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NMR Studies of the Backbone Protons and Secondary Structure of Pentapeptide and Heptapeptide Substrates Bound to Bovine Heart Protein Kinase†

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ABSTRACT: The conformations of enzyme-bound pentapeptide (Arg-Arg-Ala-Ser-Leu) and heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) substrates of protein kinase have been studied by NMR in quaternary complexes of the type

$$\begin{tikzcd}
\text{peptide} \arrow[r, dash] & \text{enzyme} \arrow[r, dash] \arrow[dr, dash] & \text{AMPPCP} \arrow[r, dash] \arrow[d, dash] & \text{Co(NH}_3)_4 \\
 & & \text{Mn}^{2+}_{\text{inh}} &
\end{tikzcd}$$

Paramagnetic effects of Mn^{2+} bound at the inhibitory site of the catalytic subunit on the longitudinal relaxation rates of backbone C_α protons, as well as on side-chain protons of the bound pentapeptide and heptapeptide substrates, have been used to determine Mn^{2+} to proton distances which range from 8.2 to 12.4 Å. A combination of the paramagnetic probe–T_1 method with the Redfield 2–1–4–1–2 pulse sequence for suppression of the water signal has been used to measure distances from Mn^{2+} to all of the backbone amide (NH) protons of the bound pentapeptide and heptapeptide substrates, which range from 6.8 to 11.1 Å. Paramagnetic effects on the transverse relaxation rates yield rate constants for peptide exchange, indicating that the complexes studied by NMR dissociate rapidly enough to participate in catalysis. Model-building studies based on the Mn^{2+}–proton distances, as well as on previously determined distances from Cr^{3+}-AMPPCP to side-chain protons [Granot, J., Mildvan, A. S., Bramson, H. N., & Kaiser, E. T. (1981) *Biochemistry 20*, 602], rule out α-helical, β-sheet, β-bulge, and all possible β-turn conformations within the bound pentapeptide and heptapeptide substrates. The distances are fit only by extended coil conformations for the bound peptide substrates with a minor difference between the pentapeptides and heptapeptides in the ϕ torsional angle at Arg$_3$C$_\alpha$ and in ψ at Arg$_2$C$_\alpha$. An extended coil conformation, which minimizes the number of interactions within the substrate, would facilitate enzyme–substrate interaction and could thereby contribute to the specificity of protein kinase.

Adenosine cyclic 3′,5′-monophosphate (cAMP)[1] dependent protein kinase plays a major role in the regulation of certain enzymes through selective phosphorylations (Krebs & Beavo, 1979). Dissociation of the regulatory subunit from the catalytic subunit, a process mediated by cAMP, has been shown to activate the catalytic subunit by allowing the binding of peptide or protein substrates (Granot et al., 1980a).

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; Co(NH$_3$)$_4$ATP, tetraammine(adenosine triphosphate-P^β,P^γ)cobalt(III); Co(NH$_3$)$_4$AMPPCP, tetraammine(adenosine β,γ-methylenetriphosphate-P^β,P^γ)cobalt(III); CrAMPPCP β,γ-bidentate, Cr^{3+}-β,γ-methylene-ATP; DTT, dithiothreitol; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; MES, 2-(N-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)methylamine; MOPS, 3-(N-morpholino)propanesulfonic acid; pH*, meter reading in ^{2}H$_2$O; Ser-pentapeptide, Arg-Arg-Ala-Ser-Leu; Ser-heptapeptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; Tyr-heptapeptide, Leu-Arg-Arg-Tyr-Ser-Leu-Gly; Ala-heptapeptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly; A/D, analogue to digital; FID, free induction decay.

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The kinetic and structural properties of bovine heart cAMP-dependent protein kinase have been studied by magnetic resonance methods (Granot et al., 1980b; Rosevear et al., 1983). The binding of metal complexes of the substrates ATP, ADP, and ATP analogues to the catalytic subunit were shown to induce the appearance of an additional binding site for a divalent cation which partially inhibited the phosphoryl transfer reaction (Armstrong et al., 1979).[2] The conformation of enzyme-bound $Co(NH_3)_4ATP$ was determined by using Mn^{2+} at this inhibitory site as a paramagnetic reference point (Granot et al., 1980b) and independently using intramolecular nuclear Overhauser effects (Rosevear et al., 1983).

Heretofore, much less was known about the conformation of enzyme-bound peptide or protein substrates. Kinetic studies with synthetic peptides in which the peptide sequence was systematically varied have established the amino acid sequence Arg-Arg-X-Ser-Y, in which X is any one of several amino acids and Y is a hydrophobic residue other than Pro, as optimal for binding and phosphorylation (Krebs & Beavo, 1979; Carlson et al., 1979; Granot et al., 1981). Since structural properties beyond the primary sequence may well play a determining role in the substrate specificity of protein kinase, Granot et al. (1981) studied the conformations of enzyme-bound peptide substrates using the paramagnetic probe–T_1 method. Distances were measured from Mn^{2+} at the inhibitory site or from the Cr^{3+} at the activating site, as in β,γ-bidentate CrAMPPCP, to protons of the Ser-heptapeptide[1] and Tyr-heptapeptide substrates and the Ala-heptapeptide substrate analogue. Assuming the three heptapeptides to have the same conformation when bound, the measured distances were found to be incompatible with the α-helical or β-pleated sheet conformations (Granot et al., 1981). However, the magnetic resonance data could not eliminate any of the eight possible β turns within the heptapeptides[3] (Granot et al., 1981). The complexity of the ^1H NMR spectra of the heptapeptides at magnetic field strengths necessary to observe measurable paramagnetic effects precluded distance measurements to many of the protons, including the important backbone atoms. Only the two terminal C_α-H resonances could be individually resolved, and lower limit distances were obtained. In order to better define the conformation of the enzyme-bound peptide, the present magnetic resonance studies were undertaken with a Ser-pentapeptide substrate, Arg-Arg-Ala-Ser-Leu. By use of this simpler substrate, distances from the inhibitory Mn^{2+} to all of the five backbone C_α protons as well as to eight carbon-bound protons of side chains could be determined. To further define the backbone conformation of the Ser-pentapeptide and the Ser-heptapeptide, distances from the inhibitory Mn^{2+} to the exchangeable amide (NH) protons were determined in H_2O by using a combination of the paramagnetic probe–T_1 method (Mildvan & Gupta, 1978) and the Redfield 2-1-4-1-2 pulse sequence to suppress the strong solvent signal (Redfield et al., 1975). Nineteen new distances from the inhibitory Mn^{2+}, including 15 backbone distances, were obtained on the Ser-pentapeptide and Ser-heptapeptide substrates. Model-building studies using the Mn^{2+} to proton distances measured on both the Ser-pentapeptide and Ser-heptapeptide substrates exclude α-helical, β-pleated-sheet, β-bulge, and all of the eight possible β-turn conformations. The distances are consistent only with an extended coil structure for the conformation of the enzyme-bound peptide substrates.

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 modifications as previously described (Armstrong et al., 1979; Bramson et al., 1982). The β,γ-bidentate complex of $Co(NH_3)_4AMPPCP$ was prepared at its isoionic pH as previously described (Cornelius et al., 1977) and characterized by ^1H NMR. No impurities were detected by thin-layer chromatography on PEI–cellulose plates developed in 1 M LiCl. Deuterated Tris base was obtained from Stohler. Dowex 50-X2 (100–200 mesh) and Chelex-100 were obtained from Bio-Rad. [γ-^{32}P]ATP was purchased from New England Nuclear. Immersible CX-10 concentrators were purchased from Millipore. PEI–cellulose plates were purchased from J. T. Baker. Poly(L-arginine)-hydrochloride (M_r ~44 000) and adenosine 5′-(β,γ-methylenetriphosphate) were obtained from Sigma. All other chemicals were of the highest purity commercially available.

The Ser-heptapeptide was prepared as described previously (Armstrong et al., 1979), and the Ser-pentapeptide was synthesized similarly. Satisfactory analytical data, including amino acid analyses, were obtained for the peptides.

Methods. The concentration of the catalytic subunit was determined spectrophotometrically by using $A^{1\%}_{280} = 14.9$, assuming a molecular weight of 40 000 (Demaille et al., 1977). The activity of the catalytic subunit was assayed by using the synthetic Ser-heptapeptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly as previously described (Witt & Roskoski, 1975; Armstrong et al., 1979). Concentrations of $Co(NH_3)_4AMPPCP$ were determined spectrophotometrically by using $\epsilon_{257} = 16.9 \times 10^3\ M^{-1}\ cm^{-1}$. All solutions used for NMR studies were passed through Chelex-100 to remove trace metal impurities. When necessary, enzyme solutions were deuterated by repeated concentration and dilution by vacuum filtration using Millipore immersible CX-10 concentrators. NMR experiments observing carbon-bound protons of the peptide substrates were performed at 25 °C. The samples contained 98–114 μM catalytic subunit, 10.0 mM peptide substrate, 2.2–2.8 mM $Co(NH_3)_4AMPPCP$, 0–154 μM $MnCl_2$, 10 mM deuterated Tris-HCl buffer, pH 7.5, 0.15 M KCl, and 0.1 mM DTT in ^2H_2O.

NMR experiments in H_2O designed to observe the exchangeable amide protons of the Ser-pentapeptide and Ser-heptapeptide were performed at 10 °C and pH 5.8 to render the NH resonances visible. Due to the irreversible denaturation and precipitation of the catalytic subunit of protein kinase at low pH, a pH of 5.8 was chosen for measuring the paramagnetic effects of Mn^{2+}, bound at the inhibitory site of the enzyme, on the relaxation rates of the amide protons of the Ser-peptide substrates in H_2O. This pH was found to be high enough for high enzyme activity and prolonged stability and low enough such that the amide proton resonances were well resolved, and their exchange rates with H_2O contributed negligibly to the paramagnetic effects on the longitudinal relaxation rates.

