titration experiment yielded a 1:1 enzyme–CrAMPPCP stoichiometry with a lower dissociation constant of 8 ± 3 μM for the complex and an enhancement factor of 1.3 ± 0.2. The effect of Mn^{2+} binding to E–CrAMPPCP on the PRR of water was studied by titrating a solution containing the enzyme (65 μM) and Mn^{2+} (88.3 μM) with an otherwise identical solution that also contained CrAMPPCP. The enhancement data due to Mn^{2+} binding (i.e., corrected for the CrAMPPCP contribution) are shown in Figure 3B. The best-fit parameters which were used to calculate the theoretical curves in Figure 3 are given in Table II. The affinity and stoichiometry are in good agreement with those previously found with MnAMPPCP (Armstrong et al., 1979a).

Additional binding studies of CrAMPPCP to protein kinase have been carried out at pH 6.0. At this lower pH CrAMPPCP is more stable than at pH 7.5 (Cleland & Mildvan, 1979). Hence, pH 6.0 is more suitable for prolonged NMR experiments. Solutions used in the titration experiments, as described above, contained 30 mM K^+–Mes buffer,

Table III: Dissociation Constants and Enhancement Factors of Protein Kinase-CrAMPPCP Complexes

complex	pH	K_D (μM)[a]	ϵ_{Cr}[b]
E-CrAMPPCP	7.5[c]	25 ± 10	1.4 ± 0.2
Mg^{2+}-E-CrAMPPCP	7.5[c]	8 ± 3	1.3 ± 0.2
E-CrAMPPCP	6.0[d]	29 ± 10	1.6 ± 0.2
peptide-E-CrAMPPCP	6.0[d]	33 ± 10	1.7 ± 0.2

[a] K_D is the dissociation constant of CrAMPPCP from the indicated complex. [b] ϵ_{Cr} is the enhancement due to binding of CrAMPPCP to the enzyme. [c] Solutions contained 50 mM Tris-HCl, pH 7.5, 150 mM KCl, and 1.0 μM dithiothreitol; T = 22 °C. [d] Conditions as in footnote *c* except that 30 mM K^+-Mes buffer, pH 6.0, was also present.

pH 6.0, 150 mM KCl, and 1.0 mM dithiothreitol. The PRR measurements were carried out in the absence and presence of a saturating level of the peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly. The binding parameters are given in Table III. The absence of significant change in the dissociation constant of the enzyme–CrAMPPCP complex between pH 7.5 and pH 6.0 is consistent with the results of comparative kinetic studies carried out at these pH values using MgATP and the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly as substrates. Within the experimental error, no changes were detected in the K_m (4.8 μM) for MgATP and in the observed rate [1850 nmol/(mg min)] between pH 7.5 and pH 6.0, at 25 °C with 2 nM enzyme.

