Nuclear Magnetic Resonance Studies of the Conformation and Kinetics of the Peptide–Substrate at the Active Site of Bovine Heart Protein Kinase†

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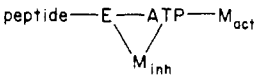
ABSTRACT: The conformation of the enzyme-bound peptide substrate on protein kinase from bovine heart has been studied by measurements of paramagnetic effects on the longitudinal relaxation rates of the protons of the peptide substrates Leu-Arg-Arg-Ala-Ser-Leu-Gly and Leu-Arg-Arg-Tyr-Ser-Leu-Gly and the analogue Leu-Arg-Arg-Ala-Ala-Leu-Gly. Two metal ions which served separately as reference points for distance measurements were Cr^{3+} at the metal activator site on the enzyme-bound nucleotide or Mn^{2+} at the inhibitory site which bridges the enzyme and the nucleotide. The relaxation rates of carbon-bound protons of the peptides measured at 100 and 360 MHz yielded distances from Cr^{3+} or Mn^{2+} to these protons (8.1 to ≥13 Å) which were used in the construction of molecular models employed in conformational studies. The measured distances are not compatible with α-helical and β-pleated-sheet conformations for the enzyme-bound heptapeptides. The two remaining classes of secondary structure, β turns and coils, are consistent with the measured distances. Kinetic measurements using a heptapeptide in which Leu^6 was replaced by Pro^6 together with data from the literature were used to exclude the obligatory requirement for any β turn possible within the heptapeptide studied, although an enzymatic preference for a β_{2-5} or β_{3-6} turn is possible. Hence if protein kinase has an absolute requirement for a specific secondary structure, then this structure must be a coil. The rate constant ($\sim 10^3$ s^{-1}) and kinetic parameters for the dissociation of the peptide from the enzyme complex were determined from the temperature dependence of the transverse relaxation rates. The rate constant for peptide binding ($\sim 10^7$ M^{-1} s^{-1}), determined from the transverse relaxation rate and the equilibrium constant, is smaller than expected for a diffusion-controlled reaction and may indicate that a conformational change occurs during peptide binding. In the phosphorylated Ser peptide the pK_a of the phosphate (5.8 ± 0.1) was found to be indistinguishable from that of phosphoserine or its di- and tripeptides, which argues against intramolecular interactions of the negatively charged phosphate with the positively charged arginyl residues of the free phosphopeptide.

Dissociation of the bovine heart protein kinase holoenzyme by cAMP has recently been shown to activate the enzyme by allowing the binding of peptide (or protein) substrates at the active site of the enzyme (Granot et al., 1980a). This protein-substrate site is blocked by the regulatory subunit when it is bound to the catalytic subunit. Kinetic studies with synthetic peptides in which the amino acid sequence was systematically varied (Krebs & Beavo, 1979; Carlson et al., 1979) have established the importance of the primary structure of the peptide or protein substrate for optimum binding and phosphorylation. The amino acid sequence -Arg-Arg-X-Ser-Y- where X and Y are hydrophobic residues was found in a number of peptides which exhibit low K_m and high V_{max} values in their reactions with protein kinases. Because of the large number of conformations that flexible polypeptides can assume and because of the selectivity of enzymes in general for specific substrate conformations (Mildvan, 1977), it is of interest to determine the secondary structure of the enzyme-bound peptide and protein substrates of protein kinase. Indeed, structural properties beyond the primary sequence may well have a role in determining the substrate specificity of protein kinase (Krebs & Beavo, 1979; Carlson et al., 1979).

In this paper, we report NMR studies of the conformations of peptide substrates (the Ser peptide[1] and the Tyr peptide) and of a substrate analogue (the Ala peptide) when bound at the active site of the catalytic subunit of protein kinase. Protein kinase has two tight metal binding sites in the presence of a nucleotide, an activating site on the enzyme-bound nucleotide and an inhibitory site which bridges the enzyme to the polyphosphate chain of the bound nucleotide (Armstrong et al., 1979a; Granot et al., 1979a):



By the appropriate use of substitution-inert complexes such as $Co(NH_3)_4ATP$, $Co(NH_3)_4AMPPCP$, and CrAMPPCP which bind at the nucleotide site, distances to the protons of peptides bound at the protein substrate site were determined from two paramagnetic reference points, namely Cr^{3+} at the activating site or Mn^{2+} at the inhibitory site. Possible conformations for the enzyme-bound peptide substrate were examined by molecular model building using the experimental internuclear distances. These studies provide insight into the secondary structural requirements for the specificity of this enzyme.

Experimental Procedures

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

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[1] Abbreviations used: $Co(NH_3)_4ATP$, tetraamminecobalt(III) β,γ-ATP; AMPPCP, β,γ-methylene-ATP; $Co(NH_3)_4AMPPCP$, tetraamminecobalt(III) β,γ-(β,γ-methylene-ATP); CrAMPPCP, chromium β,γ-(β,γ-methylene-ATP); Ser peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; Ala peptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly; Tyr peptide, Leu-Arg-Arg-Tyr-Ser-Leu-Gly; DTT, dithiothreitol.

0006-2960/81/0420-0602$01.00/0

described (Armstrong et al., 1979a). The synthetic peptides Leu-Arg-Arg-Ala-Ser-Leu-Gly (Ser peptide), Leu-Arg-Arg-Ala-Ser(PO_3)-Leu-Gly (phosphorylated Ser peptide), Leu-Arg-Arg-Ala-Ala-Leu-Gly (Ala peptide), and Leu-Arg-Arg-Tyr-Ser-Leu-Gly (Tyr peptide) were prepared as previously described (Granot et al., 1980a). The racemic β,γ-bidentate complexes $Co(NH_3)_4ATP$, $Co(NH_3)_4AMPPCP$, and CrAMPPCP were prepared as previously described (Cornelius et al., 1977; Cleland & Mildvan, 1979). [γ-^{32}P]ATP was purchased from Amersham/Searle, and deuterated Tris 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 ATP 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 nucleotide. In general, solutions used in the experiments contained 50 mM Tris-Cl, pH 7.5, 150 mM KCl, and 0.1 or 1.0 mM DTT. Unless otherwise specified, solutions used in the ^{1}H NMR experiments were prepared in deuterium oxide and contained 10 mM deuterated Tris-Cl buffer, pD 7.5, 150 mM KCl, and 0.1 mM DTT. Enzyme solutions were deuterated by repeated concentration and dilution by vacuum filtration using Millipore immersible ultrafiltration units. Other deuterated solutions were made up by repeated lyophilization in deuterium oxide. Trace metal impurities were removed from the solutions by passage through Chelex-100 (Bio-Rad).

Kinetic Studies. 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 lasting up to 12 h the enzyme was found to retain 70–90% of its activity. Other kinetic studies, such as the determination of the K_I for $Co(NH_3)_4ATP$ and the K_m and V_{max} of the Ser and Tyr peptides, were also carried out by using the above method.