To prevent denaturation and precipitation of the catalytic subunit of protein kinase when the pH was lowered from 7.0 to 5.8 for the NMR experiments, it was found necessary first to dilute the enzyme to ≤1.0 mg/mL and then rapidly to lower the pH to 5.8. When the following procedure was used, ≥95% of the total protein was consistently recovered with ≥81% of

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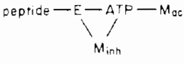
[2] The metal bound at the inhibitory site on protein kinase in the presence of a nucleotide bridges the enzyme and the triphosphate chain of the bound nucleotide (Armstrong et al., 1979; Granot et al., 1979).



$$\text{peptide}-E-\text{ATP}-M_{act} \quad (E,\ \text{ATP} - M_{inh})$$

[3] Two types of β-turn, type I and type II, at each of four positions are possible within a heptapeptide.

the initial specific activity. All components were kept at 2 °C. The stock catalytic subunit (~4 mg/mL) stored in 25 mM MOPS buffer, pH 7.0, 0.15 M KCl, and 1.0 mM DTT was diluted to 1.0 mg/mL protein with 0.15 M KCl and 0.1 mM DTT. The diluted enzyme was then added in small aliquots to an equal volume of 50 mM MES buffer, pH 5.8, 0.15 M KCl, and 0.1 mM DTT, maintaining the pH at 5.8 by 1-2-μL additions of 200 mM MES acid, pH 4.1. The catalytic subunit at pH 5.8 was then concentrated by vacuum filtration using immersible CX-10 concentrators. Concentrated catalytic subunit was then diluted 3-fold with 5 mM MES buffer, pH 5.8, 0.15 M KCl, and 0.1 mM DTT and again concentrated by vacuum filtration. These dilution and concentration steps were repeated two additional times to raise the enzyme concentration, to remove the residual MOPS buffer, and to lower the concentration of MES buffer to 5 mM in the NMR sample.

The stability of the catalytic subunit of protein kinase (1 mg/mL or 25 μM) at 10 °C in 5 mM MES buffer, pH 5.8, 0.15 M KCl, and 0.1 mM DTT was monitored over a 27-h period. No detectable loss of protein or specific activity occurred during this period. A temperature of 10 °C was chosen to help stabilize the enzyme and to further slow the amide proton exchange rates at pH 5.8. The components present in the final NMR samples were 77-102 μM catalytic subunit, 5-7.5 mM Ser-pentapeptide or heptapeptide substrate, 0.9-1.1 mM $Co(NH_3)_4$AMPPCP, 0-131 μM $MnCl_2$, 5 mM MES buffer, pH 5.8, 0.15 M KCl, 0.1 mM DTT, and 10% ^2H_2O in water to permit field/frequency locking. At the end of prolonged NMR studies which lasted up to 30 h, 60-91% of the initial enzymatic activity was retained.

NMR Measurements. ^{1}H NMR spectra were obtained on a Bruker WM 250 NMR spectrometer using 16-bit A/D conversion and quadrature phase detection. Chemical shifts are relative to external DSS. Longitudinal relaxation rates ($1/T_1$) of the carbon-bound protons were determined by using the nonselective saturation recovery method (Markley et al., 1971). Transverse relaxation rates ($1/T_2$) were calculated from line-width measurements at half-height ($\Delta\nu$) using the relation $1/T_2 = \pi\Delta\nu$.

Two-dimensional correlated spectroscopy (COSY) was used to determine the J coupling connectivities among the carbon-bound protons of the Ser-pentapeptide. The COSY spectra were obtained by using the pulse sequence [RD–90°–t_1–90°–t_2–observation pulse]$_n$ where RD is the relaxation delay, t_1 the period in which the various magnetization components are labeled with their characteristic frequencies, t_2 is the time for exchange of magnetization between J-coupled spin systems, and n is the number of scans required for the desired signal to noise (Wagner et al., 1981).

The 2-1-4-1-2 pulse sequence (Redfield et al., 1975) used as the observation pulse when amide protons in H_2O are observed, permits a full 90° observation pulse to be given to the downfield region of the spectrum without producing a strong H_2O signal. Longitudinal relaxation rates of the amide protons were measured by a selective saturation recovery method (Tropp & Redfield, 1981). The pulse sequence used for selective saturation was $[(t,\omega_A)-\tau-$observation pulse–RD$]_n$, where t is the time required for saturation of resonance A, ω_A is its frequency, τ is the variable delay between the saturation pulse and the 2-1-4-1-2 observation pulse, and RD is the relaxation delay to allow magnetic equilibration ($5T_1$'s). The decoupler was routinely used for the saturation pulse. Typically, the saturation pulse was set at 26 dB below 0.2 W for $t = 0.15$ s.

The transfer of saturation from water to each of the amide (NH) protons of the Ser-pentapeptide and heptapeptide was measured by using the following pulse sequence:

$$\{[\text{RD–preirradiate}(t,\omega_{H_2O})\text{–observation pulse}]_4\text{–}[\text{RD–preirradiate}(t,\omega_{\text{off-res}})\text{–observation pulse}]_4\}_n$$

where RD is the relaxation delay, t is the duration of preirradiation, ω_{H_2O} is the frequency of the H_2O resonance, $\omega_{\text{off-res}}$ is a control frequency, and n is the number of times the sequence is repeated to obtain the desired signal to noise. The observation pulse used was the 2-1-4-1-2 pulse sequence of Redfield et al. (1975). The pulse sequence was set up in such a way that the FID's following the irradiation at ω_{H_2O} and $\omega_{\text{off-res}}$ were collected in separate blocks. The proton decoupler was utilized to produce the selective preirradiation pulses. Typically, greater than 95% of the H_2O resonance could be saturated for a time (t) of 3.0 s by using a preirradiation pulse power of 18 dB below 0.2 W. The residual magnetization (M) of each of the amide (NH) resonances following saturation transfer from water was measured relative to its respective control magnetization (M_0) by the ratio of peak heights in the Fourier transformed spectra obtained from the FID(ω_{H_2O}) and the FID($\omega_{\text{off-res}}$). The exchange rates ($1/\tau_{ex}$) of the amide (NH) protons were measured by using eq 1 where $1/T_{1,\text{app}}$ is

$$1/\tau_{ex} = (1/T_{1,\text{app}})(1 - M/M_0) \qquad (1)$$

the selective longitudinal relaxation rate of the amide (NH) proton (Waelder & Redfield, 1977; Waelder et al., 1975). The use of eq 1 assumes complete saturation of the H_2O resonance and negligible solvent intermolecular relaxation (Waelder et al., 1975).

The longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the peptide protons were measured in titrations with $MnCl_2$ in the presence of $Co(NH_3)_4$AMPPCP and catalytic subunit. Under these conditions, Mn^{2+} is known to bind at the inhibitory site of the catalytic subunit, which bridges the enzyme to the triphosphate chain of the Co(III)–nucleotide complex (Granot et al., 1980b). The paramagnetic contributions to the longitudinal ($1/T_{1p}$) and transverse ($1/T_{2p}$) relaxation rates were calculated as the difference in rates measured in the presence and absence of Mn^{2+}. The paramagnetic contributions to the longitudinal and transverse relaxation rates were corrected for the residual outer sphere paramagnetic effects observed after the addition of saturating polyarginine, a competitive inhibitor (Demaille et al., 1977; Granot et al., 1981). Paramagnetic contributions to the relaxation rates were then used to obtain Mn^{2+} to proton distances and lower limit peptide exchange rates by using the general theory reviewed elsewhere (Mildvan & Gupta, 1978; Mildvan et al., 1980, 1983).

Results

Assignment of the Carbon-Bound Proton Resonances of the Ser-Pentapeptide. The 250-MHz ^{1}H NMR spectrum in ^2H_2O of the Ser-pentapeptide is given in Figure 1. The resonance assignments (Table I) were made by several methods. These included selective homonuclear decoupling, comparison with available chemical shift and spin-coupling data on amino acids and peptides (Wüthrich, 1976), and comparison with previous assignments for peptide substrates of protein kinase (Granot et al., 1981). In addition two-dimensional correlated spectroscopy, COSY, a useful technique for displaying the spin-coupling connectivities in biological molecules, was employed (Aue et al., 1976; Kumar et al., 1981; Wagner et al., 1981). A contour plot of the COSY ^{1}H NMR spectrum at 250 MHz

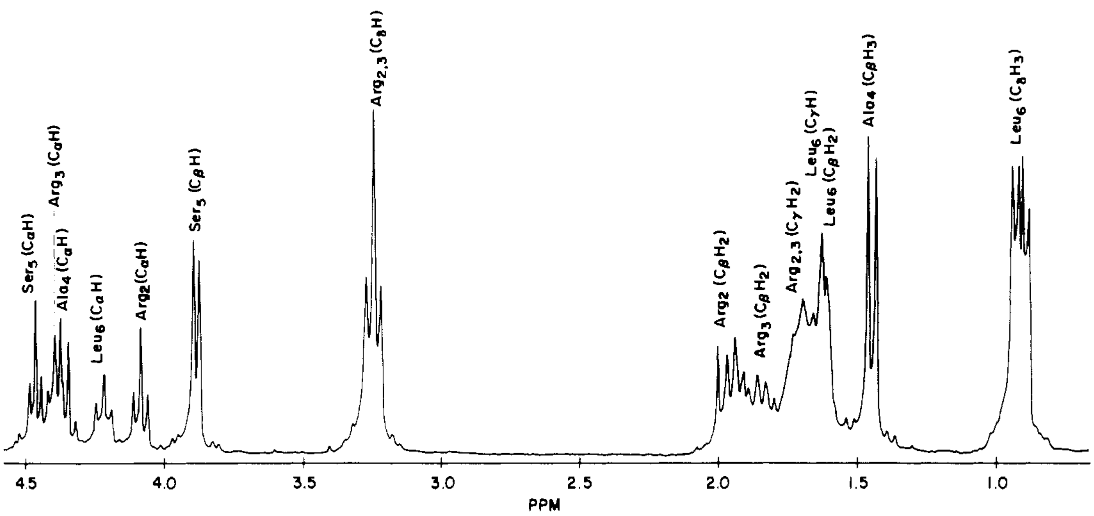


FIGURE 1: Proton NMR spectrum of the carbon-bound protons of the Ser-pentapeptide. The sample contained 15 mM Ser-pentapeptide (Arg-Arg-Ala-Ser-Leu) in ^2H_2O at pH* 6.0. The 250-MHz proton NMR spectrum was obtained with 16-bit A/D conversion using 32 transients each with 8192 data points, an acquisition time of 1.4 s, a relaxation delay of 5.0 s, a spectral width of 3012 Hz, quadrature phase correction, and a 90° pulse. T = 25 °C.