Structural Studies. (1) Determination of the Mn^{2+}-β,γ-Methylene Distance in the Mn^{2+}-E-$Co(NH_3)_4$AMPPCP Complex. In a previous study it has been shown by using $Co(NH_3)_4$ATP that the inhibitory Mn^{2+} ion which binds at the active site of protein kinase bridges the enzyme to the triphosphate chain of the nucleotide with Mn^{2+}–phosphorus distances of ~3 Å (Granot et al., 1979a). By use of $Co(NH_3)_4$AMPPCP, an additional distance of interest can be determined, i.e., between Mn^{2+} and the β,γ-methylene protons, to provide supporting information regarding the location of the inhibitory metal site. The effects of varying amounts of Mn^{2+} on the ^1H relaxation rates of the β,γ-methylene protons of $Co(NH_3)_4$AMPPCP were measured at 100 and 360 MHz in the absence and presence of enzyme. The results exemplified in Figure 4 reveal a marked enhancement of the effect of Mn^{2+} on $1/T_1$ caused by the enzyme. Bound-state values, $1/(fT_{1p})$ and $1/(fT_{2p})$, obtained by analysis of the relaxation data using the dissociation constants of Table II, and the frequency dependence ratio of T_{1p}^{-1} between 100 and 360 MHz for the binary Mn^{2+}–$Co(NH_3)_4$AMPPCP and the ternary Mn^{2+}–E–$Co(NH_3)_4$AMPPCP complexes are given in Table IV. The $1/(fT_{2p})$ values obtained for the binary and the ternary complexes set lower limits to $1/\tau_M$, the exchange rate of $Co(NH_3)_4$AMPPCP between the free and the bound states, which are six- to eightfold greater than the corresponding $1/(fT_{1p})$ values (Table V). Hence, the latter are not limited by chemical exchange and may be used for distance calculations. Assuming that the relaxation in the binary complex is dominated by the rotational correlation time, which is frequency independent, we get a value of 0.3 ± 0.1 ns for the correlation time (τ_c). For the ternary complex, by assuming either that the correlation time is frequency independent or that it has maximum frequency dependence (Bean et al., 1977), we get for the correlation time values at 360 MHz of 0.8 ± 0.3 ns and 3.0 ± 1.0 ns, respectively, with an average of 1.9 ± 1.2 ns. The Mn^{2+}–CH_2 distances calculated with the

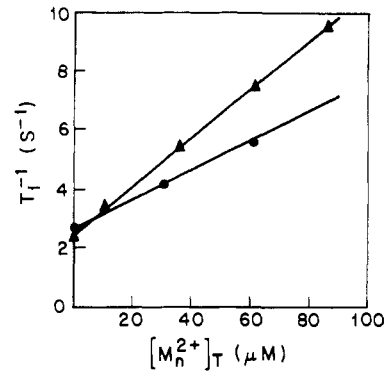


FIGURE 4: Effect of Mn^{2+} on the longitudinal relaxation rate of the β,γ-methylene protons of $Co(NH_3)_4$AMPPCP in the absence (●) and presence (▲) of protein kinase (29 μM). The $Co(NH_3)_4$AMPPCP concentrations are 8.0 (●) and 9.3 mM (▲). Other conditions were as described in Figure 2 except that the solvent was D_2O and T = 25 °C.

Table IV: Paramagnetic Bound-State Relaxation Rates of the β,γ-CH_2 Protons, Mn^{2+} Correlation Times, and Mn^{2+}–CH_2 Distances in the Mn^{2+}–$Co(NH_3)_4$AMPPCP and Mn^{2+}–E–$Co(NH_3)_4$AMPPCP Complexes

complex	$1/(fT_{1p})^a \times 10^{-3}$ (s^{-1})	$1/(fT_{2p})^a \times 10^{-3}$ (s^{-1})	$[T_{1p}(360\text{ MHz})]/[T_{1p}(100\text{ MHz})]$	τ_c^a (ns)	$R(Mn^{2+}$–$CH_2)$ (A)
Mn^{2+}–$Co(NH_3)_4$AMPPCP	1.0 ± 0.2	5.8 ± 1.0	1.4 ± 0.4	0.3 ± 0.1	7.4 ± 0.5
Mn^{2+}–E–$Co(NH_3)_4$AMPPCP	5.5 ± 1.5	44 ± 25	3.5 ± 1.5	1.9 ± 1.2	5.0 ± 0.9

[a] At 360 MHz.

Table V: Paramagnetic Effects on the Relaxation Rates at 100 MHz of the C_β–H_2 Protons of the Seryl Residue of Leu-Arg-Arg-Ala-Ser-Leu-Gly Bound to the E-CrAMPPCP and Mn^{2+}–E–$Co(NH_3)_4$AMPPCP Complexes

		concn (μM)					relaxation rates (s^{-1})					
expt	pD	enzyme	peptide	CrAMPPCP	$Co(NH_3)_4$-AMPPCP	Mn^{2+}	T_1^{-1}	T_{1p}^{-1}	$1/(fT_{1p})$	T_2^{-1}	T_{2p}^{-1}	$1/(fT_{2p})$
I	6.0[a]	72	7800				3.41			31		
				37.9			5.11	1.70	566	44	13	3800
				75.0			5.92	2.51	499	50	19	4300
				111.2[c]			3.56	0.15		33	2	
II	7.5[b]	80	7800		2400		3.05			31		
						126.0	4.81	1.76	551	38	7	2300
						251.1	5.67	2.62	529	41	10	2100
						251.1[c]	3.28	0.23		32	1	