Synthesis of the Pro^6 Heptapeptide. This peptide, Leu-Arg-Arg-Ala-Ser-Pro-Gly, was synthesized on a solid support with the aid of a Beckman Model 990 peptide synthesizer with a program slightly modified from that described by Yamashiro & Li (1974a). All solvents and reagents were purified as in Kroon & Kaiser (1978). The first amino acid, Boc-Gly, was attached to the support (chloromethylated styrene–1% divinylbenzene copolymer, Pierce) by the method of Gisin & Merrifield (1972). Subsequent couplings of Boc amino acids were performed on the Beckman synthesizer with symmetric anhydrides (Yamashiro & Li, 1974b), generated with a 65-fold molar excess of Boc amino acid and a 3.0-fold molar excess of dicyclohexylcarbodiimide. Completeness of each coupling reaction was determined by the ninhydrin test (Kaiser et al., 1970) before proceeding. The completed peptide was cleaved from the resin with hydrofluoric acid (Sakakibara et al., 1967). After the mixture was washed with ethyl acetate, the products were extracted with 10% aqueous acetic acid.

The peptide was gel filtered on a Sephadex G-15 column in 10% acetic acid and further purified by ion-exchange chromatography on a CM-Sephadex C-25 column using a 25–500 mM gradient of ammonium acetate to elute peptide. Thin layer chromatography on silica using the solvent system butanol/pyridine/water/acetic acid 15:10:13:3 (v/v) showed a single spot. The amino acid analysis gave the following values: Ala (1.00), Arg (1.94), Gly (1.11), Leu (1.04), Pro (0.94), Ser (0.96).

The sequence was established by automated Edman degradation carried out by P. Kein in the laboratory of Professor R. Heinrikson, Department of Biochemistry, University of Chicago. From 3.0 μmol of starting material the following products were obtained: Leu^1 (3.0 μmol), Arg^2 (2.2 μmol), Arg^3 (2.4 μmol), Ala^4 (3.0 μmol), Ser^5 (0.60 μmol), Pro^6 (0.90 μmol), Gly^7 (0.40 μmol). The arginines were determined by high-pressure liquid chromatography while all other amino acids were determined by gas chromatography.

NMR Measurements. ^{1}H NMR spectra of the heptapeptides were recorded on a Varian XL-100-FT spectrometer operating at 100 MHz and on a Bruker WH 180/360 spectrometer operating at 360 MHz. ^{31}P NMR spectra were recorded on a Varian XL-100-FT NMR spectrometer at 40.5 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 measurements at half-height ($\Delta\nu$) by using the relation $1/T_2 = \pi\Delta\nu$. Selective proton irradiation was used to suppress the residual water signal in the ^{1}H spectra. Unless otherwise noted, the measurements were carried out at 25 °C. Temperature control was achieved by allowing dry nitrogen to flow in the annular space between the sample tube and a dewar. For the variable temperature studies at 360 MHz the temperatures were kept constant to within ±0.5 °C by a Bruker B-ST 100/760 control unit and were calibrated by using an ethylene glycol sample.

Results

Inhibition of Peptide Phosphorylation by $Co(NH_3)_4ATP$. In a previous study (Granot et al., 1979a) we have shown that $Co(NH_3)_4ATP$ is a linear competitive inhibitor with respect to MgATP. At very high enzyme concentrations the Δ isomer of the $Co(NH_3)_4ATP$ complex was found to be a slow substrate of protein kinase with a $V_{max} \sim 10^{-3}$ times that found with MgATP (Granot et al., 1979b). Kinetic measurements of the dependence on peptide concentration of the rate of peptide phosphorylation carried out at different $Co(NH_3)_4ATP$ concentrations indicate that $Co(NH_3)_4ATP$ is a classical noncompetitive inhibitor with respect to the Ser-peptide substrate (Figure 1). The data were analyzed by a least-squares program (Cleland, 1967), yielding an average K_I value of 270 ± 40 μM from the secondary, linear plots of the slopes and intercepts vs. the $Co(NH_3)_4ATP$ concentration. Since the concentration of MgATP in the experimental solutions was 5.6 μM and its Michaelis constant has been found to be 4.2 μM (Armstrong et al., 1979b), the true K_I value of $Co(NH_3)_4ATP$ was calculated to be 120 μM, in agreement with the inhibitor constant of 151 μM obtained previously by competition against MgATP (Granot et al., 1979a). The noncompetitive inhibition by $Co(NH_3)_4ATP$ as well as our recent finding that the Δ isomer of $Co(NH_3)_4ATP$ is utilized as a substrate by protein kinase indicates that the enzyme simultaneously binds both the peptide and $Co(NH_3)_4ATP$. Therefore, in structural studies of the bound peptide, $Co(NH_3)_4ATP$ can be used to occupy the nucleotide site.

Assignment of Peptide Resonances. Effect of Mn^{2+}. The ^{1}H NMR spectra of the Ser and Ala peptides are given in Figure 2. The assignments were obtained through selective homodecoupling and by using the available chemical shift and spin-coupling data on amino acids and peptides (Wüthrich, 1976). As a control for the enzyme studies, the effect of Mn^{2+} on the relaxation rates of the Ser, Tyr, and Ala peptides were measured for peptide concentrations of 4–6 mM and Mn^{2+} concentrations up to 320 μM. It was found that within the concentration range of Mn^{2+} appropriate for the studies in the presence of the enzyme (i.e., the free Mn^{2+} did not exceed 200

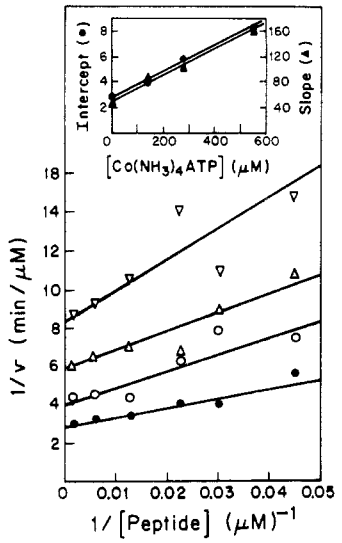

FIGURE 1: Double-reciprocal plot of the inhibition by $Co(NH_3)_4ATP$ of peptide phosphorylation by protein kinase. The assay mixture contained the catalytic subunit of protein kinase (6 nM), MgATP (5.6 μM), $MgCl_2$ (10 mM), BSA (1 mg/mL), Tris-Cl buffer (50 mM, pH 7.5), KCl (150 mM), and DTT (0.1 mM).

μM) the paramagnetic effects on both the longitudinal relaxation rates and the line widths of the peptide protons were undetectable within the experimental errors. Thus a correction for the paramagnetic contribution of the binary Mn^{2+}–peptide complex was found to be unnecessary. At higher Mn^{2+} concentrations (>200 μM) a detectable paramagnetic effect, larger on the T_1 values of Gly and Leu[6] relative to Leu[1], allowed differentiation between the C_δ-H_3 resonances of the two leucine residues (Figure 2).