Table I: Proton Chemical Shifts and Spin–Spin Coupling Constants for the Ser-Pentapeptide (Arg$_2$-Arg$_3$-Ala$_4$-Ser$_5$-Leu$_6$)[a]

amino acid resonance	chemical shifts[b] (ppm from DSS)	spin–spin coupling constants[c]		
		$^3J_{\mathrm{HN,HC}}$ (Hz)	$^3J_{\alpha,\beta}$ (Hz)	others (Hz)
Arg$_3$(NH)	8.78	6.5		
Ala$_4$(NH)	8.60	8.3		
Ser$_5$(NH)	8.28	7.0		
Leu$_6$(NH)	8.10	7.8		
Arg$_{2,3}$[N(1)H][d]	7.18			
Arg$_{2,3}$[N(2)H$_2$][d]	6.69			
Ser$_5$(C$_\alpha$H)	4.46		5.0	
Arg$_3$(C$_\alpha$H)	4.40			
Ala$_4$(C$_\alpha$H)	4.36			
Leu$_6$(C$_\alpha$H)	4.22		7.4	
Arg$_2$(C$_\alpha$H)[e]	4.09		5.8	
Ser$_5$(C$_\beta$H$_2$)	3.88		5.1	
Arg$_{2,3}$(C$_\delta$H$_2$)	3.24			$^3J_{\gamma,\delta}$ = 7.4
Arg$_2$(C$_\beta$H$_2$)	1.94		≃6.6	
Arg$_3$(C$_\beta$H$_2$)	1.85			
Arg$_{2,3}$(C$_\gamma$H$_2$)[f]	1.7			
Leu$_6$(C$_\beta$H$_2$)[f]	1.6			
Leu$_6$(C$_\gamma$H)	1.6			
Ala$_4$(C$_\beta$H$_3$)	1.45			
Leu$_6$(C$_\delta$H$_3$)	0.91, 0.94			$^3J_{\gamma,\delta} \simeq$ 5.8

[a] The numbers of the amino acid residues in the Ser-pentapeptide have been numbered so as to correspond to those in the Ser-heptapeptide. Proton chemical shifts and spin–spin coupling constants for carbon-bound proton resonances were obtained at pH* 6.0 and 25 °C. Proton chemical shifts and spin–spin coupling constants for the amide proton resonances were obtained at pH 3.8 and 25 °C. [b] Chemical Shifts are accurate to within ±0.03 ppm. [c] Coupling constants measured directly from the spectra are accurate to within ±0.5 Hz. [d] The guanidinium NH resonances of the arginine residues are numbered as follows: –C(δ)H$_2$–N(1)H–C(ϵ)–[N(2)H$_2$]$_2$. [e] The chemical shift is sensitive to pH. Increasing the pH shifts the resonance upfield. [f] Due to complex spin–spin coupling chemical shifts are accurate to only ±0.1 ppm.

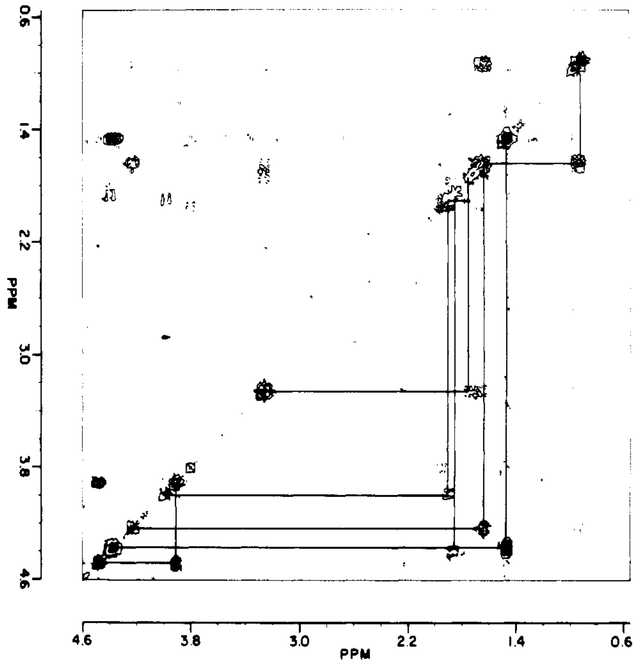


FIGURE 2: Contour plot of the 250-MHz two-dimensional J-correlated (COSY) proton NMR spectrum of the carbon-bound protons of the Ser-pentapeptide. The sample contained 5.0 mM Ser-pentapeptide (Arg-Arg-Ala-Ser-Leu) in ^2H_2O at pH* 6.0. The spectrum was obtained with 16-bit A/D conversion, a spectral width of 2000 Hz in both dimensions, 1024 data points in both dimensions, a relaxation delay of 2.9 s, t_1 and t_2 initial values of 500 μs, and 32 transients for each value of t_1. After acquisition, the data were multiplied by a sine-bell function and the 2-D transform processed to give a magnitude spectrum. T = 25 °C.

of the Ser-pentapeptide is shown in Figure 2. Contours that correspond to peaks in the normal one-dimensional spectrum (Figure 1) appear on the diagonal, with spin-coupling connectivities appearing as symmetrical cross peaks with respect to the diagonal. Spin-coupling connectivities are connected by solid lines. For example, spin-coupling between Ser(C$_\alpha$H) at 4.46 ppm and Ser(C$_\beta$H$_2$) at 3.88 ppm is manifested by symmetrical cross peaks between these two resonances (Figure 2). All of the resonance assignments made by selective homonuclear decoupling (Table I) were confirmed by inspection of the contour plots obtained from the COSY experiments. The assignment of the C$_\alpha$H resonance of the amino-terminal Arg$_2$ residue was confirmed by its upfield shift with increasing pH* from a value of 4.09 ppm at pH* 6.0 to 3.53 ppm at pH* 7.5. Use of the simpler pentapeptide substrate permitted resolution and assignment of all five of the C$_\alpha$H backbone protons. In contrast, ^{1}H NMR studies of the Ser-heptapeptide at 360 MHz could resolve only the C$_\alpha$H proton resonances of the two terminal residues (Granot et al., 1981). A total of 13 carbon-bound proton resonances of the Ser-pentapeptide could be resolved and assigned as compared to only 7 in the Ser-heptapeptide (Granot et al., 1981).

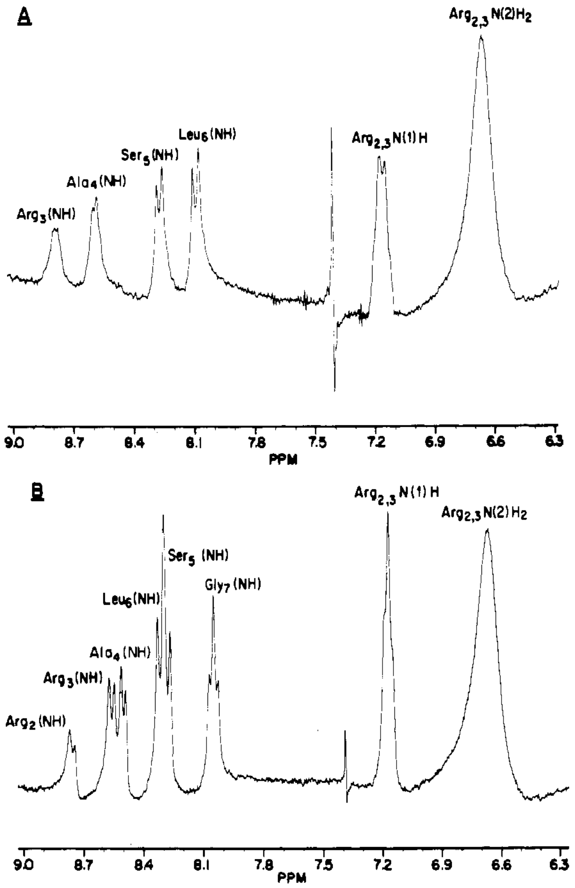


FIGURE 3: NMR spectra of the exchangeable NH protons of the Ser-pentapeptide and Ser-heptapeptide. (A) The 250-MHz NMR spectrum of the exchangeable NH protons of 5.0 mM Ser-pentapeptide (Arg-Arg-Ala-Ser-Leu) taken in H_2O at pH 3.8. Assignments are as given in Table I. (B) The 250-MHz NMR spectrum of the exchangeable NH protons of 5.0 mM Ser-heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) taken in H_2O at pH 3.8. Assignments are as given in Table II. Spectra were obtained with the 2–1–4–1–2 pulse sequence (Redfield et al., 1975) and 16-bit A/D conversion using 256 transients each with 16K data points, an acquisition time of 1.6 s, a spectral width of 5000 Hz, and a relaxation delay of 2.0 s. A line broadening of 1.0 Hz was used in the processing of the data. T = 25 °C.

Assignment of the Exchangeable NH Proton Resonances of the Ser-Pentapeptide and the Ser-Heptapeptide.[4] Paramagnetic effects of Mn^{2+} bound at the inhibitory site of protein kinase on T_1 values of the exchangeable amide NH protons were determined by using selective T_1 measurements and the 2–1–4–1–2 pulse sequence (Redfield et al., 1976). Assignments of the amide NH proton resonances were initially made at pH 3.8 and 25 °C by first assigning the $C_\alpha H$ region in ^2H_2O using selective homonuclear decoupling as previously described and then in H_2O sequentially decoupling the $C_\alpha H$ resonances from the respective amide proton resonances which were observed by using the 2–1–4–1–2 pulse sequence. By use of such specific homonuclear decoupling of the $C_\alpha H$ resonances, all four of the visible amide NH proton resonances could be assigned (Figure 3A; Table I). The $-NH_3^+$ resonance of the amino-terminal Arg was undetectable, presumably due to exchange broadening under all conditions studied. The guanidinium N(1)H resonances of both arginine residues, assigned by their chemical shifts (Klevan & Crothers, 1979) and by decoupling them from $C_\delta H_2$ (Figure 3A; Table I), were not separately resolved. The remaining exchangeable proton resonances were assigned to the overlapping guanidinium $N(2)H_2$ groups of both arginines (Figure 3A; Table I) on the basis of their chemical shifts and the detection of magnetic nonequivalence of the *E* and *Z* positions at 0 °C.