[a] Solutions in D_2O contained 10 mM K^+-Pipes, pD 6.0, 150 mM KCl, and 0.1 mM dithiothreitol. T = 25 °C. [b] Conditions as in footnote *a* except that pD was 7.5. [c] After addition of 280 μM polyarginine.

above correlation times are given in Table IV. These distances are consistent with our previous findings with $Co(NH_3)_4ATP$ using ^1H and ^{31}P NMR (Granot et al., 1979a) that Mn^{2+} binds to the adenine ring in the binary complex and to the triphosphate chain of the nucleotide in the ternary complex.

(2) Determination of the Mn^{2+}–Cr^{3+} Distance in the Mn^{2+}–E–CrAMPPCP Complex. It has been recently shown (Gupta, 1977) that the effect of cross relaxation between the unpaired spins of two nearby paramagnetic metal ions may be used to determine the intermetallic distance. This method has been applied here to the Mn^{2+}–E–CrAMPPCP system which is a favorable one since both metal ions are bound to the same polyphosphate chain of the enzyme-bound nucleotide. Indeed, comparison of the enhancement factors in Table II shows that replacing the diamagnetic Co^{3+} by the paramagnetic Cr^{3+} at the nucleotide binding sites results in a 2.5-fold decrease in the enhancement of the PRR of water due to Mn^{2+} bound at the inhibitory site. Since the rotational correlation time of the enzyme complexes is estimated by Stokes' law to be $\sim 1 \times 10^{-8}$ s, while the observed enhancement factors correspond to correlation times of $\sim 1 \times 10^{-9}$ s, the correlation time is dominated by the shorter electron spin relaxation of Mn^{2+} or Cr^{3+}. Therefore, eq 1a (Gupta, 1977) can be used to calculate the Mn^{2+}–Cr^{3+} distance (r_{Mn-Cr}):

$$r_{Mn-Cr} = C[\tau_s^{Cr}/\Delta(1/\tau_s^{Mn})]^{1/6} \quad (1a)$$

$$\Delta(1/\tau_s^{Mn}) = 1/\tau_s^{Mn-Cr} - 1/\tau_s^{Mn-Co} \quad (1b)$$

where C is a constant equal to 6.13×10^3 for the Cr^{3+}–Mn^{2+} interaction, τ_s^{Mn-Cr} and τ_s^{Mn-Co} are the electron spin relaxation times of the Mn^{2+} bound to the E–CrAMPPCP or E–Co$(NH_3)_4$AMPPCP complexes, respectively, and τ_s^{Cr} is the electron spin relaxation time of Cr^{3+} in the Mn^{2+}–E–CrAMPPCP complex.

The enhancement data can be used to calculate the electron spin relaxation times of Mn^{2+} by using the relations

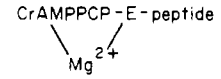
$$\epsilon_{Mn} = \frac{q}{6}\frac{F(\tau_s)}{F(\tau_r)} \quad (2a)$$

$$F(\tau) = \frac{3\tau}{1 + \omega_1^2\tau^2} + \frac{7\tau}{1 + \omega_s^2\tau^2} \quad (2b)$$

