Role of Enzyme–Peptide Substrate Backbone Hydrogen Bonding in Determining Protein Kinase Substrate Specificities†

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ABSTRACT: As part of a search for peptides that have specificity for selected protein kinases, the possibility that adenosine cyclic 3′,5′-phosphate dependent protein kinase (A-kinase) recognizes the hydrogen-bonding potential of its peptide substrates was investigated. A-Kinase catalyzes the phosphorylation of five N^{α}-methylated and four depsipeptide derivatives of Leu-Arg-Arg-Ala-Ser-Leu-Gly (peptide 1) at rates that differ by at least 7 orders of magnitude. These peptide 1 analogues each lack the ability to donate a hydrogen bond at selected positions in the peptide chain. If a particular amide hydrogen of a peptide amide is involved in hydrogen bonding, which is important for enzyme recognition, the prediction is that peptides which contain an ester or a N-methylated bond at that position in peptide 1 will be comparatively poor substrates. In contrast, if a depsipeptide has a reactivity comparable to that of peptide 1 but the analogous N-methylated peptide has a poor reactivity with A-kinase, the result might indicate that the *N*-methyl group causes unfavorable steric effects. The depsipeptide that lacks a Leu^6 amide proton is a good substrate for A-kinase, but the corresponding N-methylated peptide is phosphorylated far less efficiently. This result and others presented in this paper suggest that although enzyme–substrate hydrogen bonding may play some role in A-kinase catalysis of phosphoryl group transfer, other explanations are necessary to account for the relative reactivities of N^{α}-methylated and depsi-containing peptide 1 analogues. Alternate explanations that cannot be eliminated from the data presented here include the presence of disruptive peptide–enzyme steric interactions or intrapeptide steric interactions that might prevent a peptide 1 analogue from assuming a conformation recognizable by A-kinase. These possibilities are examined further in the following papers.

Phosphorylation and dephosphorylation of proteins are thought to exist in a dynamic equilibrium. The perturbation of this equilibrium occurs through the activation or inhibition of protein kinases, which catalyze the phosphorylation of proteins, and protein phosphatases, which catalyze phosphoprotein dephosphorylation. Several protein kinases and a few protein phosphatases have been purified and characterized (Cohen, 1985; Flockhart & Corbin, 1982). Many enzymes are known to be activated or inhibited by their phosphorylation or dephosphorylation. In addition to these enzymes with relatively well-understood biological roles, hundreds of phosphoproteins have been revealed by gel electrophoresis and their roles remain to be elucidated. Many more phosphoproteins have been found than there are known protein kinases. This discovery is in accord with the finding that some protein kinases have a broad substrate specificity. The first broad specificity protein kinase discovered was cAMP[1]-dependent protein kinase (A-kinase, EC 2.7.1.37; ATP protein phosphotransferase) (Walsh et al., 1968). This enzyme is the primary receptor for cAMP, which is the second messenger for a large number of hormones.

Often more than one protein kinase can catalyze the phosphorylation of the same protein in vitro. The study of the physiological roles of a protein kinase can be complicated by the fact that many of the protein kinases have similar in vitro substrate specificities. The following papers report on the development of an approach for targeting substrates and inhibitors to selected protein kinases. First, a thorough study of how A-kinase binds its substrates was undertaken. The effects on substrate specificity of substrate–enzyme hydrogen bonding and substrate conformation in the active site of A-kinase were investigated. The active site of G-kinase was then probed for differences between the A- and G-kinases that might be exploited in the design of inhibitors specific for G-kinase.

Peptides of the form Arg-Arg-X-Ser-Y are phosphorylated efficiently in the presence of A-kinase (Bramson et al., 1984). The important features of such peptide substrates have been shown to be the two arginine residues, a one amino acid spacer (X) that can be any one of several residues, a serine residue, and an amino acid (Y) that is preferentially a hydrophobic residue (Zetterqvist et al., 1976; Kemp et al., 1977; Feramisco et al., 1980; Ragnarsson et al., 1979) but not proline (Granot et al., 1981). In the case of the A-kinase substrate rat liver pyruvate kinase, a peptide reproducing the phosphorylation site, Leu-Arg-Arg-Ala-Ser-Val-Ala, is phosphorylated with kinetic constants that are identical within experimental error with those for the protein (Pilkis et al., 1980). From such studies and from sequences of proteins that are known to be phosphorylated in A-kinase-catalyzed reactions, it has been

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; Mops, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin; CI, chemical ionization; Tris, tris(hydroxymethyl)aminomethane; DIEA, diisopropylethylamine; DCC, dicyclohexylcarbodiamide; HPLC, high-pressure liquid chromatography.

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generalized that multiple basic residues N-terminal to the site of phosphorylation are common features of A-kinase substrates (Flockhart & Corbin, 1982; Kemp et al., 1975).