NMR Studies of the $Co(NH_3)_4ATP$–Mn^{2+}–Enzyme–Peptide Complex. (1) Ser Peptide. The longitudinal and transverse relaxation rates of the protons of the Ser peptide (5.5 mM) were measured at 360 MHz in the presence of enzyme (135 μM), racemic $Co(NH_3)_4ATP$ (2.2 mM), and varying amounts of Mn^{2+} (0–139 μM). Although this mixture constitutes a complete system for the slow phosphorylation of the Ser peptide, under the experimental conditions the maximum amount of peptide that was phosphorylated after prolonged incubation could not exceed 20% of its total concentration. Moreover, the phosphorylated peptide has been found to bind very weakly to the catalytic subunit of protein kinase ($K_I \gtrsim$ 30 mM; H. Kondo and E. T. Kaiser, unpublished results), thus constituting, at best, a weak competitor with respect to the Ser peptide. Hence the relaxation data given in Table I could be used to obtain reasonably accurate bound-state longitudinal relaxation rates ($1/fT_{1p}$), which are summarized in Table II. In the analysis of the relaxation data, the concentration of the enzyme–peptide–$Co(NH_3)_4ATP$–Mn^{2+} complex was calculated by using the dissociation constants obtained previously (Armstrong et al., 1979a), making the reasonable assumption that the enzyme is saturated with the peptide [(K_D = 180 μM; Feramisco (1979)] at a 1:1 stoichiometry. Transverse relaxation rates and their bound-state values ($1/fT_{2p}$) are exemplified in Table III for the Ala[4] residue. The other protons showed similar $1/fT_{2p}$ values.

Further structural studies were carried out by using substitution-inert complexes of AMPPCP rather than ATP to avoid reaction of the Ser peptide in the presence of the enzyme. By use of CrAMPPCP or Mn^{2+} together with $Co(NH_3)_4$AMPPCP, distances were determined from either of two paramagnetic reference points which are 4.8 Å apart (Granot et al., 1980b), i.e., the Cr^{3+} and Mn^{2+} ions, both of which are coordinated to the enzyme-bound nucleotide (Granot et al., 1979a). Binding studies of the complexes $Co(NH_3)_4$AMPPCP and CrAMPPCP to the enzyme and intersubstrate distances along the reaction coordinate for phosphoryl transfer indicate active-site binding of these nucleotide complexes (Granot et al., 1980b). Since CrAMPPCP is more stable at pH 6.0, deuterated solutions used in measurements with this complex contained 10 mM bis-tris-Cl or K^+Pipes buffer, pD 6.0, 150 mM KCl, and 0.1 mM DTT. The effects on $1/T_1$ of the peptide protons in the presence of enzyme of varying amounts of the paramagnetic CrAMPPCP complex, or of Mn^{2+} in the presence of $Co(NH_3)_4$AMPPCP, are summarized in Table I. Displacement studies, using polyarginine as a competitor [K_I = 0.76 μM; Demaille et al. (1977)], resulted in almost complete disappearance of the paramagnetic effects on both $1/T_1$ and $1/T_2$ of the Ser peptide in the above experiments. This result established the binding of the peptide at the active site of the enzyme. In the absence of enzyme no significant effects of CrAMPPCP or Mn^{2+} on the relaxation rates of the peptide protons were detected. Calculated $1/fT_{1p}$ values are given in Table II.

(2) Ala Peptide. Additional NMR studies were carried out in which phosphoryl transfer in the experimental mixture was prevented by using an unreactive analogue of the peptide substrate. A tight-binding analogue, with $K_I \sim$ 500 μM, was found to be the one in which the serine was replaced by alanine (Feramisco & Krebs, 1978). The longitudinal relaxation rates of the protons of the Ala peptide (4.2, 6.4 mM) in the presence of enzyme (68, 165 μM), $Co(NH_3)_4ATP$ (2.8, 3.0 mM), and varying amounts of Mn^{2+} (0–323 μM) were measured at 100

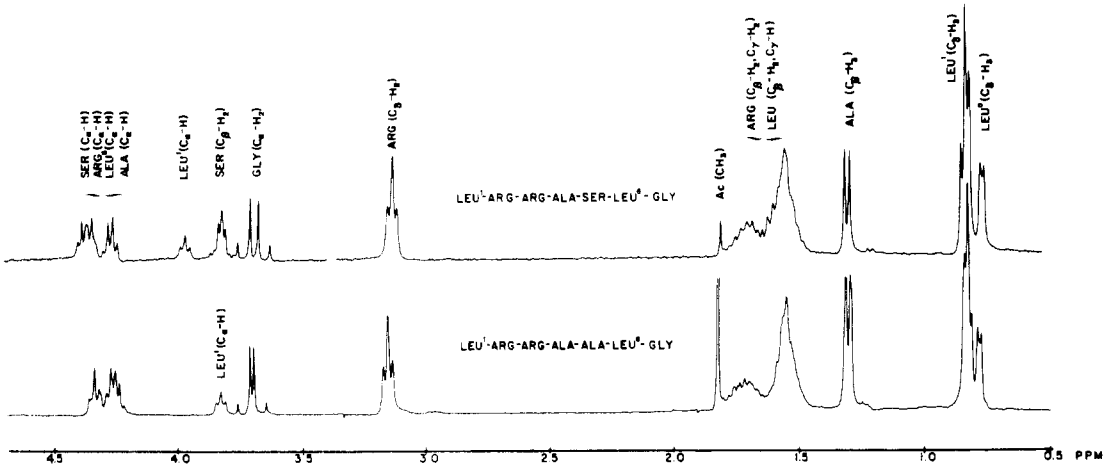

FIGURE 2: ^1H NMR spectra (at 360 MHz) and assignments of the Ser peptide (pD 6.0) and Ala peptide (pD 7.5).

Table I: Paramagnetic Effects on the Longitudinal Relaxation Rates of the Proton Resonances of Peptide Substrates and Analogue in the Presence of Enzyme–Metal–Nucleotide Complexes