In eq 2a ϵ_{Mn} is the enhancement factor due to enzyme-bound Mn^{2+} of the PRR of water in the ternary complex, $\tau_r = 3 \times 10^{-11}$ s is the rotational correlation time for the water molecules in the aquo–Mn^{2+} complex, and q is the number of water molecules coordinated to the enzyme-bound Mn^{2+}. Since Mn^{2+} has a coordination number of 6 and, when bound to protein kinase, it is coordinated by three oxygens of the triphosphate chain of the nucleotide (Granot et al., 1979a) and by one or two ligands from the enzyme, q can be either 2 or 1, yielding $\tau_s^{Mn-Co} = 2.0 \pm 0.8$ ns and $\tau_s^{Mn-Cr} = 0.74 \pm 0.25$ ns. Similarly, using eq 2 with a value of 1.4×10^{-10} s for the Cr^{3+} electron spin relaxation time found for the unbound CrATP complex (Gupta et al., 1976) and the measured enhancement factor of 1.4 for the enzyme-bound CrATP, we get $\tau_s^{Cr} = 2.0 \times 10^{-10}$ s, making the reasonable assumptions that the coordination sphere of Cr^{3+} does not change upon binding of the Cr^{3+}–nucleotide complex to the enzyme (Cleland & Mildvan, 1979) and that the effect of Mn^{2+} on the electron spin relaxation time of Cr^{3+} is negligible (Gupta, 1977). Substituting the above values for the correlation times in eq 1, we get $r_{Mn-Cr} = 4.8 \pm 0.4$ Å. This result agrees well with the intermetallic distances obtained on a molecular model in which two metal ions were coordinated to the same triphosphate chain (5.2 Å) and from the best-fit conformation found by a computer search program for Mn^{2+}–Co$(NH_3)_4$ATP bound to protein kinase [4.8 Å (Granot et al., 1979a)].

(3) Determination of the Intersubstrate Distance on Protein Kinase. Using the two paramagnetic reference points at the nucleotide site of protein kinase, i.e., Cr^{3+} on the nucleotide and Mn^{2+} at the inhibitory site, intersubstrate distances to the bound peptide substrate can be determined. From these values, the distance along the reaction coordinate for phosphoryl transfer, namely, that between the γ-phosphorus of the bound nucleotide and the serine hydroxyl oxygen of the bound peptide substrate, can be estimated.

The effects of varying amounts of CrAMPPCP on the relaxation of the serine methylene protons (C_β–H_2)[3] of the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly, in the presence of enzyme, at 100 MHz, are given in Table V. In the absence of enzyme no effect was detected, within the experimental error, of CrAMPPCP on the relaxation rates of the peptide protons. The observed enhancement of the relaxation rates of the serine methylene protons in the presence of enzyme and CrAMPPCP was largely abolished by addition of polyarginine (cf. Table V), a competitive inhibitor with respect to the peptide (Demaille et al., 1977), thus establishing that the observed effects are due to binding at the active site. A T_{1p}/T_{2p} ratio of ~ 8 (Table V) indicates that $1/(fT_{1p})$ is not limited by chemical exchange. Additional measurements at 360 MHz yielded a frequency dependence of T_{1p}^{-1}, $[T_{1p}^{-1}(100\ MHz)]/[T_{1p}^{-1}(360\ MHz)] = 1.8 \pm 0.5$, from which we estimate a correlation time of $(4.3 \pm 1.5) \times 10^{-10}$ s for Cr^{3+} at 100 MHz, which is in the range of previously determined values for Cr^{3+} (Gupta et al., 1976; Cleland & Mildvan, 1979). This correlation time, together with an average bound state value for the longitudinal relaxation rate of 530 ± 150 s^{-1} (from Table V), yields a Cr^{3+} to Ser (C_β–H_2) distance of 8.1 ± 0.8 Å. Addition of a saturating level of Mg^{2+} (5.8 mM $MgCl_2$) to form the complex



did not change $1/(fT_{1p})$ of the peptide protons. Hence, the binding of a divalent cation at the inhibitory site does not significantly affect the intersubstrate distance or arrangement, although it lowers the dissociation constant of the metal–nucleotide–enzyme complex.