Table II: Amide Proton Chemical Shifts and Spin–Spin Coupling Constants for the Ser-Heptapeptide (Leu_1-Arg_2-Arg_3-Ala_4-Ser_5-Leu_6-Gly_7)[a]

amino acid resonance	chemical shift[b] (ppm from DSS)	spin–spin coupling constants[c] $^3J_{HN,HC}$ (Hz)
$Arg_2(NH)$	8.76	6.4
$Arg_3(NH)$	8.56	6.5
$Ala_4(NH)$	8.50	5.7
$Leu_6(NH)$	8.32	8.5
$Ser_5(NH)$	8.29	6.1
$Gly_7(NH)$	7.94	5.6
$Arg_{2,3}[N(1)H]$[d]	7.19	
$Arg_{2,3}[N(2)H_2]$[d]	6.69	

[a] Amide proton chemical shifts and spin–spin coupling constants obtained at pH 3.8 and 25 °C. [b] Chemical shifts are accurate to within ±0.03 ppm. [c] Coupling constants are accurate to within ±0.5 Hz. [d] The guanidinium NH resonances of the arginine residues are numbered as follows: $-C(\delta)H_2-N(1)H-C(\epsilon)-[N(2)H_2]_2$.

Table III: Steady-State Kinetic Parameters of the Ser-Peptides under Conditions of the NMR Experiments[a]

conditions		Ser-pentapeptide		Ser-heptapeptide	
temp (°C)	pH	K_M (μM)	V_{Max} [μmol/(mg·min)]	K_M (μM)	V_{Max} [μmol/(mg·min)]
10	5.8	90 ± 7	0.42 ± 0.07	23 ± 2	0.68 ± 0.05
25	7.5	37 ± 4	1.71 ± 0.06	10.7 ± 0.5	2.40 ± 0.06

[a] The experiments at pH 5.8 and 10 °C contained 5 mM MES buffer, pH 5.8, 0.15 M KCl, 0.1 mM DTT, 0.5 mg/mL BSA, 100 μM $MnCl_2$, 50 μM ATP, and 12 peptide concentrations over the range 5–200 μM. Those at pH 7.5 and 25 °C contained 10 mM Tris-HCl, pH 7.5, instead of MES but were otherwise identical.

All of the exchangeable amide and guanidinium protons of the Ser-heptapeptide substrate were also assigned by specific homonuclear decoupling of the $C_\alpha H$ and $C\delta H_2$ resonances, respectively, and by comparison with the exchangeable proton region of the Ser-pentapeptide substrate (Figure 3B; Table II). Again, the NH_3^+ resonance of the amino-terminal Leu was undetectable under all conditions studied.

Steady-State Kinetic Studies of the Ser-Peptides. For comparison with the NMR studies, the kinetic parameters of the two Ser-peptides were determined under conditions closely approximating those of the NMR experiments (Table III). The pentapeptide is seen to have a (3.9 ± 0.7)-fold greater K_M and (1.6 ± 0.5)-fold lower V_{max} than those of the heptapeptide. Such small differences in kinetic parameters have previously been found in the interaction of similar penta- and heptapeptides with the cAMP-dependent protein kinase from rat liver (Zetterqvist et al., 1976). Changing the temperature and pH (Table III) is also seen to have relatively small effects on the kinetic constants.

Relaxation Rates of Carbon-Bound Protons of the Ser-Pentapeptide in the Peptide–Enzyme–$Co(NH_3)_4$AMPPCP–Mn^{2+} Complex. Nuclear relaxation studies were carried out by using saturating levels of $Co(NH_3)_4$AMPPCP to create the inhibitory Mn^{2+} site on protein kinase.[2] This nucleotide was used rather than $Co(NH_3)_4$ATP to avoid the slow phosphorylation of the peptide substrates (Granot et al., 1980c, 1981). The longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the carbon-bound protons of the Ser-pentapeptide (10 mM)

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[4] For ease in comparing amino acid positions corresponding to those of the heptapeptide, the pentapeptide residues are numbered as Arg_2-Arg_3-Ala_4-Ser_5-Leu_6.

were measured at 250 MHz in the presence of enzyme (100 μM), $Co(NH_3)_4AMPPCP$ (2.2 mM), and varying amounts of Mn^{2+} (0–154 μM). The relaxation rates in the absence of Mn^{2+} served as diamagnetic controls. The paramagnetic effects of Mn^{2+} at the inhibitory site on $1/T_1$ of the carbon-bound protons of the Ser-pentapeptide in two separate experiments are given in Table IV. From the dissociation constants of the binary enzyme–$Co(NH_3)_4AMPPCP$ and ternary enzyme–$Co(NH_3)_4AMPPCP$–Mn^{2+} complexes (Granot et al., 1980b) it could be shown that the catalytic subunit was >90% saturated with nucleotide under the experimental conditions. Making the reasonable assumption that the enzyme was also saturated with the Ser-pentapeptide at the high concentrations used, the concentration of the pentapeptide–enzyme–$Co(NH_3)_4AMPPCP$–Mn^{2+} complex was calculated by using the dissociation constant of Mn^{2+} from the inhibitory site in the presence of enzyme and $Co(NH_3)_4AMPPCP$ (172 μM; Granot et al., 1980b).[5] From the concentration of enzyme-bound Mn^{2+}, the normalized paramagnetic effects on the longitudinal relaxation rates $[1/(fT_{1p})]$ were calculated (Table IV).

To establish active site binding of the Ser-pentapeptide in the NMR experiments and to estimate the outer sphere contributions to $1/(fT_{1p})$ (Granot et al., 1981; Mildvan et al., 1980, 1983), displacement of the Ser-pentapeptide was carried out with the tight binding competitive inhibitor polyarginine (K_I = 0.76 μM; Demaille et al., 1977) (Table IV). From the K_I of polyarginine and the dissociation constants of heptapeptide substrates and inhibitors (0.2–0.5 mM; Feramisco & Krebs, 1978; Feramisco, 1979) displacement of ~90% of the Ser-pentapeptide by polyarginine would be expected under these conditions. The significant decreases in the paramagnetic effects on $1/T_1$ after the addition of polyarginine (Table IV) indicate active site binding of the Ser-pentapeptide in the NMR experiment. The residual paramagnetic effects were found to be insensitive to further increases of the polyarginine concentrations over the range 215–423 μM, indicating no further displacement by polyarginine and an outer-sphere contribution to $1/(fT_{1p})$ as previously found (Granot et al., 1981). These residual effects on $1/T_1$ at maximal levels of polyarginine were used to correct for the outer-sphere contributions to the $1/(fT_{1p})$ values (Table V). The presence of polyarginine obscured some resonances of the Ser-pentapeptide, precluding measurement of the outer-sphere contribution to their rates. In such cases, the average of the residual paramagnetic effects on the observed resonances was used to correct the $1/(fT_{1p})$ values (Table V). The ultimate effect of such corrections was to increase the Mn–proton distances by only 12 ± 6%.

The normalized paramagnetic effects on the transverse relaxation rates $[1/(fT_{2p})]$ of the $Ala(C_\beta H_3)$ and $Ser(C_\beta H_3)$ protons were calculated in a manner entirely analogous to those of $1/(fT_{1p})$. Their values, corrected for the measured outer-sphere contribution (18%), are given in Table V. Complex spin–spin coupling precluded accurate $1/T_2$ measurements of the other carbon-bound proton signals by the line-broadening method.

In the absence of enzyme, negligible paramagnetic effects of Mn^{2+} on $1/T_1$ and $1/T_2$ of the carbon-bound protons of the Ser-heptapeptide were detected under these conditions (Granot et al., 1981). Hence, no further corrections to $1/(fT_{1p})$ or to $1/(fT_{2p})$ for the weak binary Mn^{2+}–peptide interaction were necessary.

Determination of Distances from Mn^{2+} Bound at the Inhibitory Site of Protein Kinase to the Carbon-Bound Protons of the Ser-Pentapeptide. From the corrected $1/(fT_{1p})$ values (Table V) the distances (r) between the Mn^{2+} bound at the inhibitory site and the carbon-bound protons of the Ser-pentapeptide on the enzyme may be calculated by using the relations

$$1/(fT_{1p}) = 1/(T_{1M} + \tau_M) \qquad (2)$$

$$r = C\left[qT_{1M}\left(\frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2\tau_c^2}\right)\right]^{1/6} \qquad (3)$$

where τ_M is the lifetime of the peptide–enzyme–$Co(NH_3)_4AMPPCP$–Mn^{2+} complex, T_{1M} is the relaxation time of a peptide proton within this complex, q is the relative stoichiometry of bound Mn^{2+} and bound Ser-pentapeptide, τ_c is the correlation time for the Mn^{2+}–proton dipolar interaction, ω_I and ω_S are the nuclear and electron precession frequencies, and C is a constant, numerically equal to 812 Å/s$^{1/3}$ for Mn^{2+}–proton interactions. Peptide (Feramisco & Krebs 1978; Feramisco et al., 1979) and Mn^{2+} binding studies (Armstrong et al., 1979; Granot et al., 1980b, 1981) indicate a relative stoichiometry, q, of 1. As discussed elsewhere (Mildvan et al., 1980), the largest value of $1/(fT_{2p})$ sets a lower limit on the rate of dissociation ($1/\tau_M$) of a ligand from a paramagnetic complex. In the present case, since the measured $1/(fT_{2p})$ value of the $Ser(C_\beta H_2)$ protons exceeds by at least an order of magnitude all of the $1/(fT_{1p})$ values (Table V), it can be concluded that τ_M contributes little to fT_{1p}. Hence, the $1/(fT_{1p})$ values are dominated by $1/T_{1M}$ and can therefore be used in eq 3 to calculate distances. The correlation time (τ_c) for the Mn^{2+}–proton dipolar interaction in protein kinase complexes was previously determined for the Ala-heptapeptide–enzyme–$Co(NH_3)_4ATP$–Mn^{2+} system as $(1.7 \pm 1.2) \times 10^{-9}$ s at 360 MHz on the basis of the frequency dependence of $1/(fT_{1p})$ of the protons of the Ala-heptapeptide over the frequency range 100–360 MHz (Granot et al., 1981). This τ_c value, which represents the average of the frequency-independent and frequency-dependent correlation times (Bean et al., 1977), agrees closely with that of the enzyme–$Co(NH_3)_4AMPPCP$–Mn^{2+} complex [$(1.9 \pm 1.2) \times 10^{-9}$ s; Granot et al., 1980b], indicating that τ_c is not very sensitive to the precise nature of the nucleotide or to the presence or nature of the bound peptide. At 250 MHz, the frequency-independent and frequency-dependent correlation times, calculated from the previously determined frequency dependence of $1/(fT_{1p})$ (Granot et al., 1981), are 0.7×10^{-9} and 1.3×10^{-9} s, respectively. The average of these limiting values [$(1.0 \pm 0.3) \times 10^{-9}$ s] yields an average value of the correlation function, defined as the terms in parentheses of eq 3, of $(8.6 \pm 1.1) \times 10^{-10}$ s at 250 MHz. This value was used in eq 3 to calculate the distances from the enzyme-bound Mn^{2+} to the carbon-bound protons of the Ser-pentapeptide (Table V). The errors in the absolute distances (Table V) reflect those in both $1/(fT_{1p})$ and in $f(\tau_c)$.