Despite its similarity to the above substrate, the peptide Leu-Arg-Arg-Ala-Ser-Pro-Gly is at best an extremely poor substrate for A-kinase (Granot et al., 1981). This unexpected result demonstrates that there are factors influencing the binding of substrates to A-kinase that are not yet understood. Possibly, because proline lacks an amide proton, this peptide might be unable to form an essential hydrogen bond at the proline position. Since biological specificity is thought to be determined, in part, by hydrogen bonding, the absence of a hydrogen bond may account for the inability of a peptide to be recognized by the enzyme. In addition to elucidating the mechanism of binding, the manipulation of hydrogen bonding might provide a means of targeting inhibitors for specific protein kinases. Also, in order to probe later the conformational implications of peptide 1 backbone modifications, it was first necessary to identify peptide backbone structural features that are crucial for enzyme–substrate interactions.

The ability of biomolecules to hydrogen bond is thought to be important for the formation of several structural motifs such as DNA double helices, α-helices, and β-sheets. The interfaces between protein subunits and protein domains are often extensions of β-sheets (Schulz & Schirmer, 1979), emphasizing the importance of hydrogen bonding in protein–protein interactions. Similarly, hydrogen bonding is thought to play a role in contributing to enzyme specificity and to the process of catalysis itself (Fersht, 1985). Opinions differ as to whether hydrogen bonding provides a driving force for binding, but it is often stated to be responsible for specificity of binding. Such specificity might occur because hydrogen-bond acceptors or donors left unpaired when two proteins interact might be energetically disruptive. Because catalysis might be aided by differential hydrogen bonding to the transition state vs. the substrate (Jencks, 1969; Fersht, 1985), a detailed understanding of enzyme–substrate hydrogen bonding would aid mechanistic studies. In summary, the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly might utilize its hydrogen-bond donors and acceptors to form a secondary structural motif, to participate in bonds with cAMP-dependent protein kinase, or for transition-state stabilization. Hydrogen bonding between peptide substrates and the catalytic subunit of A-kinase was thus investigated.

Experimental Procedures

Materials. Number 1 cellulose phosphate paper P81 was from Whatman. All Sephadex resins were from Sigma or Pharmacia. Polystyrene cross-linked with 1% divinylbenzene was from Pierce, as were solutions of 4 N HCl in dioxane. All amino acid derivatives were from Peninsula or Bachem, and all N-methylated amino acids except Boc(*N*-Me)Arg(Tos) were from Bachem or Swiss Bachem. [γ-^{32}P]ATP was from New England Nuclear. ATP, DTT, Mops, and Tris were from Sigma. The syntheses of Leu-Arg-Arg-Ala-Ser-Leu-Gly (peptide 1) and Leu-Arg-Arg-Ala-Ser-Pro-Gly (peptide 12) have been reported before (Granot et al., 1981). All other chemicals were of reagent grade or a higher quality.

Methods. The catalytic subunit of this enzyme from bovine heart muscle was purified according to the method of Demaille et al. (1977) with the modifications previously described (Bramson et al., 1982). The concentrations of catalytic subunit and ATP were determined spectrophotometrically by using A_{280nm} = 14.9 for a 1% solution of enzyme (Peters et al., 1977) and ϵ_{260nm} = 15.4 mM^{-1}, respectively. Peptide concentrations were determined by amino acid analysis. Ultraviolet spectra were measured with Perkin-Elmer Lambda 5 or Cary 219 spectrometers. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Fourier-transformed NMR spectra were obtained with a Nicolet 360-MHz spectrometer.

Peptide phosphorylation was monitored by three different methods, as has been described before (Bramson et al., 1985). ATP concentrations were varied from 1 to 100 μM, and peptide concentrations were varied as follows: peptide 1, 1–110 μM; peptide 7, 20–1000 μM; peptide 8, 3–230 μM; peptide 9, 20–1000 μM; peptide 10, 2–200 μM; peptide 11, 1–100 μM. Initial rate data were analyzed with an Apple IIe microcomputer in three ways: (1) linear least-squares fit to the Lineweaver–Burk double reciprocal plots; (2) fit to a hyperbolic velocity vs. [substrate] plot using the iterative program obtained from Professor John Westley of the Department of Biochemistry, The University of Chicago, which follows the methodology of Wilkinson (1961); and (3) fit to the rate equations using the curve-fitting program MULTI of Yamaoka et al. (1981).

N^{α}-Boc(N^{α}-Me)(N^{g}-Tos)Arg. The general procedure of Quitt et al. (1963) for the synthesis of N^{α}-Me(N^{g}-NO_2)Arg was utilized with the following modifications. (N^{g}-Tos)Arg was treated 4 times rather than 2 times with benzaldehyde followed by borohydride. After each of the three final borohydride treatments, the reaction was worked up according to the procedure of Quitt et al. (1963). The final product was recrystallized from boiling water to obtain a yield of 41%. This amino acid moved as a single spot (R_f = 0.49) when chromatographed on Merck silica gel 60 plates in chloroform/methanol/acetic acid (16:4:1 v/v). The following peaks were observed in the NMR spectrum for N^{α}-Bzl(N^{g}-Tos)Arg at 360 MHz (CD_3OD): δ 7.74 (d, 2), 7.49 (m, 5), 7.30 (d, 2), 4.18 (m, 2), 3.53 (m, 1), 3.15 (m, 2), 2.40 (s, 3), 1.88 (m, 2), and 1.61 (m, 2).