The effects of Mn^{2+} on the relaxation rates of the serine methylene protons in the presence of enzyme and Co$(NH_3)_4$AMPPCP at 100 MHz are given in Table V. No significant effect of Mn^{2+} on the relaxation rates was observed in the absence of enzyme. A T_{1p}/T_{2p} ratio of ~ 4 may indicate that $1/(fT_{1p})$ has a small contribution (<25%) due to chemical exchange of the peptide between free and enzyme-bound states. This effect, which is within the experimental error of $1/(fT_{1p})$, would have a negligible contribution to the calculated distance. Using an average value of $1/(fT_{1p}) = 540 \pm 150$ s^{-1} from Table V and the correlation time, obtained above, for Mn^{2+} in the Mn^{2+}–E–Co$(NH_3)_4$AMPPCP complex, we get a Mn^{2+} to Ser (C_β–H_2) distance of 9.1 ± 0.9 Å.

Figure 5 depicts the arrangement of the substrates and metal ions at the active site of protein kinase, based on the present intersubstrate distances and previous studies of the conformation and metal coordination of the enzyme-bound nucleotide (Granot et al., 1979a,b). Using Dreiding molecular models,

[3] The assignment of the proton resonances of the peptide substrate and analogues has been described elsewhere (Granot et al., 1980).

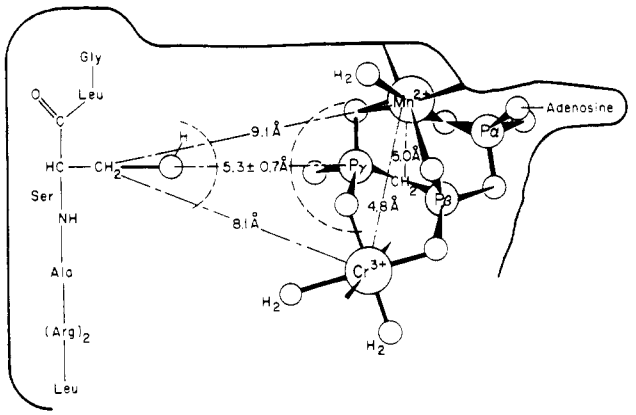


FIGURE 5: Arrangement of substrates and metal ions at the active site of protein kinase. CrAMPPCP and Leu-Arg-Arg-Ala-Ser-Leu-Gly are bound at the substrate sites, and Mn^{2+} is bound at the inhibitory site. The distance of 5.3 ± 0.7 Å along the reaction coordinate for phosphoryl transfer was obtained by model building using the indicated experimental distances. The dashed arcs show the van der Waals radii of the serine hydroxyl oxygen and the γ-phosphorus atom of ATP.

together with the measured distances between Cr^{3+} and Mn^{2+} and the serine methylene, assuming that the active substrates and their analogues bind in an identical manner, we can estimate a distance between the γ-phosphorus of the nucleotide and the serine hydroxyl oxygen of 5.3 ± 0.7 Å. This distance along the reaction coordinate for phosphoryl transfer is significantly greater than that predicted for van der Waals contact between phosphorus and oxygen (3.3 Å) (Figure 5) and is in a range which would permit a dissociative mechanism [≥4.9 Å (Mildvan, 1979)] or even a phosphoenzyme intermediate. However, the finding that the substitution-inert complex β,γ-bidentate $Co(NH_3)_4ATP$ (Δ isomer) is a substrate for protein kinase (Granot et al., 1979b) and the absence of ping-pong kinetics for peptide phosphorylation by MgATP (Bolen et al., 1980) argue against an E–P intermediate. From recent model studies of phosphoryl transfer reaction mechanisms, the coordination of the γ-phosphate by a single metal, the activating divalent cation, would not be expected to prevent the formation of the metaphosphate intermediate in a dissociative mechanism (Satterthwait & Westheimer, 1980). However, coordination of the γ-phosphate by a second metal would be expected to inhibit metaphosphate formation (Benkovic & Schray, 1973). A dissociative mechanism (Figure 6A) is therefore further supported by the strong inhibition of the phosphoryl transfer produced by the coordination of the γ-phosphoryl group of ATP by a second metal ion (Armstrong et al., 1979a; Granot et al., 1979a) and the present finding that this inhibitory metal ion does not alter the intersubstrate distance or geometry. A previous kinetic observation (Armstrong et al., 1979b) that the rates of both the ATPase and the peptide kinase reactions catalyzed by protein kinase decrease by the same factor (~18-fold) when Mg^{2+} is replaced by Mn^{2+}, even though the ATPase reaction is more than 2 orders of magnitude slower than the peptide kinase reaction, suggests that bond breaking between ADP and the γ-P is not concerted with bond formation between the γ-P and the entering nucleophile. Since an E–P intermediate is rendered unlikely by the arguments given above, a reasonable explanation for these kinetic data is the existence of a metaphosphate intermediate. The finding that the ATPase reaction rate catalyzed by another protein kinase, from bovine brain, is not inhibited but actually increased by the presence of the histone substrate (Moll & Kaiser, 1976) is also compatible with a reaction coordinate distance between ATP and serine exceeding molecular contact and with a dissociative mechanism.