Relaxation Rates of the Exchangeable Amide Protons of the Ser-Pentapeptide in the Peptide–Enzyme–$Co(NH_3)_4AMPPCP$–Mn^{2+} Complex. The 250-MHz ^1H NMR spectrum of the Ser-pentapeptide (7.5 mM) in H_2O in the

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[5] We have previously shown that the binding of the regulatory subunit, which occupies the peptide binding site of the catalytic subunit, does not significantly alter the affinity of the catalytic subunit–ADP complex for Mn^{2+} at the inhibitory site (Granot et al., 1980a). Kinetic data indicate that the analogue AMPPCP does not alter the affinity of the enzyme for the Ser-heptapeptide (Whitehouse & Walsh, 1983; Whitehouse et al., 1983). Hence, it is reasonable to assume that small peptides would have little effect on the dissociation constant of Mn^{2+} or nucleotide from the enzyme–$Co(NH_3)_4AMPPCP$ complex.

Table IV: Paramagnetic Effects of Mn^{2+} at the Inhibitory Site of Protein Kinase on the Longitudinal Relaxation Rates and the Normalized Paramagnetic Contribution to the Longitudinal Relaxation Rates of the Carbon-Bound Proton Resonances of Arg_2-Arg_3-Ala_4-Ser_5-Leu_6[a]

	concn (mM)				resonance[b]											
					$Arg_2(C_\alpha H)$		$Arg_3(C_\alpha H)$		$Ala_4(C_\alpha H)$		$Ser_5(C_\alpha H)$		$Leu_6(C_\alpha H)$		$Arg_2(C_\beta H_2)$	
expt	[pentapeptide]	[enzyme]	$[Co(NH_3)_4$ AMP-PCP]	$[Mn^{2+}]$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$
I	10.1	0.11	2.2	0	3.39		1.12		0.96		0.86		0.84		4.65	
				0.053	5.56	1134	2.17	548	1.82	449	1.64	407	1.67	433	6.25	836
				0.090	6.45	1022	3.13	671	2.70	581	2.13	424	2.13	434	6.67	675
				0.154	8.33	1087	3.45	513	3.33	521	2.44	348	2.78	427	7.41	607
				0.126[c]	*e*	*e*	*e*	*e*	*e*	*e*	*e*	*e*	*e*	*e*	*e*	*e*
II	10.0	0.098	2.8	0	2.56		1.45		1.18		0.83		1.11		4.08	
				0.053	5.26	1567	2.44	574	1.96	452	1.59	440	2.22	645	5.71	947
				0.123	7.41	1413	3.64	637	3.03	539	2.67	535	3.03	560	6.58	728
				0.117[d]	4.00	431	*e*	*e*	*e*	*e*	*e*	*e*	*e*	*e*	*e*	*e*

	concn (mM)				resonance[b]											
					$Arg_{2,3}(C_\gamma H_2)$, $Arg_3(C_\beta H_2)$		$Arg_{2,3}(C_\delta H_2)$		$Ala_4(C_\beta H_3)$		$Ser_5(C_\beta H_2)$		$Leu_6(C_\beta H_2)$, $Leu_6(C_\gamma H)$		$Leu_6(C_\delta H_3)$	
expt	[pentapeptide]	[enzyme]	$[Co(NH_3)_4$ AMP-PCP]	$[Mn^{2+}]$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$
I	10.1	0.11	2.2	0	3.77		3.03		2.70		3.33		2.78		1.89	
				0.053	4.65	459	4.00	507	3.03	172	4.55	637	3.23	235	2.22	172
				0.090	*f*	*f*	4.55	508	3.33	210	5.26	645	3.85	357	2.33	147
				0.154	*f*	*f*	5.00	433	3.77	235	6.76	755	4.55	389	2.56	147
				0.126[c]	*e*	*e*	*e*	*e*	3.13	105	4.88	379	*e*	*e*	2.13	59
II	10.0	0.098	2.8	0	3.70		3.03		2.56		3.23		2.86		1.67	
				0.053	4.44	432	4.26	710	3.23	386	4.26	594	3.33	272	2.30	365
				0.123	5.71	587	4.88	538	4.39	532	5.56	678	3.92	309	2.50	242
				0.117[d]	*e*	*e*	*e*	*e*	4.00	342	4.17	280	*e*	*e*	2.13	138

[a] Proton NMR spectra were obtained at 250 MHz in 10 mM deuterated Tris-HCl buffer, pH 7.5, 0.15 M KCl, and 0.1 mM DTT in ^2H_2O by using 16 transients with 16K data points, a spectral width of 3000 Hz, an acquisition time of 2.8 s, 16-bit A/D conversion, quadrature detection, a saturation recovery pulse sequence, and saturation of the residual H_2O signal by irradiation. $T = 25$ °C. The errors in $1/T_1$ are ±10%. Those in $1/(fT_{1p})$ are somewhat greater since differences in measured $1/T_1$ values are taken. [b] The Ser-pentapeptide is numbered according to the Ser-heptapeptide nomenclature as follows: Arg_2-Arg_3-Ala_4-Ser_5-Leu_6. [c] After the addition of 0.330 mM polyarginine. [d] After the addition of 0.423 mM polyarginine. [e] Resonance obscured by the addition of polyarginine. [f] Resonances obscured due to paramagnetic line broadening at higher Mn^{2+} concentrations.

Table V: Paramagnetic Bound State Proton Relaxation Rates and Metal–Proton Distances from Mn^{2+} Bound at the Inhibitory Site of Protein Kinase to the Ser-Pentapeptide and Ser-Heptapeptide Substrates[a]

	Ser-pentapeptide			Ser-heptapeptide		
amino acid[c] resonance	$1/(fT_{1p})$[b] (s^{-1})	$1/(fT_{2p})$ (s^{-1})	r (Å)	$1/(fT_{1p})$ (s^{-1})	$1/(fT_{2p})$ (s^{-1})	r (Å)
Arg_2(NH)				550 ± 170	1600 ± 600	8.7 ± 0.9
Arg_3(NH)	2600 ± 350		6.8 ± 0.6	160 ± 70		10.8 ± 1.2
Ala_4(NH)	240 ± 60	11500 ± 4700	10.1 ± 0.7	130 ± 40		11.1 ± 0.8
Ser_5(NH)	280 ± 120	8000 ± 3500	9.8 ± 0.9	130 ± 40	1900 ± 400	11.1 ± 0.8
Leu_6(NH)	200 ± 80	7800 ± 3500	10.3 ± 1.6	180 ± 60		10.6 ± 1.2
Gly_7(NH)				170 ± 40		10.7 ± 0.7
$Leu_1(C_\alpha H)$						>13[d]
$Arg_2(C_\alpha H)$	810 ± 250		8.2 ± 0.6			
$Arg_3(C_\alpha H)$	300 ± 40		9.7 ± 0.4			
$Ala_4(C_\alpha H)$	250 ± 40		10.0 ± 0.5			
$Ser_5(C_\alpha H)$	220 ± 40		10.2 ± 0.5			
$Leu_6(C_\alpha H)$	260 ± 40		9.9 ± 0.4			
$Gly_7(C_\alpha H_2)$						>13[d]
$Leu_1(C_\delta H_3)$						>13[d]
$Arg_2(C_\beta H_2)$	380 ± 30		9.3 ± 0.3			
$Arg_3(C_\beta H_2)$, $Arg_{2,3}(C_\gamma H_2)$	≤240		≥10.0			
$Arg_{2,3}(C_\delta H_2)$	280 ± 40		9.8 ± 1.0			>12[d]
$Ala_4(C_\beta H_3)$	130 ± 60[e]	3800 ± 1200[e]	11.1 ± 1.2[e]	220 ± 70[e]	2000 ± 300[e]	10.2 ± 0.9[e]
	110 ± 40[f]	6000 ± 1000[f]	11.4 ± 1.0[f]			10.4 ± 1.6[d]
$Ser_5(C_\beta H_2)$	350 ± 30	9900 ± 2000	9.4 ± 0.3			9.1 ± 1.3[d]
$Leu_6(C_\beta H_2)$, $Leu_6(C_\gamma H)$	≤160		≥10.8			
$Leu_6(C_\delta H_3)$	70 ± 40[e]		12.4 ± 1.4[e]	100 ± 40[e]		11.8 ± 1.0[e]
	130 ± 60[f]		11.1 ± 1.3[f]			10.8 ± 1.6[d]

[a] Paramagnetic effects and distances to amide proton (NH) resonances were measured at pH 5.8 and 10 °C. All other measurements were made at pH 7.5 and 25 °C. Distances to $Ala(C_\beta H_3)$ and $Leu(C_\delta H_3)$ were measured at both pH 5.8 and 10 °C and pH 7.5 and 25 °C for comparison. [b] Bound state proton relaxation rates were corrected by subtracting the residual normalized paramagnetic effects in the presence of saturating polyarginine from the average normalized paramagnetic effects. [c] Amino acid resonances are labeled by using the nomenclature for the Ser-heptapeptide (Leu_1-Arg_2-Arg_2-Ala_4-Ser_5-Leu_6-Gly_7). The Ser-pentapeptide is thus labeled Arg_2-Arg_3-Ala_6-Ser_5-Leu_6 for ease of comparison with the Ser-heptapeptide. [d] Distances from Mn^{2+} bound at the inhibitory site of protein kinase to amino acid resonances of the Ser-heptapeptide taken from Granot et al. (1981). [e] Measured at pH 5.8 and 10 °C. [f] Measured at pH 7.5 and 25 °C.