N^{α}-Bzl(N^{α}-Me)(N^{g}-Tos)Arg was obtained according to the method of Quitt et al. (1963) and then debenzylated in methanol by treatment with Pd/C and cyclohexene (10 equiv) at 65 °C. The product, obtained after filtration and evaporation, was treated with di-*tert*-butyl dicarbonate (Pierce), and N^{α}-Boc(N^{α}-Me)(N^{g}-Tos)Arg was extracted according to the general protocol of Moroder et al. (1976). After the resultant product was dried over sodium sulfate and the solvent was evaporated, the yield was 31% [from N^{α}-Bzl(N^{g}-Tos)Arg]. The clear oil chromatographed as a single spot on Merck silica gel 60 plates developed in chloroform/methanol/acetic acid (16:4:1 v/v). The CI low-resolution mass spectrum was consistent with the expected product; 360-MHz NMR ($CDCl_3$) δ 7.72 (d, 2), 7.27 (m, 2), 6.50 (br, 1), 4.68 + 4.42 (br, 1), 3.20 (m, 2), 2.74 (s, 3), 2.37 (s, 3), 1.95 (m, 2), 1.74 (m, 2), and 1.48 (m, 9). Anal. Calcd for $C_{19}H_{30}N_4O_6S$: C, 51.6; H, 6.83; N, 12.66. Found: C, 51.25; H, 6.97; N, 12.19.

Synthesis of Leu-Arg-(N^{α}-Me)Arg-Ala-Ser-Leu-Gly (Peptide 7), Leu-Arg-Arg-(N-Me)Ala-Ser-Leu-Gly (Peptide 8), Leu-Arg-Arg-Ala-(N-Me)Ser-Leu-Gly (Peptide 9), and Leu-Arg-Arg-Ala-Ser-(N-Me)Leu-Gly (Peptide 10). N-Methylated peptides were synthesized as was described before (Bramson et al., 1985), except as noted. Boc(*N*-Me) amino acids were coupled to the growing resin-bound peptide as hydroxybenzotriazole active esters in the manner described by Yamashiro and Li (1978). These residues were deprotected with 4 N HCl in dioxane and were not preneutralized. The formation of N-methylated amides was accomplished by direct DCC coupling, employing a ratio of amino acid to DCC to resin-bound peptide of 3:3:1, respectively. Two equivalents of DIEA was added only after the coupling reaction had begun.

Table I: Identities of Peptides 1–12

peptide no.	peptide sequence[a]	type of bond (underlined)
1	Leu-Arg-Arg-Ala-Ser-Leu-Gly	
2	AcLeu-Arg-Arg-Ala-Ser-Leu-GlyOEt	
3	AcLeu-(argininic acid)-Arg-Ala-Ser-Leu-GlyOEt	ester
4	AcLeu-Arg-(argininic acid)-Ala-Ser-Leu-GlyOEt	ester
5	AcLeu-Arg-Arg-[(*S*)-(+)-lactic acid]-Ser-Leu-GlyOEt	ester
6	AcLeu-Arg-Arg-Ala-Ser-(α-hydroxyisocaproic acid)-GlyOEt	ester
7	Leu-Arg-(*N*-Me)Arg-Ala-Ser-Leu-Gly	N-methylated
8	Leu-Arg-Arg-(*N*-Me)Ala-Ser-Leu-Gly	N-methylated
9	Leu-Arg-Arg-Ala-(*N*-Me)Ser-Leu-Gly	N-methylated
10	Leu-Arg-Arg-Ala-Ser-(*N*-Me)Leu-Gly	N-methylated
11	Leu-Arg-Arg-Ala-Ser-Leu-(*N*-Me)Gly	N-methylated
12	Leu-Arg-Arg-Ala-Ser-Pro-Gly	

[a] The ester-containing peptides have been described before.

Each peptide was gel filtered and purified by ion-exchange chromatography as described earlier (Bramson et al., 1985). Peptides 7–10 were analyzed by HPLC and were eluted as a single peak from an Altex analytical C18 column with a gradient from 10% to 60% CH_3CN in 0.2 M sodium phosphate, pH 2.1, buffer. Amino acid analysis; for peptide 7, Ala(1) 1.00, Arg(1) 0.95, Gly(1) 1.04, Leu(2) 1.94, Ser(1) 0.92; for peptide 8, Arg(2) 2.18, Gly(1) 1.00, Leu(2) 1.90, Ser(1) 0.89; for peptide 9, Ala(1) 1.00, Arg(2) 2.23, Gly(1) 1.03, Leu(2) 1.91; for peptide 10, Ala(1) 1.00, Arg(2) 2.16, Gly(1) 1.02, Leu(1) 1.02, Ser(1) 0.88. The molecular weight of each peptide was confirmed by fast atom bombardment mass spectrometry.