			concentrations (μM)				$1/T_1$ (s^{-1})								
expt	complex	frequency (MHz)	[Enz]	[M^{3+}–nucleotide]	[peptide]	[Mn^{2+}]	Leu[1] (C_α-H)	Leu[1] (C_δ-H_3)	Arg[2,3] (C_δ-H_2)	Ala[4,5] (C_β-H_3)	Ser[5] (C_β-H_2)	Leu[6] (C_δ-H_3)	Gly[7] (C_α-H)	Tyr[4] (C_2-H, C_6-H)	Tyr[4] (C_3-H, C_5-H)
I	Ser peptide–Enz–$Co(NH_3)_4ATP$–Mn^{2+}	360	135	2200	5500	0	1.56	1.92	2.86	2.38	2.43	1.92	1.54		
						88	1.67	2.04	2.97	2.86	3.22	2.32	1.67		
						139	1.72	2.11	3.15	2.94	3.86	2.54	*a*		
II	Ser peptide–Enz–$Co(NH_3)_4AMPPCP$–Mn^{2+}	100	80	2400	7800	0	*a*	*b*	*a*	3.12	3.05	*b*	1.97		
						126	*a*	*b*	*a*	3.90	4.81	*b*	2.16		
						251	*a*	*b*	*a*	4.40	5.67	*b*	2.25		
III	Ser peptide–Enz–CrAMPPCP	360	59	0	8200		1.40	2.08	2.80	2.43	*b*	2.08	1.59		
				48			1.59	2.29	3.19	2.86	*b*	2.14	1.67		
				77			1.63	2.27	3.29	2.98	*b*	2.18	1.70		
IV	Ser peptide–Enz–CrAMPPCP	100	72	0	7800		*b*	*b*	*a*	3.33	3.31	*b*	1.95		
				38			*b*	*b*	*a*	3.91	5.01	*b*	2.13		
				75			*b*	*b*	*a*	4.21	5.82	*b*	2.23		
V	Ala peptide–Enz–$Co(NH_3)_4ATP$–Mn^{2+}	360	165	3000	6400	0	*b*	2.56	3.89	3.05		2.56	*a*		
						217	*b*	2.64	4.30	4.03		3.04	*a*		
						323	*b*	2.78	4.47	4.24		3.22	*a*		
VI	Ala peptide–Enz–$Co(NH_3)_4ATP$–Mn^{2+}	100	165	3000	6400	0	*b*	2.56	3.89	3.05		*b*	*a*		
						110	*b*	3.56	4.46	4.62		*b*	*a*		
						217	*b*	4.17	5.15	5.95		*b*	*a*		
VII	Ala peptide–Enz–$CO(NH_3)_4ATP$–Mn^{2+}	360	68	2800	4200	0	1.70	2.04	2.97	2.49		2.04	1.58		
						250	1.95	2.11	3.23	3.24		2.38	1.71		
VIII	Tyr peptide–Enz–$Co(NH_3)_4AMPPCP$–Mn^{2+}	100	119	2600	7700	0	*b*	2.48	4.17		3.05	*b*	*a*	2.35	1.63
						83	*b*	2.97	5.09		4.21	*b*	*a*	4.72	5.20
						144	*b*	3.31	5.86		4.89	*b*	*a*	6.32	8.00
						244	*b*	3.40	6.56		*b*	*b*	*a*	7.00	9.33

[a] Obscured by buffer resonance. [b] Resonance unresolvable. The errors in the $1/T_1$ measurements are between 5 and 10%.

Table II: Paramagnetic Bound-State Proton Longitudinal Relaxation Rates and Metal–Proton Distances of Peptides Bound to Enzyme–Metal–Nucleotide Complexes

	Ser peptide–Enz–$Co(NH_3)_4ATP$–Mn^{2+}		Ser peptide–Enz–CrAMPPCP		Ala peptide–Enz–$Co(NH_3)_4ATP$–Mn^{2+}		Tyr peptide–$Co(NH_3)_4AMPPCP$–Mn^{2+}	
resonance	$1/fT_{1p}$ (s^{-1})[a]	r (Å)	$1/fT_{1p}$ (s^{-1})[a]	r (Å)	$1/fT_{1p}$ (s^{-1})[a]	r (Å)	$1/fT_{1p}$ (s^{-1})[b]	r (Å)
Leu[1] (C_α-H)	$\lesssim$19	$\gtrsim$13	47 ± 21	11.0 ± 1.4	$\lesssim$29	$\gtrsim$12		
Leu[1] (C_δ-H_3)	$\lesssim$22	$\gtrsim$13	$\lesssim$30	$\gtrsim$12	$\lesssim$15	$\gtrsim$14		
Arg[2,3] (C_δ-H_2)	$\lesssim$33	$\gtrsim$12	100 ± 35	9.7 ± 1.0	37 ± 20	12.0 ± 1.6	330 ± 151	10.0 ± 1.2
Ala[4,5] (C_β-H_3)	74 ± 52	10.7 ± 1.6 (10.4[c])	108 ± 35	9.6 ± 1.0	85 ± 25	10.5 ± 1.2		
Ser[5] (C_β-H_2)	150 ± 77	9.5 ± 1.3 (9.1[c])	300 ± 80	8.1 ± 0.9			370 ± 145	9.8 ± 1.0
Leu[6] (C_δ-H_3)	70 ± 45	10.8 ± 1.6	$\lesssim$20	$\gtrsim$13	43 ± 16	11.7 ± 1.4		
Gly[7] (C_α-H_2)	$\lesssim$19	$\gtrsim$13 ($\gtrsim$13[c])	$\lesssim$21	$\gtrsim$13	$\lesssim$16	$\gtrsim$14		
Tyr[4] (C_2-H,C_6-H)							740 ± 160	8.7 ± 0.9
Tyr[4] (C_3-H,C_5-H)							1180 ± 250	8.1 ± 0.8

[a] At 360 MHz. [b] At 100 MHz. [c] In the Ser peptide–Enz–$Co(NH_3)_4AMPPCP$–Mn^{2+} complex. The errors in r are ±10%.

Table III: Paramagnetic Effects on the Relaxation Rates of the Carbon-Bound Protons of the Middle Residues of Peptide Substrates and Analogue Bound to Enzyme-$Co(NH_3)_4$Nucleotide-Mn^{2+} Complexes

complex	protons	frequency (MHz)	concentrations (μM) [Enz]	[Co^{3+}-$(NH_3)_4$Nuc]	[peptide]	[Mn^{2+}]	relaxation rates (s^{-1}) T_1^{-1}	T_{1p}^{-1}	$1/fT_{1p}$	T_2^{-1}	T_{2p}^{-1}	$1/fT_{2p}$
Ser peptide-Enz-$Co(NH_3)_4$ATP-Mn^{2+}	Ala^4 (C_β-H_3)	360	135	2200	5500	0	2.38			34		
						88	2.86	0.48	81	41	9	1580
						139	2.94	0.56	66	46	12	1360
Ala peptide-Enz-$Co(NH_3)_4$ATP-Mn^{2+}	$Ala^{4,5}$ (C_β-H_3)	360	165	3000	6400	0	3.05			34		
						217	4.03	0.98	84	88	54	5820
						323	4.24	1.20	81	105	71	4900
						323[a]	3.15	0.10		43	9	
Ala peptide-Enz-$Co(NH_3)_4$ATP-Mn^{2+}	$Ala^{4,5}$ (C_β-H_3)	100	165	3000	6400	0	3.05			34		
						110	4.62	1.57	223	75	41	4610
						217	5.95	2.90	249	91	57	4840
						323[a]	3.25	0.20		43	9	
Ala peptide-Enz-$Co(NH_3)_4$ATP-Mn^{2+}	$Ala^{4,5}$ (C_β-H_3)	360	68	2800	4200	0	2.49			39		
						250	3.24	0.75	90	85	46	5420
Tyr peptide-Enz-$Co(NH_3)_4$AMPPCP-Mn^{2+}	Tyr^4 (C_3-H, C_5-H)	100	119	2600	7700	0	1.63			35		
						83	5.20	3.57	1120	60	25	7840
						144	8.00	6.37	1290	76	41	8300
						244	9.33	7.70	1150	85	50	7490
						244[a]	2.45	0.82		41	6	

[a] After addition of 333 μM polyarginine.

and 360 MHz. The longitudinal relaxation rates are given in Table I, and the calculated average $1/fT_{1p}$ values are given in Table II. The paramagnetic contributions to $1/T_1$ and $1/T_2$ of the peptide protons were abolished by the addition of the polyarginine (333 μM), establishing active-site binding of the Ala peptide (Table III).