A P–O distance significantly less than 4.9 Å would suggest an associative mechanism (Figure 6B) for the phosphoryl transfer (Mildvan et al., 1976; Li et al., 1979). Although the present distance of 5.3 ± 0.7 Å may be too large for an associative mechanism, the fact that the activating metal is β,γ coordinated (Granot et al., 1979b) raises the possibility that the mechanism may have some associative character (Mildvan, 1979). Indeed, an associative mechanism remains possible because the intersubstrate distances may decrease as the transition state is approached.

Conclusion

The present work demonstrates the use of substitution-inert complexes of Co^{3+} and Cr^{3+} in studies of protein kinase. The similarity in stoichiometry, affinity, and structure of the AMPPCP complexes of protein kinase to those of the corresponding ATP complexes strongly supports active-site binding of these nucleotide analogues. The use of the AMPPCP complexes rather than those of ATP has the advantage that it prevents the occurrence of phosphoryl transfer when the enzyme and a phosphoryl acceptor substrate are present. Another advantage of $Co(NH_3)_4AMPPCP$ is the larger ^{31}P chemical-shift separation between the phosphorus resonances relative to $Co(NH_3)_4ATP$, which permits better resolution even at lower magnetic fields. Furthermore, the β,γ-methylene group that is incorporated into the polyphosphate chain of ATP

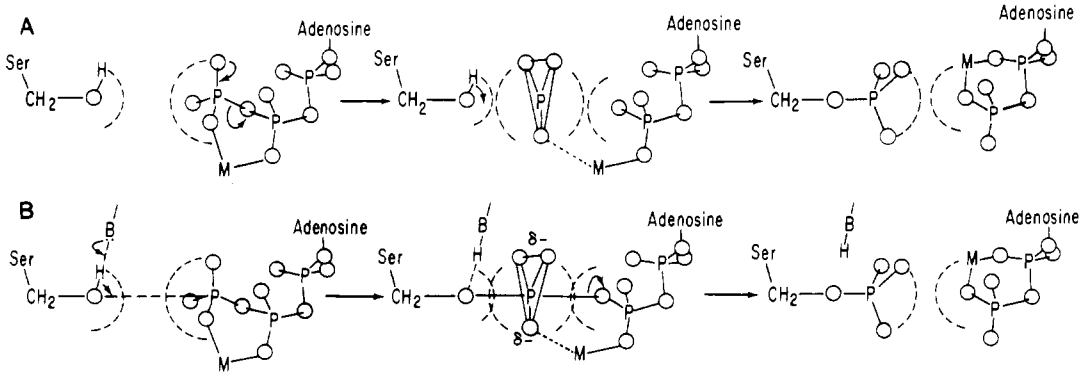


FIGURE 6: Possible mechanisms of phosphoryl transfer catalyzed by protein kinase. The dissociative mechanism (A) is shown with a reaction coordinate distance of 5.3 Å, and the associative mechanism (B) is shown with the lower limit reaction coordinate distance of 4.6 Å in accord with the geometry of Figure 5. In the intermediates, the metal is shown as coordinated to the phosphoryl group undergoing transfer, although the alternative possibility that the metal is no longer so coordinated at this stage cannot be ruled out. The associative mechanism (B) requires a base while the dissociative mechanism (A) does not necessarily require one.