Synthesis of Leu-Arg-Arg-Ala-Ser-Leu-(N-Me)Gly (Peptide 11). Peptide 11 was synthesized according to the procedure described for the synthesis of the other N-methylated peptides with the following alterations. The Boc group of the second amino acid was removed with 4 N HCl in dioxane, and the BocSer(Bzl) was coupled to the free amine according to the procedure described for the formation of a N-methylated amide. This method was adopted to avoid *N*-alkyldiketopiperazine formation, which has been noted before (Khosla et al., 1972; Gisin & Merrifield, 1972). The peptide was purified by gel filtration, ion-exchange chromatography, and HPLC using an Altex C18 semipreparative column and the elution conditions noted earlier for analytical HPLC. Further analysis by HPLC using a C18 analytical column detected no impurities. Amino acid analysis: for peptide 11, Ala(1) 0.94, Arg(2) 2.00, Leu(2) 2.00, Ser(1) 0.95. The molecular weight was confirmed by fast atom bombardment mass spectroscopy.

Results

A series of peptides based on the sequence of peptide 1 in which amide protons were systematically eliminated by replacing single peptide bonds with N-methylated bonds was synthesized. In all cases the amino acid side chains were left unchanged. These peptides are listed in Table I, together with several depsipeptides [which contain esters in place of amides, as described in Bramson et al. (1985)]. The depsipeptides are acetylated at the N-terminus and esterified at the C-terminus, which simplified their synthesis. An acetylated and esterified version of peptide 1 was synthesized as a standard for kinetic measurements on this series of peptides.

Both an ester bond and a *N*-methylamide bond lack the amide hydrogen that might be involved in hydrogen bonding. In addition, the *N*-methyl bond has a methyl group in place of the amide hydrogen, and this methyl group can cause steric hindrance with the enzyme or intrapeptide steric hindrance. N-Methylation of amides in peptides restricts allowed conformations due to the greater size of a methyl group vs. a hydrogen. There are, of course, no hydrogens in an ester, and these bonds are not subject to the steric hindrance encountered with N-methylated peptides. The rationale for synthesizing and testing these two series of peptides together is as follows. If N-methylated peptide 1 analogues are poor substrates for A-kinase due to steric hindrance, then the depsipeptide analogues may be good substrates. If both *N*-methyl and ester analogues are poor substrates, then a simple interpretation is that enzyme–substrate or substrate–substrate hydrogen bonding is important.

The phosphorylation of each peptide was studied in the presence of the A-kinase catalytic subunit, which exhibits the full phosphotransferase activity of this enzyme. Various concentrations of [γ-^{32}P]ATP as the phosphoryl group donor were employed, and peptide concentrations were varied about 100-fold. The data for these peptides were analyzed in two ways. Initial studies established approximate Michaelis constants for the peptides, which were then divided into two groups on the basis of whether the Michaelis constant was above or below 500 μM. For peptides that had apparent Michaelis constants of over 500 μM, the total time courses for peptide phosphorylation were measured and the data were analyzed as shall be described later. The initial rates for phosphorylation of peptides that had K_m values below 500 μM were analyzed according to steady-state sequential Bi-Bi and rapid-equilibrium random Bi-Bi mechanisms (Cook et al., 1982; Whitehouse & Walsh, 1983; Whitehouse et al., 1983). Both mechanisms yield the same form of rate equation, differing only in their definition of the constants in the equation. The constants in this study were labeled according to the steady-state sequential Bi-Bi mechanism (see Scheme I), which is constant with the data of Whitehouse and co-workers.

Scheme I

$$E + ATP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{–}ATP + P \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{–}ATP\text{–}P \xrightarrow{k_5} E + \text{products}$$

In Scheme I, E is enzyme, E–ATP is an enzyme–ATP noncovalent complex, P is peptide, and E–ATP–P is an enzyme–ATP–peptide noncovalent complex. The rate equation for Scheme I is

$$v = \frac{k_5[E]_0}{1 + \frac{K_{m,ATP}}{[ATP]} + \frac{K_{m,peptide}}{[P]} + \frac{K_{d,ATP}K_{m,peptide}}{[ATP][P]}} \quad (1)$$

where the subscript 0 denotes initial concentration, $K_{m,ATP} = k_5/k_1$, $K_{m,peptide} = (k_4 + k_5)/k_3$, and $K_{d,ATP} = k_2/k_1$. The Michaelis constants measured for each substrate were found to increase approximately 3-fold as the second substrate concentration was increased about 100-fold (data not shown). For example, the apparent K_m describing peptide 1 phosphorylation was calculated to be 4 and 14 μM, at fixed ATP concentrations of 1 and 100 μM, respectively. In an analogous fashion, the apparent $K_{m,ATP}$ was found to vary from 5 to 14 μM, when fixed concentrations of peptide 1 from 1 to 110 μM were included in the assays. This relatively low variation of the apparent Michaelis constants indicates that the steady-state ordered and rapid-equilibrium mechanistic schemes are reasonable approximations of the enzymatic mechanism but that there are complexities in the enzymatic mechanism that are not fully accounted for by either mechanistic scheme.