(3) Tyr Peptide. Another peptide substrate was used to gain further insight into the conformation of the peptide bound at the active site of protein kinase. In this peptide the alanine residue (Ala^4) of the Ser peptide was replaced by tyrosine. This substitution introduces a bulky and internally inflexible ring in close proximity to the site of phosphorylation, which is advantageous for structural studies. Parallel kinetic studies with the Ser peptide and Tyr peptide under the conditions described under Methods yielded K_m values of 20.8 and 18.5 μM, respectively, and V_{max} values of 380 and 520 min^{-1}, respectively. The K_m and V_{max} values of the Ser peptide agree with those previously found at the level of Mg^{2+} used (Armstrong et al., 1979a). The Tyr peptide is thus found to be a slightly better substrate than the Ser peptide. The nonreactive $Co(NH_3)_4$AMPPCP complex was used to avoid phosphorylation of the Tyr peptide in the NMR studies. The longitudinal relaxation rates of the protons of the Tyr peptide (7.7 mM) in the presence of enzyme (119 μM), $Co(NH_3)_4$AMPPCP (2.6 mM), and varying amounts of Mn^{2+} (0–224 μM) were measured at 100 MHz. The relaxation data are given in Table I. As with the Ser and Ala peptides, polyarginine (333 μM) removed the paramagnetic effects, establishing active-site binding (Table III). The calculated average $1/fT_{1p}$ values are given in Table II.

Determination of Correlation Times and Distances. From known $1/fT_{1p}$ values the distances (r) between Mn^{2+} and the proton nuclei of the peptides may be calculated by using the relations

$$1/fT_{1p} = 1/(T_{1M} + \tau_M) \quad (1)$$

$$r = C[T_{1M}f(\tau_c)]^{1/6} \quad (2)$$

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

where τ_M is the mean lifetime of the peptide in the paramagnetic environment, ω_I and ω_S are the nuclear and electron Larmor frequencies, and C is a constant equal to 812 or 705 for Mn^{2+}–^1H or Cr^{3+}–^1H interactions, respectively. For calculation of the internuclear distances, the exchange contribution to $1/fT_{1p}$ has to be evaluated and the correlation time (τ_c) which governs the longitudinal relaxation has to be determined. Typical longitudinal and transverse relaxation data, exemplified for protons of the middle residue of the three peptides studied, are shown in Table III. The largest value of the paramagnetic contribution to the transverse relaxation rate within the enzyme complex sets a lower limit on the rate of dissociation ($1/\tau_M$) of the peptide from that complex. From Table III the ratio $(1/fT_{2p})/(1/fT_{1p})$ is between 7 and 60.[2] Hence it can be concluded that the longitudinal relaxation rates are not limited by chemical exchange and may be used for distance calculations. From the frequency dependence of the paramagnetic contribution to the longitudinal relaxation rates of the Ala peptide due to enzyme-bound Mn^{2+}–$Co(NH_3)_4$ATP an average ratio $[1/fT_{1p}(100\ MHz)]/[1/fT_{1p}(360\ MHz)] = 3.0 \pm 0.4$ is found. This value is similar to that found for the enzyme–Mn^{2+}–$Co(NH_3)_4$ATP complex (Granot et al., 1979a), indicating that the binding of the peptide does not significantly alter the correlation time of the inhibitory Mn^{2+} ion. As described previously, (Granot et al., 1979a), the above ratio corresponds to a correlation time of 1.7 ± 1.2 ns (at 360 MHz). Correlation times of 1.9 ± 1.2 ns (at 360 MHz) for enzyme-bound Mn^{2+}–$Co(NH_3)_4$AMPPCP and (4.3 ± 1.5) × 10^{-10} s (at 100 MHz) for enzyme-bound CrAMPPCP were determined previously from the frequency dependence of $1/fT_{1p}$ (Granot et al., 1980b). Distances calculated with the above correlation times are given in Table II.

Kinetic Properties of the System. In the presence of the catalytic subunit, $Co(NH_3)_4$ATP, and Mn^{2+}, the transverse relaxation rates of the protons of the Ala-peptide are similar

[2] The reason for the large $(1/fT_{2p})/(1/fT_{1p})$ ratio at 100 MHz is unclear, considering the observed τ_c obtained from the frequency dependence of $1/fT_{1p}$. It should be pointed out that longitudinal relaxation is sensitive only to fast motion of the order of ω_I, while transverse relaxation is sensitive to slow motion as well. Hence different mechanisms, not presently studied, may contribute to $1/fT_{2p}$, increasing it to the point of exchange limitation. However, even if the maximal $(1/fT_{2p})/(1/fT_{1p})$ ratios are used to estimate τ_c, the resulting distances are within the reported errors.

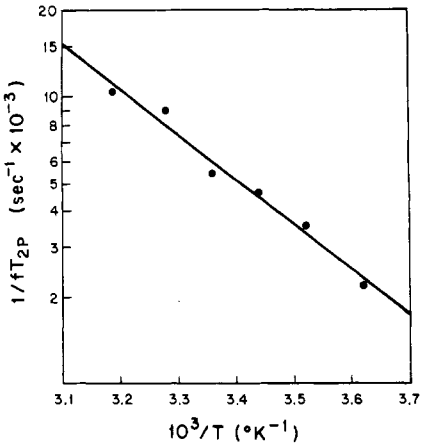

FIGURE 3: Effect of temperature on $1/fT_{2p}$ of the C_β-H_3 protons of the alanine residues of the Ala peptide bound to the enzyme–Co-$(NH_3)_4$ATP–Mn^{2+} complex. The experimental solutions contained the enzyme (68 μM), Ala peptide (4.2 mM), $Co(NH_3)_4$ATP (2.8 mM), and either zero or 250 μM $MnCl_2$.