can provide additional structural information from ^1H NMR studies. The binding constants of CrAMPPCP are similar to those of MnAMPPCP (cf. Table II). However, while with Mn^{2+} and AMPPCP two metal ions can bind simultaneously to the enzyme-bound nucleotide, thus leading to ambiguity in the analysis of the Mn^{2+} effects, the use of the exchange-inert CrAMPPCP complex overcomes this problem and may be useful in obtaining structural information with the paramagnetic reference point being only at the nucleotide metal binding site. The results obtained by using CrAMPPCP and Mn^{2+}–$Co(NH_3)_4$AMPPCP are mutually complementary, and the use of both is therefore recommended whenever possible, with enzymes which bind two metal ions at the active site.

The experimental results presented in this work support the previously proposed location for the inhibitory metal on protein kinase, i.e., as a bridge between the enzyme and the triphosphate chain of the enzyme-bound nucleotide. The finding that the distance between the γ-phosphorus of the enzyme-bound nucleotide and the serine hydroxyl oxygen of the enzyme-bound peptide is larger than 4.6 Å suggests a dissociative mechanism for the phosphoryl transfer reaction, provided that no further motion of the substrates occurs as the transition state is approached.

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Kinetic and Chemical Mechanisms of Yeast Formate Dehydrogenase†

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ABSTRACT: Yeast formate dehydrogenase has an ordered kinetic mechanism with NAD adding before formate. NAD analogues are substrates, but formate is the only molecule oxidized. Anions are competitive inhibitors vs. formate and bind only to E–NAD. Linear triatomic anions are the best inhibitors, with azide (K_i = 7 nM) showing tight binding inhibition behavior and appearing to be a transition-state analogue. pH profiles of the kinetic parameters show that a group in E–NAD with a pK of 8.3 must be protonated for binding of azide and formate. Since the pK is elevated to 9.8 upon formate binding, it is probably a cationic acid involved in substrate binding. A group with pK = 6.4 and no temperature dependence must be ionized for binding of azide and formate, and another group with pK = 5.9 must be ionized for catalysis, but the roles of these groups in the mechanism are not clear. A ^{13}C isotope effect of 1.043 with either unlabeled or deuterated formate shows that formate has a very low commitment to catalysis, and all isotope effects observed are intrinsic ones on the chemical reaction. The deuterium isotope effect on $V/K_{formate}$ varies with the nature of the nucleotide, presumably as the result of changes in transition-state structure. The value of 2.8 with NAD probably results from a late transition state, while the values of 4.4 for thio-NAD, 6.9 for acetylpyridine-NAD, and 3.8 for pyridinecarboxaldehyde-NAD represent progressively earlier transition states. By contrast, the ^{13}C isotope effect drops slightly to 1.036 with acetylpyridine-NAD. These data are explained if both a higher redox potential (that is, more electrophilic C-4) for the nucleotide and an increased distance between formate and nucleotide in the transition state (indicated by a lower V_{max} and increased activation energy for the reaction) cause the transition state to become earlier.

Yeast formate dehydrogenase (formate:NAD oxidoreductase, EC 1.2.1.2) catalyzes the oxidation of formate by NAD as shown in eq 1. The enzyme will utilize a number of NAD

$$NAD^+ + HCO_2^- \rightarrow CO_2 + NADH \quad (1)$$

analogues but is apparently specific for formate. Because the reaction is essentially irreversible and involves the simple transfer of hydrogen from formate to NAD, one can simul-

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