Table II: Kinetic Constants for the A-Kinase-Catalyzed Phosphorylation of Peptides 1–12[a]

peptide no.	$k_{cat}/K_{m,peptide}$ (min^{-1} μM^{-1})	k_{cat} (min^{-1})	$K_{m,peptide}$ (μM)	$K_{m,ATP}$ (μM)
1	68.2 ± 0.09	911 ± 53	13.3 ± 1.4	12.6 ± 1.4
2	136 ± 14	500 ± 55	3.68 ± 0.39	6.7 ± 1.5
3	289 ± 40	535 ± 75	1.80 ± 0.19	10 ± 2
4	0.378 ± 0.041	24.5 ± 2.0	64.8 ± 6.8	4.8 ± 0.2
5	14.2 ± 2.7	186 ± 30	13.3 ± 3.1	3.2 ± 1.0
6	12.0 ± 2.5	31.5 ± 1.9	2.63 ± 0.20	8.4 ± 1.0
7	0.366 ± 0.011			
8	11.4 ± 0.3	796 ± 67	69.7 ± 7.7	14.7 ± 1.4
9	$\leq(1.5 \pm 0.3) \times 10^{-5}$			
10	0.111 ± 0.001			
11	66.5 ± 1.0	1040 ± 50	16.1 ± 2.1	22.0 ± 1.4
12	$(3 \pm 1) \times 10^{-5}$			

[a] These kinetic constants were measured in 50 mM Mops buffer, pH 7.0, containing 0.15 M KCl, 1 mM DTT, 0.2 mg/mL BSA, and 1–1500 nM enzyme at 30 °C. The kinetic values for peptides 2–6 have been reported before (Bramson et al., 1985).

Since we found that high concentrations of peptide substrates inhibit the enzymatic phosphotransferase activity, concentrations of peptides over 2 mM were not employed. Therefore, accurate Michaelis constants for peptide of over 1 mM cannot be obtained by using initial rate kinetics. Peptides 7, 9, 10, and 12 fall into the category of substrates with Michaelis constants greater than 1 mM, and the complete time course for the phosphorylation of each of these peptides was measured by using peptide concentrations less than the Michaelis constant. Under conditions of ATP in large excess, the phosphorylation of peptide can be treated as a single substrate reaction, and the data can be analyzed according to

$$v = k_{cat}[E]_0[P]/(K_{m,peptide} + [P]) \quad (2)$$

where v is velocity, k_{cat} is the rate constant for the slowest step, $K_{m,peptide}$ is the Michaelis constant for peptide, P is peptide, and the subscript 0 denotes initial concentration. Under conditions of $[P] \ll K_{m,peptide}$, the data can be analyzed graphically in terms of a pseudo-first-order reaction according to

$$\ln([P]_0/[P]) = ([E]_0 k_{cat}/K_{m,peptide})T \quad (3)$$

where T is time. The slope of the plot of $\ln([P]_0/[P])$ vs. T gave the parameter $k_{cat}/K_{m,peptide}$. All of these plots were linear over the time period considered, indicating that no significant amounts of ATP were consumed through the ATPase activity inherent to the enzyme (Armstrong et al., 1979a).

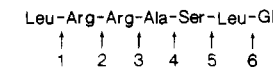
The kinetic parameters for the phosphorylation of peptides 2–6, 8, and 11 that were obtained from secondary Lineweaver–Burk plots and the simultaneous analysis of all data using the multiple iterative curve-fitting routine of Yamaoka et al. (1981) are presented in Table II (both methods yielded equivalent results). In all cases $K_{d,ATP}$ is approximately equal to $K_{m,ATP}$ and is insensitive to peptide concentration (data not shown). The result is reasonable since it has been demonstrated previously that the value for K_d measured by equilibrium dialysis for MgATP binding to the catalytic subunit is experimentially indistinguishable from the value for $K_{m,ATP}$ determined from the reaction kinetics for phosphorylation of peptide 1 (Armstrong et al., 1979b). The values listed in Table II for the kinetic parameter $k_{cat}/K_{m,peptide}$ that describes the phosphorylation of peptides 7, 9, 10, and 12 were obtained by a graphical analysis of total time course data, as was described under Methods.