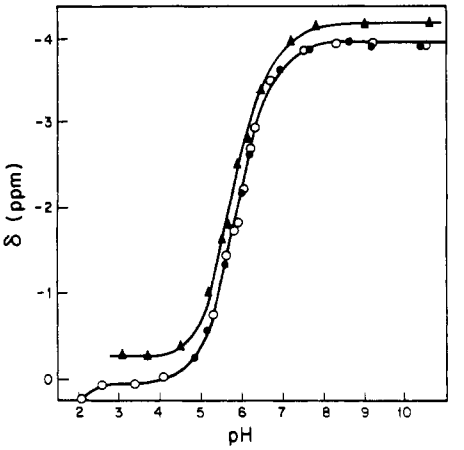

FIGURE 4: The pH dependence of the ^{31}P chemical shifts of the phosphorylated Ser peptide [4.4 mM (▲)] and *O*-phospho-L-serine [11.6 mM (●), 4.1 mM (○)]. Shifts were measured relative to H_3PO_4 external reference. The solid lines through the experimental points are the result of least-squares analyses.

and show little or no frequency dependence between 100 and 360 MHz (as exemplified in Table III), implying that they are limited by k_{off} (= $1/\tau_M$), the rate constant for dissociation of the peptide from the paramagnetic complex. In order to establish this point, the effect of temperature on the transverse relaxation rates were studied with the Ala peptide (4.2 mM) in the presence of enzyme (68 μM) and $Co(NH_3)_4$ATP (2.8 mM), and in the absence or presence of 250 μM Mn^{2+}. An Arrhenius plot of the net paramagnetic contributions to the transverse relaxation rate of the alanine C_β–H_3 resonances of the Ala peptide as a function of temperature (Figure 3) is typical of exchange-limited relaxation. The data of Figure 3 were fitted by least-squares analysis to the Arrhenius equation to obtain E_{act} = 7.3 ± 0.5 kcal/mol. This value and the k_{off} value for the Ala peptide (below) yielded absolute reaction rate parameters of $\Delta H^\ddagger$ = 6.7 ± 0.5 kcal/mol, $T\Delta S^\ddagger$ = −5.8 kcal/mol, and $\Delta F^\ddagger$ = 12.5 ± 1.3 kcal/mol (at 25 °C). The relatively large and negative entropy implies a high degree of ordering in the transition state for peptide dissociation. From Table III, the rate constant for dissociation (k_{off}) of the Ala peptide at 25 °C is 5.1 × 10^3 s^{-1} and is independent of peptide concentration. This value together with the dissociation constant for this peptide [(K_I = 500 μM; Feramisco & Krebs (1978)] yields k_{on} ~1.0 × 10^7 M^{-1} s^{-1}. As found with the Ala peptide, the $1/fT_{2p}$ values of the carbon-bound protons of the Ser peptide at 360 MHz are found to be similar, suggesting exchange limitation here as well. Assuming this to be the case, we estimate k_{off} to be 1.5 × 10^3 s^{-1} and k_{on} ~0.8 × 10^7 M^{-1} s^{-1} at 25 °C for the Ser peptide. This k_{on} value agrees with that of the Ala peptide. Hence the 3-fold greater affinity of the enzyme for the Ser peptide than for the Ala peptide is the result primarily of the slower rate of dissociation of the Ser peptide.

Effect of pH on the ^{31}P Chemical Shift of Phosphorylated Ser Peptide. The negatively charged phosphoserine residue of the phosphorylated Ser peptide is adjacent to three positively charged groups, i.e., the two arginyls and the terminal ammonium. For examination of the possible interaction between these groups in the phosphopeptide, the ^{31}P chemical shifts of the phosphorylated Ser peptide were measured as a function of pH and were compared to that of *O*-phospho-L-serine. The data (Figure 4) were analyzed by a least-squares fitting method, yielding pK_a values of 5.8 ± 0.1 and 5.9 ± 0.1 for the phosphorylated peptide and *O*-phospho-L-serine, respectively. These values agree well with previously published pK_a values for *O*-phosphoserine (pK_a = 5.9) and its di- and tripeptides such as glycyl-DL-phosphoserine (pK_a = 6.0) and glycyl-DL-phosphoserylglycine [pK_a = 5.8; Osterberg (1962)]. The absence of significant differences between the pK_a's of the phosphorylated peptide and other phosphoserine compounds suggests little or no interaction between the charged groups of the phosphopeptide.

Kinetic Studies with the Pro^6 Heptapeptide. Substitution of Pro^6 for Leu^6 in the Ser peptide substrate resulted in a peptide which showed little or no substrate activity. Comparative kinetic studies of the Pro^6 peptide (0.5 mM) with the Ser peptide (0.5 mM) under the same standard assay conditions with 2.5 mM catalytic subunit indicated the former peptide to be phosphorylated ≤0.02% relative to the latter. No inhibition by the Pro^6 peptide (8–400 μM) was detected in competition with the Ser peptide (32 μM), indicating the K_I of the former to be considerably greater than 400 μM.

Discussion

The rate constant for dissociation of the peptide from its complex with the enzyme (~10^3 s^{-1}; vide supra) as well as the rate constant for dissociation of the metal–nucleotide–E complex [~10^6 s^{-1}; Granot et al. (1979a)] is much larger than the rate constant for the enzyme-catalyzed phosphorylation of the peptide [~10 s^{-1}; Armstrong et al. (1979a)], indicating that the complexes detected by NMR are kinetically competent to function in catalysis. These rate constants also imply that the rate-determining step in the protein kinase reaction is either the phosphoryl transfer or a conformational change within the active quaternary complex.

The enthalpy barrier to dissociation of the enzyme–peptide complex ($\Delta H^\ddagger$ = 6.7 kcal/mol) suggests the breaking of 3–4 hydrogen bonds while the entropy barrier ($-T\Delta S^\ddagger$ = 5.8 kcal/mol) implies a higher degree of ordering as the transition state is approached. The entropy change could be the result of the solvation of the hydrophilic side chains of the peptide (e.g., the Arg side chains) as these groups begin to dissociate from the enzyme. The present and previous results provide information on the overall kinetic scheme of the protein kinase reaction. Since a nucleotide can bind to the enzyme in the absence of peptide (Armstrong et al., 1979a), an ordered reaction in which the peptide binds first is excluded. A rapid equilibrium ordered reaction with the nucleotide binding first may be ruled out since such a mechanism will yield competitive behavior of $Co(NH_3)_4$ATP vs. the peptide (Segel, 1975, p 329), contrary to the data shown in Figure 1. Although the

data of Figure 1 are consistent with a rapid equilibrium random scheme (Segel, 1975, p 287), this mechanism may also be ruled out since K_m is not equal to K_D for the peptide (Feramisco & Krebs, 1978) and K_m of the peptide is unaffected by the binding of $Co(NH_3)_4ATP$ (i.e., the lines in Figure 1, when extrapolated, intersect on the 1/[peptide] axis). Hence two remaining probable schemes, neither of which can at present be ruled out, are the steady-state-random and the steady-state-ordered mechanisms, with the nucleotide binding first.

The second-order rate constants for the binding of peptides to protein kinase ($k_{on} \sim 10^7$ M^{-1} s^{-1}) are somewhat smaller than expected for a diffusion-controlled reaction, suggesting the possibility of selection by the enzyme of a minor conformational form of the peptide or a conformation change upon formation of the enzyme–peptide complex. Such a conformation change may occur on the enzyme or may be induced by the enzyme in the secondary structure of the peptide or protein substrate to one which is appropriate for catalysis. A conformation change of the peptide, induced by binding to the enzyme, would be consistent with the structural studies discussed below.

The secondary structures of peptide and proteins include four main classes of backbone conformations. The α helix, the β pleated sheet, and the β turn are conformations in which the carbonyl oxygens and amide protons are involved in specific and regular hydrogen-bonding patterns. The fourth class consists of coils, which are definite structures, although lacking regular intramolecular backbone hydrogen bonding (Schulz & Schirmer, 1978; Chou & Fasman, 1978). Examples of each of these four classes of secondary structure can be found on the surfaces of proteins (Richardson, 1980) where residues therein would be accessible to phosphorylation by protein kinase. Hence each class was taken into consideration in attempting to fit the experimental internuclear distances of Table II to a structural model of the bound peptide substrate of protein kinase. In our model building, the reasonable assumption was made that the conformations of the three peptides studied, when bound to the enzyme, are the same.