presence of protein kinase (77 μM) and $Co(NH_3)_4AMPPCP$ (1.0 mM) at pH 5.8 and 10 °C is shown in Figure 4A, and the assignments of the exchangeable NH protons are given in Table I. At the lower temperature and pH all four of the amide (NH) resonances are clearly resolved. Since the K_M of the pentapeptide increased only 2.4-fold under these conditions (Table III), the enzyme may be assumed to have similar affinities for the pentapeptide and the heptapeptide. At the high pentapeptide concentrations used, it is therefore reasonable to assume that the enzyme is saturated with the pentapeptide. The adenine H2 and H8 resonances of $Co(NH_3)_4AMPPCP$ are also visible at 8.10 and 8.32 ppm, respectively (Figure 4A). Higher concentrations of $Co(NH_3)_4AMPPCP$ were avoided since the adenine H8 resonance appears on the shoulder of the Ser_5(NH) resonance.[4] These lower concentrations of $Co(NH_3)_4AMPPCP$ resulted in 82–84% of the binding seen at saturation of the enzyme with the nucleotide, which needed to be taken into consideration in calculating the normalized paramagnetic effects of Mn^{2+} on the relaxation rates. The paramagnetic effects of Mn^{2+} at the inhibitory site of protein kinase on $1/T_1$ of the amide (NH), $Ala_4(C_\beta H_3)$, and $Leu_6(C_\delta H_3)$ protons of the Ser-pentapeptide from two experiments are summarized in Table VI. Because of the subsaturating level of $Co(NH_3)_4AMPPCP$ present, simultaneous equations based on the dissociation constants of $Co(NH_3)_4AMPPCP$ from the enzyme (281 μM) and of Mn^{2+} from the enzyme–$Co(NH_3)_4AMPPCP$ complex (172 μM) at pH 7.5 (Granot et al., 1980b) were used to calculate the concentration of the peptide–enzyme–$Co(NH_3)_4AMPPCP$–Mn^{2+} complex and the normalized relaxation rates (Tables V and VI).[5] This correction resulted in a small (~3%) decrease in the distances, well within their errors. As described above, the outer-sphere contributions to the relaxation rates were evaluated by the addition of a high level of the competitive inhibitor polyarginine (370 μM) which significantly reduced the paramagnetic effects on $1/T_1$ by 33–100% (Table VI). The $1/(fT_{1p})$ and $1/(fT_{2p})$ values, corrected for outer-sphere relaxation, are summarized in Table V.

In a separate experiment, negligible paramagnetic effects of $MnCl_2$ (0–338 μM) on $1/T_1$ or $1/T_2$ of the amide (NH) protons of the Ser-pentapeptide (5.1 mM) were detected in the absence of protein kinase and $Co(NH_3)_4AMPPCP$. Hence, as previously found for the carbon-bound protons of the Ser-heptapeptide in the presence of Mn^{2+} (Granot et al., 1981), no corrections for such binary interactions were necessary in the present case.

To examine the possibility that the paramagnetic effects of Mn^{2+} on $1/T_1$ of the amide (NH) protons might result from chemical exchange with more rapidly relaxing water protons, rather than by a direct dipolar effect, the exchange rates of the (NH) protons of the pentapeptide with H_2O were measured by the transfer of saturation from water under the conditions of the NMR experiment. Three of the four amide protons, those of Ala_4, Ser_5, and Leu_6, exchanged protons with water at rates <0.2 s^{-1} while that of Arg_3 exchanged at 8.5 ± 1.0 s^{-1}. All of these exchange rates constitute negligible contributions to $1/(fT_{1p})$ well below the errors in this parameter (Tables V and VI).

Since the largest $1/(fT_{2p})$ value, that of Ala_4(NH) at pH 5.8 and 10 °C, exceeds by at least an order of magnitude the $1/(fT_{1p})$ values of the Ala_4(NH), Ser_5(NH), Leu_6(NH), $Ala_4(C_\beta H_3)$, and $Leu_6(C_\delta H_3)$ protons (Table V), these $1/(fT_{1p})$ values are not limited by the rate of chemical exchange be-

Table VI: Paramagnetic Effects of Mn^{2+} at the Inhibitory Site of Protein Kinase on the Longitudinal Relaxation Rates and the Normalized Paramagnetic Contribution to the Longitudinal Relaxation Rates of the Amide (NH) and Other Proton Resonances of the Ser-Peptides in H$_2$O[a]

		concn (mM)				resonances[b]															
						Arg$_2$(NH)		Arg$_3$(NH)		Ala$_4$(NH)		Ser$_5$(NH)		Leu$_6$(NH)		Gly$_7$(NH)		Ala$_4$(C$_\beta$H$_3$)		Leu$_6$(C$_\delta$H$_3$)	
expt	Ser-peptide	[peptide]	[enzyme]	[Co(NH$_3$)$_4$AMPPCP]	[Mn^{2+}]	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$	$1/T_1$	$1/(fT_{1p})$
I	penta	7.5	0.077	1.1	0			18.18		4.17		3.64		3.08				2.63		2.22	
					0.019			17.54	0	4.35	281	4.00	562	3.23	235			2.63	0	1.54	0
					0.046			21.50	2330	4.55	268	4.48	588	3.39	217			3.13	350	2.56	238
					0.099			25.00	2503	5.26	400	4.76	411	3.62	199			3.85	447	2.75	194
					0.089[c]			8.33	0	*e*	*e*	*e*	*e*	3.45	144			3.33	273	3.45	144
II	penta	5.1	0.102	1.1	0			13.51		4.76		4.08		3.18				3.64		2.86	
					0.039			20.83	3262	5.40	287	4.46	171	3.64	205			4.22	258	2.98	55
					0.097			25.32	2366	5.71	190	5.00	184	4.35	237			4.65	202	3.31	90
					0.088[d]			12.66	0	4.22	0	3.13	0	3.85	134			4.35	93	2.85	0
III	hepta	5.0	0.100	1.0	0	20.41		5.00		4.83		4.65		3.92		2.26		3.33		3.03	
					0.034	22.22	932	5.56	288	5.26	222	5.12	242	4.08	82	3.28	526	3.87	278	3.33	155
					0.065	21.74	379	6.14	324	5.40	162	5.21	160	4.44	148	4.08	517	4.26	264	3.37	95
					0.094	24.69	824	6.12	216	5.65	158	5.40	145	4.72	154	5.78	679	5.56	429	3.55	100
					0.089[f]	18.87	0	*e*	*e*	*e*	*e*	5.00	71	3.85	0	4.17	390	4.17	171	3.03	0
IV	hepta	5.1	0.090	0.94	0	18.52		5.44		4.44		4.33		3.89		2.56		3.77		2.90	
					0.056	20.00	539	5.95	186	4.88	160	4.93	218	4.64	263	3.48	335	4.71	343	3.30	146
					0.131	21.28	501	6.10	120	5.95	274	5.56	223	5.08	216	5.18	476	4.85	196	3.51	110
					0.120[g]	19.23	135	*e*	*e*	*e*	*e*	4.65	61	4.81	174	3.85	244	3.20	0	3.20	57

[a] Conditions are given in Figure 4 and in the text. The errors in $1/T_1$ are ±10%. Those in $1/(fT_{1p})$ are somewhat greater since differences in measured $1/T_1$ values are taken. [b] The Ser-pentapeptide is numbered according to the Ser-heptapeptide nomenclature as follows: Arg$_2$-Arg$_3$-Ala$_4$-Ser$_5$-Leu$_6$. [c] After the addition of 0.370 mM polyarginine. [d] After the addition of 0.384 mM polyarginine. [e] Resonance obscured by the addition of polyarginine. [f] After the addition of 0.418 mM polyarginine. [g] After the addition of 0.370 mM polyarginine.

Table VII: Deviations between 31 Measured Distances and Distances Obtained from Models of Heptapeptides of Various Secondary Structure[a]

structure	no. of deviating distances	range of deviations (Å)	$\sum$ of deviations (Å)	structure	no. of deviating distances	range of deviations (Å)	$\sum$ of deviations (Å)
α helix[b]	11	2.4–6.4	42.7	β_{2-5} turn I	12	1.7–4.8	20.2
β-sheet[c]	8	1.5–5.3	23.5	β_{2-5} turn II	12	1.2–4.8	21.0
β_{4-5} bulge[d]	10	1.5–6.8	32.4	β_{3-6} turn I	14	1.2–5.9	29.6
β_{5-6} bulge[e]	11	1.8–5.7	36.8	β_{3-6} turn II	13	1.2–5.9	27.7
β_{1-4} turn I[f]	14	1.0–6.3	33.8	β_{4-7} turn I	13	1.2–6.1	24.5
β_{1-4} turn II	14	1.0–5.8	34.1	β_{4-7} turn II	13	1.0–6.1	25.6

[a] Absolute deviations were calculated from the experimental distances to the Ser-pentapeptide, when available. Secondary structures were chosen on the basis of those most frequently found in peptides and proteins (Richardson, 1981). Distances from the Mn^{2+} bound at the inhibitory site or from the metal of CrAMPPCP at the active site were measured to the various amino acid protons of a given structure by first fixing the position of the Mn^{2+} and Cr^{3+} using three or four experimental distances. Distances that were measured by model building had an ±0.2-Å error. The distance between the inhibitory Mn^{2+} and the Cr^{3+} of CrAMPPCP was set at 4.8 Å (Granot et al., 1980c). [b] Right-handed α helix. For model building the inhibitory Mn^{2+} was placed on the left side of the α helix when Arg$_2$(NH$_3^+$) of the Ser-heptapeptide is at the lower left corner of the helix. The Mn^{2+} could not be positioned on the right side of the α helix due to steric interactions with the Arg$_3$ and Leu$_6$ R groups. [c] Antiparallel β-pleated sheet of the Ser-heptapeptide. [d] β bulge of the Ser-heptapeptide. Positions 1 and 2 of the β bulge, defined by Richardson (1981), correspond to Ala$_4$ and Ser$_5$. [e] β bulge of the Ser-heptapeptide. Positions 1 and 2 of the β bulge, defined by Richardson (1981), correspond to Ser$_5$ and Leu$_6$. [f] Eight β turns possible within the Ser-heptapeptide. The I and II refer to type I and type II β turns, respectively.