Discussion

Evidence exists for the occurrence of substrate–enzyme hydrogen bonding in several systems. For example, X-ray crystallographic data are consistent with the postulate that significant hydrogen bonding occurs when the pancreatic enzymes trypsin and chymotrypsin bind substrates or inhibitors. In these cases a three hydrogen bond extended coil is thought to be formed between the amide backbones of the substrate and the enzyme (Steitz et al., 1969). Fersht and co-workers have investigated hydrogen bonding in the active site of tyrosyl-tRNA synthetase (Fersht et al., 1985; Wells & Fersht, 1985). These researchers have systematically altered, by site-specific mutagenesis, enzyme residues suggested by the X-ray crystal structure to be within hydrogen-bonding distance of the substrate. The results are consistent with the postulate that hydrogen bonding is important for enzymic substrate and transition-state binding. Ingles and Knowles (1968) investigated chymotrypsin-catalyzed hydrolysis of substrates that have methylene, ester, or N-methylated bonds in place of the amide N-terminal to the scissile bond and concluded that this amide proton was involved in hydrogen bonding to the enzyme. This postulate was later found to be consistent with the X-ray crystallographic studies mentioned above. Interestingly, the absence of this hydrogen bond compromised the enzyme's ability to distinguish between L and D amino acids. The conclusion of this work was that this hydrogen bond plays an important role in the transition state for catalysis.

In a hydrogen bond, the distance from the polar hydrogen atom to the acceptor atom is less than the sum of their van der Waals radii. The van der Waals radius of a hydrogen atom is 1 Å, while that of an acceptor atom is ≤2 Å. If a hydrogen involved in hydrogen bonding is replaced by a methyl group as in the N-methylated peptides, the latter will not participate in that hydrogen bond due to the low polarity of a methyl group. In addition, the methyl group might cause steric problems due to the 0.8 Å greater van der Waals radius than that of a hydrogen atom. Therefore, a peptide analogue containing a *N*-methyl group in place of an amide hydrogen that hydrogen bonds to the enzyme should be a poorer substrate than the corresponding amide. A depsipeptide also cannot donate a proton for hydrogen bonding, but ideally produces fewer steric problems, and so is expected to have a reactivity lower than the amide but higher than the corresponding N-methylated peptide. The possibility that a hydrogen-bond acceptor in A-kinase has the flexibility to move away from and accommodate an *N*-methyl amide group on a peptide seems unlikely. In the work of Ingles and Knowles (1968), the substrate N-methylated at a hydrogen-bonding position was hydrolyzed at a substantially slower rate than the corresponding ester substrate.

The kinetic data for the depsipeptide and N-methylated peptide analogues and proline-containing peptides are summarized with respect to bond numbering:



The amide protons at positions 1, 3, 5, and 6 are not crucial for recognition of peptide by A-kinase. Elimination of the hydrogens at positions 1 and 6 causes no decrease in the kinetic parameter $k_{cat}/K_{m,peptide}$ for peptide phosphorylation. The peptide with an ester bond at position 3 is phosphorylated 10-fold less well than the parent peptide, possibly indicating that a hydrogen bond causes a 10-fold increase in the parameter $k_{cat}/K_{m,peptide}$. However, in contrast, the corresponding N-methylated peptide is only 5 times less reactive than peptide

1, and further the peptide Leu-Arg-Arg-Pro-Ser-Leu-Gly has full substrate reactivity, judging by the kinetic parameter $k_{cat}/K_{m,peptide}$ (Feramisco et al., 1979). Thus, the hydrogen bond at position 3 is unimportant, and other factors must cause the poorer reactivity of the depsipeptide. A possibility that might account for this effect is that the different geometries of an ester and an amide (Pauling, 1960; Brant et al., 1969; Corey et al., 1985) lead to slightly different peptide conformations (Goodman et al., 1974). Alternatively, the lower reactivity of the depsipeptide could be due to the lower basicity of an ester carbonyl vs. an amide carbonyl. Introduction of an ester bond at position 5 causes a similar 10-fold loss in reactivity. Whether this effect is due to the loss of a hydrogen bond is unclear. However, even if hydrogen bonding occurs at this position, an upper limit of its contribution to catalysis has been established as a 10-fold increase in $k_{cat}/K_{m,peptide}$. This effect is small in comparison to the 840-fold decreased binding of a phosphonate ester vs. a phosphonamidate analogue to the enzyme thermolysin (Bartlett & Marlowe, 1987).

Each peptide analogue that does not contain an amide proton at position 2 or 4 is a far worse substrate for A-kinase than is peptide 1. Placing an ester or an N-methylated bond at position 2 yields peptides that are 400- and 200-fold worse substrates, respectively, than peptide 1, when judged by the kinetic parameter $k_{cat}/K_{m,peptide}$. This result could be due in part to the removal of a hydrogen bond. However, the fact that the N-methylated peptide has higher reactivity than the depsipeptide, unlike the results of the study of Ingles and Knowles (1968) in which the N-methylated substrate was orders of magnitude worse than the corresponding ester substrate, would argue that other factors play a significant role in the low reactivity of the depsipeptides with an ester bond at position 2. Likewise, the 10^7-fold lower reactivity of Leu-Arg-Arg-Ala-(*N*-Me)Ser-Leu-Gly (peptide 12) might suggest that the amide hydrogen at position 4 is important for effective interactions with the enzyme. This possibility at present cannot be eliminated; however, an analogue of peptide 12, which contains a Gly in place of Ala[4], is phosphorylated at least 37-fold more rapidly than peptide 12 in the presence of enzyme (Bramson et al., 1987), indicating that not all of the loss in peptide reactivity upon N-methylation at this position is due to a loss of a hydrogen bond.