By use of Dreiding molecular models it became apparent that an α-helical conformation of the peptide is inconsistent with the experimentally measured distances. For example, when the Ser peptide is built into an α helix and the distances from Mn^{2+} to the Ser (C_β-H_2), Ala (C_β-H_3), and Leu^6 (C_δ-H_3) protons are fixed according to their experimental values (Table II), the distances on the α-helical model from Mn^{2+} to the two Arg (C_δ-H_2) protons were found to be in the range 15.8 to 18.7 Å which greatly exceeds the experimentally measured average distance of 11.0 ± 2.0 Å. Similarly when Cr^{3+} is built into its appropriate position based on its experimental distances from the Ser (C_β-H_2) and the Ala (C_β-H_3) protons and its known coordination to the β- and γ-phosphoryl groups of AMPPCP, a large discrepancy is found between the measured distances from Cr^{3+} to the Arg (C_δ-H_2) protons (9.7 ± 1.0 Å) and those on the α-helical model (15.2–18.0 Å). Alternative starting positions for Mn^{2+} or Cr^{3+} using the α-helical model of the heptapeptide lead to even greater differences between observed and measured distances. Therefore an α-helix may be excluded. Similarly, while the strand of a β-pleated-sheet conformation for the heptapeptide is consistent with the measured distances from Mn^{2+} and Cr^{3+} to the protons of the Ser and Ala peptides, such a conformation is clearly inconsistent with the distances to the Tyr peptide (Table II). This inconsistency results from the alternating orientations of the peptide side chains in each strand of a β-pleated sheet. On a molecular model such a conformation places the tyrosyl protons further away from the nucleotide-bound Mn^{2+} than those of serine which is adjacent to the tyrosine and is also near the polyphosphate chain of the nucleotide (Granot et al., 1980), yielding distances of 16–18 Å between the Mn^{2+} and the tyrosine ring protons. The observation that the tyrosyl ring is actually closer to the Mn^{2+} ion than the seryl side chain, at distances of 8–9 Å (Table II), thus argues strongly against a β-pleated-sheet conformation. Recently Feramisco et al. (1979) have found that when the Ala^4 residue in the Ser peptide is replaced by proline, the resulting Pro^4 heptapeptide is actually improved as a substrate with a 2.6-fold lower K_m and the same V_{max}. Since proline is known to be a "strong breaker" of both α-helix and β-sheet structures (Chou & Fasman, 1978), these findings agree with our exclusion of these conformations.

The β turn, a conformation which appears very frequently on the surface of globular proteins, involves a sequence of four consecutive amino acids with a hydrogen bond between the carbonyl oxygen of the first amino acid and the N–H of the fourth amino acid (Chou & Fasman, 1978). The occurrences of β-turn structures near or at sites of phosphorylation in proteins have been suggested (Daile et al., 1975; Small et al., 1977; Matsuo et al., 1978; Graves et al., 1978) from the primary structures of phosphorylated sites by using the empirical model of Chou and Fasman for predicting β turns in proteins. On the enzyme-bound Ser heptapeptide four such β turns are possible, namely, Leu^1-Arg^2-Arg^3-Ala^4 (β_{1-4}), Arg^2-Arg^3-Ala^4-Ser^5 (β_{2-5}), Arg^3-Ala^4-Ser^5-Leu^6 (β_{3-6}), and Ala^4-Ser^5-Leu^6-Gly^7 (β_{4-7}). None of these can be ruled out by the measured distances of Table II. An example of such a conformation, the β_{3-6} turn, which is compatible with the NMR distances, is shown in Figure 5A. However, the occurrence of each of the above β turns can be assessed by examining comparative kinetic data on peptide substrates and by sequence data on phosphorylated proteins from the literature. Thus the β_{1-4} and the β_{4-7} turns can be ruled out as obligatory by the high substrate activities of the hexapeptides lacking either Leu^1 or Gly^7, respectively (Kemp et al., 1977).[3] The β_{1-4} turn is also incompatible with the substrate activity of the Pro^4 heptapeptide (Feramisco et al., 1979) since proline cannot donate an N–H for a hydrogen bond. Similarly the observation that the heptapeptide in which the Ser^5 has been replaced by Hyp^5 is a substrate provides evidence against the β_{2-5} turn as an obligatory conformation. This peptide is phosphorylated on the hydroxyproline residue, albeit with very poor kinetic parameters (Feramisco et al., 1979), which might indicate a preference for a β_{2-5} turn. The low K_m and excellent substrate activity of the Pro^4 heptapeptide (Feramisco et al., 1979) provide further evidence against an obligatory β_{2-5} turn since the sequence Arg^2-Arg^3-Pro^4-Ser^5 is found to have a very low probability of occurrence in a β turn ($P_t = 0.13 \times 10^{-4}$, compared to the cutoff value of 1.0×10^{-4}), using the conformational parameters of Chou & Fasman (1979). This low probability results mainly from Pro^4 which may be considered a "β-turn breaker" when in the third position. With regard to the remaining β_{3-6} turn, our kinetic measurements on the Pro^6 heptapeptide show that its dissociation constant must be

[3] In these studies, the catalytic subunit of bovine skeletal muscle protein kinase, a type I kinase, was used. We assume here the similarity of its substrate specificity to that of the catalytic subunit of bovine skeletal muscle protein kinase, a type II kinase. Supporting evidence for the assumption of similar substrate specificities for the catalytic subunits of type I and type II kinases has come from studies of kinases isolated from several different sources (Armstrong et al., 1979a; Kemp et al., 1977; Pomerantz et al., 1977; Zetterqvist et al., 1976).