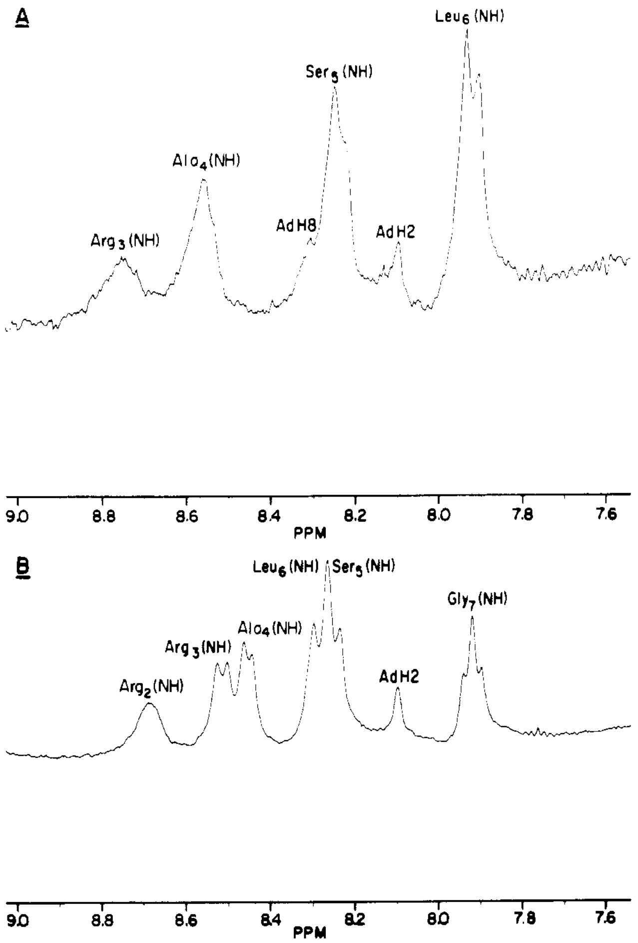

FIGURE 4: NMR spectra of the amide (NH) protons of the Ser-pentapeptide and Ser-heptapeptide in the presence of the catalytic subunit of protein kinase and Co((NH$_3$)$_4$AMPPCP. (A) The 250-MHz NMR spectrum of 7.5 mM Ser-pentapeptide (Arg-Arg-Ala-Ser-Leu) in the presence of 77 μM catalytic subunit of protein kinase, 1.1 mM Co(NH$_3$)$_4$AMPPCP, 5 mM MES buffer, pH 5.8, 0.15 M KCl, and 0.1 mM DTT in H_2O. (B) The 250-MHz NMR spectrum of 5.0 mM Ser-heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) in the presence of 100 μM catalytic subunit of protein kinase, 1.0 mM Co(NH$_3$)$_4$AMPPCP, 5 mM MES buffer, pH 5.8, 0.15 M KCl, and 0.1 mM DTT in H_2O. The spectra were obtained by using the 2–1–4–1–2 pulse sequence (Redfield et al., 1975) and 16-bit A/D conversion using 32 transients for spectrum A and 64 transients for spectrum B each with 16K data points, an acquisition time of 1.6 s, a spectral width of 5000 Hz, and a relaxation delay of 1.0 s. A line broadening of 1.0 Hz was used in processing all data. T = 10 °C.

tween free and bound peptide ($1/\tau_M$) and can be utilized in eq 3 for the calculation of distances. By use of the correlation time at 250 MHz [(1.0 ± 0.3) × 10^{-9} s], calculated from the $1/(fT_{1p})$ values measured as a function of frequency (Granot et al., 1981), distances were calculated by using eq 3. The errors in the reported distances reflect those in both $1/(fT_{1p})$ and the correlation time (Table V). The lower limit of the largest observed $1/(fT_{2p})$ value, 6.8 × 10^3 s^{-1} (Table V), sets a lower limit to $1/\tau_M$ which exceeds the observed $1/fT_{1p})$ value for the Arg$_3$(NH) by only 2.6-fold. Thus, the $1/(fT_{1p})$ value of Arg$_3$(NH) may be partially limited by the exchange rate of free and bound peptide. By use of eq 2 and the lower limit value of $1/\tau_M$, an upper limit $1/T_{1M}$ value of 4200 s^{-1} is calculated. The resulting uncertainty of the $1/T_{1M}$ value for the Arg$_3$(NH) proton (2600–4200 s^{-1}) is reflected in the somewhat larger error in the Mn^{2+} to Arg$_3$(NH) distance (Table V).

Paramagnetic Effects of Mn^{2+} at the Inhibitory Site of Protein Kinase on the Exchangeable Amide Protons of the Ser-Heptapeptide. The 250-MHz ^{1}H NMR spectrum of the Ser-heptapeptide (5.0 mM) at pH 5.8 and 10 °C in the presence of protein kinase (100 μM) and Co(NH$_3$)$_4$AMPPCP (1.0 mM) is shown in Figure 4B. All six of the amide (NH) protons resonances of the Ser-heptapeptide are resolved. Under these conditions, the adenine H2 resonance of the Co(NH$_3$)$_4$AMPPCP was clearly visible at 8.10 ppm (Figure 4B). However, the adenine H8 resonance of Co(NH$_3$)$_4$AMPPCP was not resolved and found to be under the Leu$_6$(NH) resonance. To prevent the adenine H8 resonance from interfering with the measurement of the relaxation rates of the Leu$_6$(NH) proton, the concentration of Co(NH$_3$)$_4$AMPPCP was kept at 1.0 mM (Table VI). Under these conditions 82–84% of the nucleotide binding sites were occupied. Hence, simultaneous equations were used as described above to calculate the concentrations of bound Co(NH$_3$)$_4$AMPPCP and bound Mn^{2+}. The effects of Mn^{2+} at the inhibitory site of protein kinase on $1/T_1$ of the amide (NH), Ala$_4$(C$_\beta$H$_3$), and Leu$_6$(C$_\delta$H$_3$) protons of the Ser-heptapeptide, from two experiments, are given in Table VI. The average residual paramagnetic effect on $1/(fT_{1p})$ after the addition of saturating polyarginine was 33% (Table VI), indicating active site binding of the heptapeptide and a significant outer-sphere contribution to $1/(fT_{1p})$. Residual paramagnetic effects on $1/(fT_{1p})$ and $1/(fT_{2p})$ in the presence of polyarginine were used, as described above, to obtain corrected $1/(fT_{1p})$ and $1/(fT_{2p})$ values (Table V).

As with the pentapeptide, the exchange rates of the amide (NH) protons of the heptapeptide with water were found, by transfer of saturation, to be negligible compared to their respective $1/(fT_{1p})$ values. Thus, the amide protons of Arg$_3$, Ala$_4$, Ser$_5$, Leu$_6$, and Gly$_7$ exchanged with water protons at rates ≤0.1 s^{-1} while that of Arg$_2$ exchanged at a rate of 7.5 ± 1.0 s^{-1}.

Since the maximal $1/(fT_{2p})$ value (Table V) exceeds the $1/(fT_{1p})$ values of Arg$_3$(NH), Ala$_4$(NH), Ser$_5$(NH), Leu$_6$(NH), and Gly$_7$(NH) by at least an order of magnitude, the $1/(fT_{1p})$ values are not limited by chemical exchange and may be utilized in eq 3 for the calculation of Mn^{2+} to proton distances (Table V). The lower limit to the exchange rate ($1/\tau_M \geq 1.7 \times 10^3$ s^{-1}), based on the lower limit of the maximum $1/(fT_{2p})$ value, exceeds the $1/(fT_{1p})$ value of Arg$_2$(NH), 550 s^{-1} (Table V), by only 3.1-fold. As described above, this lower limit to $1/\tau_M$ leads, from eq 2, to an upper limit of 810 s^{-1} for $1/T_{1M}$ of Arg$_2$(NH). The wide range of values of $1/T_{1M}$ for the Arg$_2$(NH) proton (550–810 s^{-1}) slightly increases the error in the Mn^{2+} to Arg$_2$(NH) distance (Table V).

Kinetic Properties of the Complexes Studied by NMR. The largest values of $1/(fT_{2p})$ set lower limits to the exchange rates of the Ser-peptides out of their respective enzyme complexes of ~6.8 × 10^3 s^{-1} for the pentapeptide and ~1.7 × 10^3 s^{-1} for the heptapeptide (Table V). These values exceed by at least 2 orders of magnitude the catalytic rate of protein kinase, indicating that the complexes studied by NMR are kinetically competent to participate in catalysis. The 4-fold greater limiting values for the exchange of the pentapeptide over that of the heptapeptide may reflect its greater rate of dissociation from and weaker binding to the enzyme.

Discussion

Previously determined distances from Mn^{2+} at the inhibitory site and from Cr^{3+} at the activating site of protein kinase to carbon-bound protons of heptapeptide substrates and analogues were found to be incompatible with α-helical or β-pleated-sheet conformations (Granot et al., 1981). However, no other classes of secondary structure could be ruled out by these distance measurements. The present studies of the simpler pentapeptide substrate permitted distances to be measured to all five of the backbone C_α protons and to all four of the backbone amide protons, as well as to eight side-chain protons. The combination of the paramagnetic probe–T_1 method with the Redfield method for suppression of the water proton signal also permitted measurement of distances to all six of the backbone amide protons of the Ser-heptapeptide substrate. These 15 new backbone distances and 4 new side-chain distances permitted a more precise determination of the secondary structure of the enzyme-bound peptide substrate.

Our conclusions on the conformation of the enzyme-bound peptide substrate of protein kinase are applicable not only to the Ser-pentapeptide, based on the distances obtained with this substrate, but also to the heptapeptide substrate, using all of the available distances. The appropriateness of using distances measured with both the pentapeptide and heptapeptide substrates, and under two sets of experimental conditions, to derive the heptapeptide conformation may be shown as follows. With two minor exceptions discussed below, the distances from Mn^{2+} at the inhibitory site to corresponding protons of both the pentapeptide and heptapeptide substrates were in excellent agreement within their experimental errors (Table V), suggesting very similar conformations for the two bound substrates. Especially important is the agreement of the distances from Mn^{2+} to the Ser($C_\beta H_2$) group which is directly bonded to the site of phosphorylation by ATP. These agreements are consistent with the similar kinetic properties of the two peptides (Table III). The exceptional distances are those to Arg_3(NH) and $Arg_{2,3}$($C_\delta H_2$), which are greater by 4.0 ± 1.3 and ≥1.2 Å, respectively, in the heptapeptide. These differences may be due to the closer proximity of the Arg residues to the N-terminus in the pentapeptide. Moreover, in the heptapeptide the latter distance was estimated only as a lower limit (Granot et al., 1981). Changing the experimental conditions from pH 7.5, 25 °C, to pH 5.8, 10 °C, produced no detectable changes in distances (Table V) in accord with the small differences in kinetic behavior of the peptides under these two sets of conditions (Table III).