In summary, peptide analogues containing backbone modifications have been studied with A-kinase. Hydrogen bonding between the peptides and the catalytic subunit alone does not adequately explain the relative reactivities of peptides 2–12. Alternate explanations include the presence of disruptive peptide–enzyme steric interactions and intrapeptide steric interactions that prevent the peptide from assuming a conformation recognizable by A-kinase. Steric interactions will be explored further in the following paper.

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REFERENCES

Armstrong, R. N., Kondo, H., & Kaiser, E. T. (1979a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 722–725.

Armstrong, R. N., Kondo, H., Granot, J., Kaiser, E. T., & Mildvan, A. S. (1979b) *Biochemistry 18*, 1230–1238.

Bartlett, P. A., & Marlowe, C. K. (1987) *Science (Washington, D.C.) 235*, 569–571.

Bramson, H. N., Thomas, N., Matsueda, R., Nelson, N. C., Taylor, S. S., & Kaiser, E. T. (1982) *J. Biol. Chem. 257*, 10575–10581.

Bramson, H. N., Kaiser, E. T., & Mildvan, A. S. (1984) *CRC Crit. Rev. Biochem. 15*, 93–124.

Bramson, H. N., Thomas, N. E., & Kaiser, E. T. (1985) *J. Biol. Chem. 260*, 15452–15457.

Bramson, H. N., Thomas, N. E., Miller, W. T., Fry, D. C., Mildvan, A. S., & Kaiser, E. T. (1987) *Biochemistry* (following paper in this issue).

Brant, D. A., Tonelli, A. E., & Flory, P. F. (1969) *Macromolecules 2*, 228–235.

Cohen, P. (1985) *Eur. J. Biochem. 151*, 439–448.

Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, R., Jr. (1982) *Biochemistry 21*, 5794–5799.

Corey, E. J., Engler, T. A., & Berg, J. M. (1985) *Acta Crystallogr., Sect. C: Cryst. Struct. Commun. C41*, 289–292.

Demaille, J. G., Peters, K. A., & Fischer, E. H. (1977) *Biochemistry 16*, 3080–3086.

Feramisco, J. R., Kemp, B. E., & Krebs, E. G. (1979) *J. Biol. Chem. 254*, 6987–6990.

Feramisco, J. R., Glass, D. B., & Krebs, E. G. (1980) *J. Biol. Chem. 255*, 4240–4245.

Fersht, A. (1985) *Enzyme Structure and Mechanism*, W. H. Freeman, New York.

Fersht, A. R., Wilkinson, A. J., Carter, P., & Winter, G. (1985) *Biochemistry 24*, 5858–5861.

Flockhart, D. A., & Corbin, J. D. (1982) *CRC Crit. Rev. Biochem. 12*, 133–186.

Gisin, B. F., & Merrifield, R. B. (1972) *J. Am. Chem. Soc. 94*, 3102–3106.

Goodman, M., Gilon, C., Palumbo, M., & Ingwall, R. T. (1974) *Isr. J. Chem. 12*, 67–77.

Granot, J., Mildvan, A. S., Bramson, H. N., Thomas, N., & Kaiser, E. T. (1981) *Biochemistry 20*, 602–610.

Ingles, D. W., & Knowles, J. R. (1968) *Biochem. J. 108*, 561–569.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York.

Kemp, B. E., Bylund, D. B., Huang, T. S., & Krebs, E. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3448–3452.

Kemp, B. E., Graves, D. J., Benjamini, E., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 4888–4894.

Khosla, M. C., Smeby, R. R., & Bumpus, F. M. (1972) *J. Am. Chem. Soc. 94*, 4721–4724.

Moroder, L., Hallet, A., Wuensch, E., Keller, O., & Wersin, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1651–1653.

Pauling, L. (1960) *The Nature of the Chemical Bond*, 3rd ed., Cornell University Press, Ithaca, NY.

Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691–5697.

Pilkis, S. J., El-Maghrabi, M. R., Coven, B., Claus, T. H., Tager, H. S., Steiner, D. S., Keim, P. S., & Heinrikson, R. L. (1980) *J. Biol. Chem. 255*, 2770–2775.

Quitt, P., Hellerbach, J., & Vogler, K. (1963) *Helv. Chim. Acta 46*, 327–333.