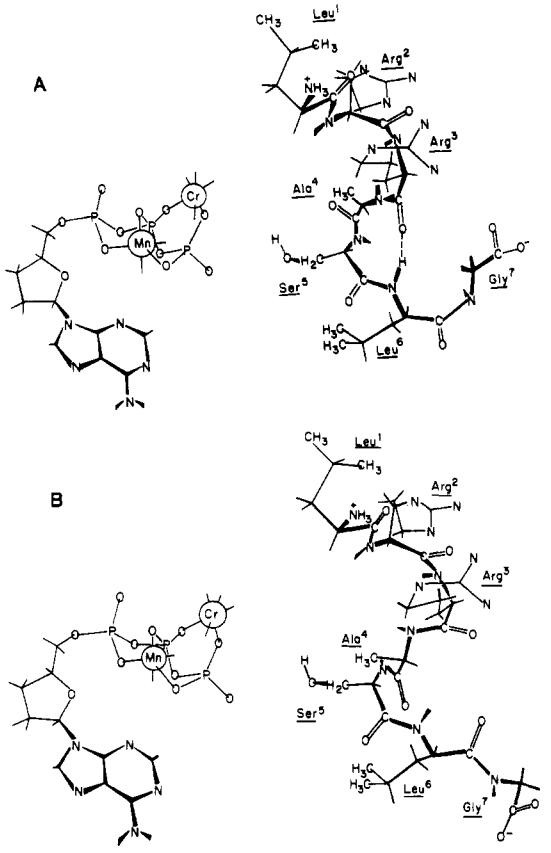

FIGURE 5: Arrangement and conformations of substrates bound at the active site of protein kinase. The β_{3-6} turn (A) and coil (B) conformations of the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly are based on the distances of Table II. As discussed in the text, both peptide conformations are consistent with the NMR data. Although all four β turns within the heptapeptide may be excluded as obligatory enzyme-bound conformations by kinetic and primary sequence data, the β_{3-6} or β_{2-5} turns may actually be preferred by the enzyme. The conformation of the enzyme-bound metal–ATP complex is based on a previous NMR study (Granot et al., 1979a). The paramagnetic probe Cr^{3+} occupies the activating metal site while Mn^{2+} occupies the inhibitory site.

appreciably greater than 400 μM. Under conditions where kinetic measurements can be made with confidence, a comparison of the reactivity of the Pro^6 heptapeptide relative to that of the Leu^6 substrate (Ser peptide) shows the former peptide to be at least 500 times less reactive. Our results may suggest that a β_{3-6} turn is preferred, although other explanations are possible. Recent results of Kemp et al. (1979) which detect low substrate activity of synthetic peptides with a phosphorylatable Thr residue followed by a proline, together with the substrate activity of two proteins containing similarly located proline residues (Cohen et al., 1977; Sung & Dixon, 1970), indicate that the β_{3-6} turn is not obligatory.

By ruling out the α helix, the β sheet, and the obligatory requirement for a β turn, we are left with the coil as the only secondary structure that could be obligatory for the bound heptapeptide substrate on protein kinase. Indeed, if protein kinase has an absolute requirement for a specific secondary structure, then this structure must be a coil.[4] An example of a coil structure consistent with all of the distances of Table II is given in Figure 5B. Inspection of sequences in proteins and peptides near phosphoserines and phosphothreonines reveals numerous variants from the optimum sequence which nevertheless get phosphorylated. Since the secondary structure is determined mainly by the primary structure (Epstein et al., 1963), this variability in sequence implies a variety of local secondary structures of protein substrates prior to their binding to protein kinase.[5] Upon binding, the enzyme may induce conformational changes in the substrate, primarily at the expense of binding affinity (Jencks, 1975), to one which is preferred or required for catalysis.

[4] Interestingly, the conformation of the portion of phosphorylase *a* containing the phosphoserine residue is clearly a coil as determined by X-ray analysis (Fletterick et al., 1979). However, this protein is phosphorylated by a different kinase and cannot be phosphorylated by cAMP-dependent protein kinase (Carlson et al., 1979). Moreover, this coil structure is already a phosphorylated product and is not enzyme bound. Hence the relevance of this particular coil structure to the conformation of the enzyme-bound peptide substrate on cAMP-dependent protein kinase is at present unclear.

[5] For example, the variable position of the phosphorylated residue within or near predicted β turns in protein substrates (Small et al., 1977) suggests that, if such structures indeed exist in the free substrate, upon binding to the enzyme these structures would have to be altered to provide a unique position for the serine residue in the active site. A conformational change to a coil would be energetically and structurally facile from such a wide variety of β turns.

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Diffusion-Enhanced Energy Transfer Shows Accessibility of Ribonucleic Acid Polymerase Inhibitor Binding Sites†

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ABSTRACT: Rifamycin and Cibacron Blue F3GA are powerful inhibitors of *Escherichia coli* deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase. In addition, both inhibitors strongly absorb visible light, making them suitable for use as acceptors in energy-transfer experiments. Transfer of energy to these acceptors from *freely diffusing* energy donors with long excited-state lifetimes ($\approx 10^{-3}$ s) depends strongly on whether donor and acceptor can make direct intermolecular contact. We observe that the rate constant for energy transfer from a small terbium chelate to enzyme-bound rifamycin is 1×10^7 M^{-1} s^{-1}, which is about *half* as large as the rate constant observed for *free* rifamycin in solution. This relatively small change upon binding indicates that enzyme-bound rifamycin is highly accessible to small molecules in the solvent. In the case of Cibacron Blue, under conditions where ~90% of this inhibitor is bound to RNA polymerase, the small amount of unbound inhibitor accounts for practically all of the observed energy transfer. This implies that enzyme-bound Cibacron Blue is relatively inaccessible to energy donors in the solution. The dependence of energy transfer on the accessibility of the acceptor is illustrated by using simple geometric models. Synthesis of a stable, electrically neutral terbium chelate which can be efficiently excited with UV radiation is also described.

Deoxyribonucleic acid (DNA)[1] dependent RNA polymerase (EC 2.7.7.6) from *Escherichia coli* is a complex enzyme composed of five subunits, $\beta'\beta\alpha_2\sigma$ (Burgess & Jendrisak, 1975), with a composite molecular weight of 4.8×10^5. The synthesis of RNA on a DNA template by this enzyme is strongly inhibited by the antibiotic rifamycin, whose binding site is thought to be on the β subunit of the enzyme (Heil & Zillig, 1970). Affinity-labeling studies indicate, however, that the bound inhibitor is within ~1 nm of nearly all the subunits (Stender et al., 1975; Rice & Meares, 1978). The rifamycin binding site on RNA polymerase appears to lie in the path of the growing RNA chain, since only di- and trinucleotides may be formed by the enzyme in the presence of rifamycin (McClure & Cech, 1978; McClure, 1980).

Another RNA polymerase inhibitor is the dye Cibacron Blue, which is thought to bind to the "dinucleotide fold" of a variety of nucleotide-binding enzymes (Thompson et al., 1975). Evidently, Cibacron Blue and rifamycin have separate binding sites on RNA polymerase (Kumar & Krakow, 1977; L. S. Rice, unpublished observations).

As part of understanding the mechanism of their effect on RNA polymerase, it is important to know whether these inhibitors lie on the outer surface of the enzyme or buried among the subunits. Since both inhibitors absorb visible light, a means of doing this was suggested to us by the experiments of Thomas

† From the Department of Chemistry, University of California, Davis, California 95616. *Received June 9, 1980.* Supported by Research Grant GM 25909 from the National Institute of General Medical Sciences, Research Career Development Award CA 00462 from the National Cancer Institute to C.F.M., and a University of California graduate fellowship to L.S.R.

[1] Abbreviations used: HED3A, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; Tb-HED3A, the terbium(III) chelate of HED3A; Bzl-HED3A, (*S*)-*N*-[2-[bis(carboxymethyl)amino]-3-phenylpropyl]-*N*-(2-hydroxyethyl)glycine (see Figure 2); Tb-Bzl-HED3A, terbium(III) chelate of Bzl-HED3A (see Figure 2); DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DEAE, diethylaminoethyl.

0006-2960/81/0420-0610$01.00/0