To avoid the slow phosphorylation of the peptides during the NMR studies, the analogue $Co(NH_3)_4$AMPPCP rather than $Co(NH_3)_4$ATP was used to create the inhibitory Mn^{2+} binding site on protein kinase. The possibility of structural differences between complexes of ATP analogues and those of ATP is raised by recent kinetic and binding studies with AMPPNP (Whitehouse & Walsh, 1983; Whitehouse et al., 1983). While AMPPNP competes for the ATP binding site of protein kinase, unlike ATP the analogue does not raise the affinity of the enzyme for the Ser-heptapeptide substrate above that found in the absence of nucleotides. Our studies with both $Co(NH_3)_4$AMPPCP and $Co(NH_3)_4$ATP revealed no differences in their binding properties (Granot et al., 1980b) and no differences in distances from Mn^{2+} to the carbon-bound protons of the Ser-heptapeptide, indicating no differences in the peptide conformation when either nucleotide was present (Granot et al., 1981).

In model-building studies to derive the heptapeptide and pentapeptide conformations, the major classes of secondary structure found on the surfaces of proteins were considered: the α helix, the antiparallel β-pleated sheet, β bulges, and β turns. In each of these structures, specific and regular hydrogen bonding patterns are observed between carbonyl oxygens and amide protons (Richardson, 1981). Skeletal models of a heptapeptide of each of these classes were constructed and were fitted to several measured Mn^{2+} to proton distances (Table V) and to two measured Cr^{3+} to proton distances, setting the intermetal distance at 4.8 Å (Granot et al., 1980b). Distances from Mn^{2+} and Cr^{3+} were then measured to other CH and NH protons in the model and compared with those determined by nuclear relaxation (Table V). The deviations, summarized in Table VII, clearly permit the exclusion not only of the α-helix and β-sheet conformations but also of both β bulges and all of the eight possible β turns within the bound heptapeptide substrate. The distances determined by NMR are consistent only with extended coil structures as determined by building both skeletal and space-filling models. Two such coil structures, derived from the measured distances by positioning the metals on either side of the heptapeptide chain, are shown in Figure 5. The differences in their C_α backbone structures are more clearly shown in Figure 6.

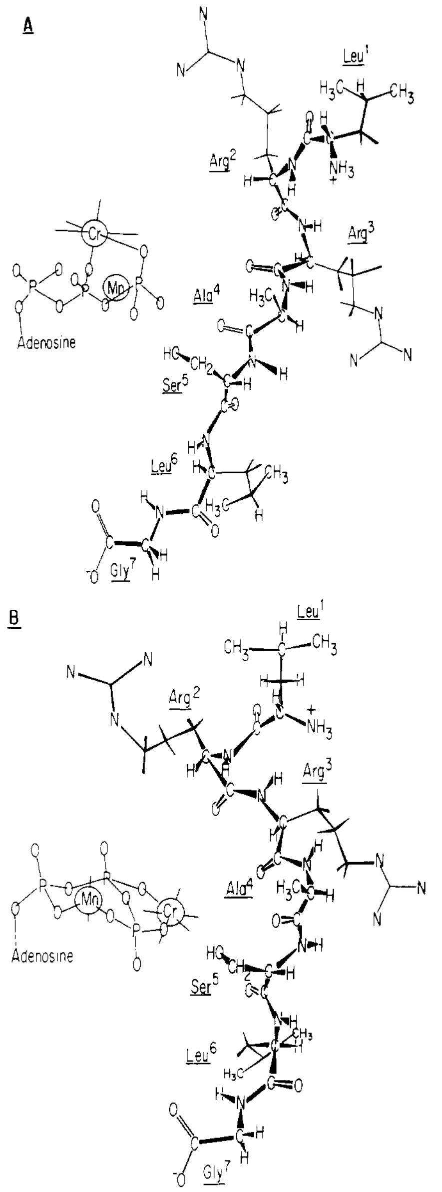

FIGURE 5: Alternative coil conformations for the enzyme-bound heptapeptide substrate of protein kinase. Models consistent with all of the measured distances were built with the Mn^{2+} and Cr^{3+} reference points either on the left (A) or on the right (B) of the peptide chain. For comparison, the models are shown in a similar orientation with respect to the metals, with the backbone bonds highlighted.

The use of distances determined only with the pentapeptide also yields extended coils and permits the exclusion of all other major classes of secondary structure. Shortening the distance between Mn^{2+} and Arg_3 (NH) from that found in the heptapeptide to that found in the pentapeptide changes the torsional angles ψ at Arg_2 from −40° to +60° and ϕ at Arg_3 from

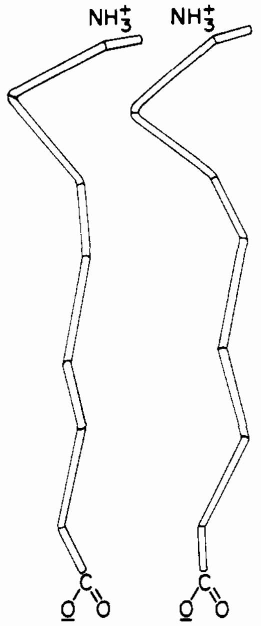

FIGURE 6: Comparison of the C_α backbone structures of the alternative coils shown in Figure 5. The structure on the left corresponds to Figure 5A and that on the right to Figure 5B.

+22° to −130° but preserves the highly extended structure. The small decrease in distances from Mn^{2+} to $Arg_{2,3}(C_\delta H_2)$ found in the pentapeptide had no effect on the backbone conformation. No other structural differences were observed between the two peptide substrates. Our finding of an extended coil structure for the bound peptide substrates of protein kinase may be compared with the predominantly extended conformations of bound peptide and protein substrates on other enzymes. The tripeptide glutathione, on glyoxalase I, is highly extended as determined by nuclear relaxation studies (Sellin et al., 1982),[6] as is the conformation of oxidized glutathione on glutathione reductase based on X-ray crystallography (Schulz et al., 1978). Extended, predominantly coil conformations of the contact regions of bound polypeptide inhibitors are found by X-ray analysis on each of the four classes of proteases, serine proteases (Robertus et al., 1972; Ruhlmann et al., 1973; Segal et al., 1971; Sweet et al., 1975), thiol proteases (Drenth et al., 1971), metalloproteases (Matthews, 1977; Rees & Lipscomb, 1982), and acid proteases (Bott et al., 1982; James et al., 1982). On serine proteases, the bound peptides contain a short and possibly distorted strand of β-pleated sheet, which is nevertheless highly extended (Segal et al., 1971).

In contrast with protease–substrate complexes, the cytochrome *c* peroxidase–cytochrome *c* complex may involve, in part, a helix–helix interaction, based on the optimum positioning of the two separately determined X-ray structures (Poulos & Kraut, 1980). Protein–protein interactions other than enzyme–substrate complexes such as intersubunit and interdomain contacts within individual proteins involve all types of secondary structure, with extended β-strand–β-strand interactions to form sheets predominating (Richardson, 1981).

The universality of highly extended substrate conformations on proteases is not surprising since these enzymes operate on the protein backbone, catalyzing cleavage at a specific amide bond after having recognized a local amino acid sequence. The present finding of an extended coil conformation for the bound substrate on a protein kinase is of special interest since it is not the peptide backbone which is covalently modified by this class of enzymes but a seryl side chain which is phosphorylated. Two possible advantages of such a conformation may be considered. First, such a structure could allow flexibility of the bound substrate, permitting it to achieve an optimal geometry for reaction. Second, when the number of interactions within the substrate is minimized, an extended coil conformation might permit greater interaction with the enzyme, thereby contributing to the specificity of the enzyme.

Acknowledgments

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Registry No. Arg-Arg-Ala-Ser-Leu, 90083-77-5; Leu-Arg-Arg-Ala-Ser-Leu-Gly, 65189-71-1; Mn, 7439-96-5; L-arginine (homopolymer), 25212-18-4; protein kinase, 9026-43-1; L-arginine (SRU), 24937-47-1.

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Side-Chain Motions in Peptides Bound to Elastase. NMR Relaxation Investigation on Carbon-13 Specifically Enriched Trifluoroacetyl-Tripeptide Inhibitors[†]

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ABSTRACT: Kinetic, ^{19}F NMR, and recently X-ray data have shown that CF_3CO-tripeptides and dipeptide anilides bind in a unique mode to elastase, the CF_3CO group interacting with a specific site on the enzyme. We report the results of an NMR study of the interaction with elastase of the two CF_3CO-tripeptides, CF_3CO-Ala_3 and CF_3CO-Lys-Ala_2, in which the N-terminal residue has been uniformly enriched with ^{13}C. We first show that neglecting cross-relaxation and cross-correlation effects should lead at 25 and 50 MHz to small errors in ^{13}C relaxation times T_1 of uniformly enriched molecules bound to elastase. Under these conditions, the model-free analysis [Lipari, G., & Szabo, A. (1982) *J. Am. Chem. Soc. 104*, 4546–4559] of the ^{13}C relaxation times of the two enzyme–inhibitor complexes obtained at two frequencies of observation by using the C_α carbon as a probe of the protein motion provides estimations of the generalized order parameters S^2 of each ^{13}C atom. These estimations allow calculation with a good precision of the rotation angles γ_{0i} about each $C_{i-1}C_i$ bond, selecting restricted multiple rotation as a model for the side-chain motion and assuming that the lowest energy conformation of the lysine side chain in solution is trans as observed in the crystal. Angles γ_{0i} once obtained have then been used to estimate the correlation times τ_i of rotation about each $C_{i-1}C_i$ bond, from a complete analysis of the experimental T_1. The results show that the $C_\beta H_3$ of [^{13}C]Ala is still freely rotating in the complex. In contrast the side chain of [^{13}C]Lys is found to be highly immobilized. Furthermore, the difference of orientation of the Ala and Lys side chains is suggested by the different chemical shift behaviors of the C_β of these amino acids during complexation. The similar affinity of both peptides is interpreted in light of these NMR data.

The peptide CF_3CO-Lys-Ala-NH-C_6H_4-*p*-CF_3 is a strong inhibitor of elastase. The structure of its complex with porcine pancreatic elastase has been recently determined at 2.5-Å resolution by X-ray crystallography (Hughes et al., 1982). This resolution is sufficient to observe close contacts between the peptide and the protein and to estimate the local mobilities through isotropic atomic temperature coefficients. On the other hand, enzyme kinetics and ^{19}F NMR have shown that CF_3CO-tripeptides and dipeptide anilides bind to the enzyme in a single mode involving the interaction of the trifluoroacetyl group at a specific site of the protein (Dimicoli et al., 1979, 1980). Their affinity for elastase critically depends, however, on the nature of each amino acid and also on the substituents

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