Ragnarsson, U., Zetterqvist, O., Titanji, V., Edlund, B., & Engstrom, L. (1979) *Peptides 1979* (Siemion & Kupryszewski, Eds.) Wroclaw University Press, Wroclaw, Poland.

Schulz, G. E., & Schirmer, R. H. (1979) *Principles of Protein Structure* (Cantor, C. R., Ed.) pp 102–103, Springer-Verlag, New York.

Steitz, T. A., Henderson, R., & Blow, D. M. (1969) *J. Mol. Biol. 46*, 337–348.

Walsh, D. A., Perkins, J. P., & Krebs, E. G. (1968) *J. Biol. Chem. 243*, 3763–3765.

Wells, T. N. C., & Fersht, A. R. (1985) *Nature (London) 316*, 656-657.
Whitehouse, S., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3682-3692.
Whitehouse, S., Feramisco, J. R., Casnellie, J. E., Krebs, E. G., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3693-3701.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324-332.
Yamaoka, K., Tanigawara, Y., Nakagawa, T., & Uno, J. (1981) *J. Pharmacobio. Dyn. 4*, 879-885.
Yamashiro, D., & Li, C. H. (1978) *J. Am. Chem. Soc. 100*, 5174-5179.
Zetterqvist, O., Ragnarsson, U., Humble, E., Berglund, L., & Engstrom, L. (1976) *Biochem. Biophys. Res. Commun. 70*, 696-703.

Conformation of Leu-Arg-Arg-Ala-Ser-Leu-Gly Bound in the Active Site of Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase†

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ABSTRACT: Studies utilizing NMR spectroscopy have shown that adenosine cyclic 3′,5′-phosphate dependent protein kinase (A-kinase) probably binds Leu-Arg-Arg-Ala-Ser-Leu-Gly (peptide 1) in one of two extended coil conformations (A or B). The relative reactivities of a series of N-methylated peptides based on the structure of peptide 1 might, therefore, be related to how well each can assume the A or B conformation. From estimates of the magnitude of steric interactions that would be induced by N-methylation of an amide in peptide 1 that is locked in either conformation, the ability of each peptide to form that conformation was predicted. The ability of A-kinase to catalyze phosphorylation of the N-methylated peptides correlated well with the ability of each peptide to form conformation A, but not conformation B. In accord with these findings, the reactivity of an unreactive N-methylated peptide was partially restored by a second change, which allowed the peptide to assume conformation A. These results suggest that, when bound in the enzymatic active site, peptide 1 has a conformation that resembles structure A much more closely than structure B.

Although a motif of multiple basic residues is commonly found in cAMP[1]-dependent protein kinase substrates and inhibitors, this alone is not sufficient to account for the specificity of this enzyme. Additional factors must account for the increased affinity of the protein inhibitors over the short peptides. The studies of Scott et al. (1985) and Cheng et al. (1986) illustrate that larger (15-20 residues) but not smaller (5-10 residues) peptides mimic the tight binding of the heat-stable protein inhibitor for A-kinase. Whether this increased affinity with longer sequences is due to the inclusion of additional primary sequence recognition elements or to conformational effects is as yet not understood.

Other studies have indicated that substrates, as well as inhibitors, are influenced by conformational and/or distal effects. In the case of protein phosphatase inhibitor I, a physiological substrate for the A-kinase, the peptides Arg-Arg-Pro-Thr-Pro-Ala and Arg-Arg-Arg-Arg-Pro-Thr-Pro-Ala, which reproduce the phosphorylation site of the protein, are not good substrates for A-kinase (Chessa et al., 1983). Possibly, the enzyme preferentially recognizes a particular conformation that this sequence forms in the protein but that is not adequately simulated in the peptide. By analogy, perhaps certain of the N-methylated derivatives of peptide 1 may not be good substrates for A-kinase because, due to steric hindrance, they might be unable to adopt a conformation recognized by the enzyme. Enzyme–substrate hydrogen bonding, though well documented for other enzymes (Bartlett & Marlowe, 1987; Fersht, 1985), does not adequately explain the different reactivities of the depsi and N-methylated analogues of peptide 1 as substrates of A-kinase (Thomas et al., 1987a). In this paper, the potential intrapeptide steric interactions introduced by peptide backbone N-methylation are investigated with the aid of conformational considerations based on structures A and B (Figures 1 and 2), alternative models of enzyme-bound peptide 1 that are consistent with the data from NMR experiments (Rosevear et al., 1984).

Experimental Procedures[2]

Distance Measurements. The distances between peptide atoms were estimated from stick and space-filling models built to be consistent with data from paramagnetic probe–T_1 NMR experiments (Rosevear et al., 1984). Depending on which side of the mirror plane through the peptide the paramagnetic reference points where placed, two alternative conformations were found to be consistent with the NMR data, and they are

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; Mops, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin; DCC, dicyclohexylcarbodiamide; CM, carboxymethyl.

[2] The materials used and many of the methods employed were described in the preceding paper (Thomas et al., 1987a).

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