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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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referred to as conformation A and conformation B (Figures 1 and 2). The van der Waals radii used were those of Pauling (1960).

Synthesis of Leu-Arg-Arg-Gly-Ser-Leu-Gly (Peptide 15) and Leu-Arg-Arg-Ala-Ser-Gly-Gly (Peptide 17). The carboxyl-terminal amino acid BocGly was attached to the chloromethylated styrene–1% divinylbenzene copolymer support by the method of Horiki et al. (1978). Subsequent couplings of Boc amino acids were performed on a Beckman synthesizer using symmetric anhydrides that were generated immediately before use by employing a 6.5-fold molar excess of Boc amino acid and a 3.0-fold molar excess of dicyclohexylcarbodiimide (DCC). The completeness of each coupling reaction was determined by the ninhydrin test (Kaiser et al., 1970) before proceeding. Each completed peptide was cleaved from the resin with hydrofluroic acid (Sakakibara et al., 1967). After the mixture was washed with ethyl acetate, the products were extracted with 10% aqueous acetic acid. The material extracted in the acetic acid was gel filtered on a 2.6 × 90 cm bed of Sephadex G-15, eluting with 1% acetic acid. Peak fractions were further purified by ion-exchange chromatography using 20 mL of CM-Sephadex C25, which was eluted with a 25–250 mM pH 7.0 ammonium acetate gradient. Desalting was accomplished by gel filtration using the G-15 column already mentioned. Amino acid analysis: for peptide 15, Arg(2) 2.12, Gly(2) 2.00, Leu(2) 1.90, Ser(1) 0.88; for peptide 17, Ala(1) 1.00, Arg(2) 2.16, Gly(2) 2.05, Leu(1) 0.96, Ser(1) 0.86. Elution of these peptides from an Altex analytical C18 reverse-phase column with a gradient from 10% to 60% CH_3CN in 0.2 M sodium phosphate, pH 2.1, buffer revealed only a single peak.

Synthesis of Arg-(N^{α}-Me)Arg-Ala-Ser-Leu-Gly (Peptide 14) and Leu-Arg-Arg-Gly-(N-Me)Ser-Leu-Gly (Peptide 16). These N-methylated peptides were synthesized as was described for peptide 17, except as noted for the preparation of peptides 7–10 in the preceding paper (Thomas et al., 1987a). Peptide 14 was purified to apparent homogeneity as were peptides 7–10, and peptide 16 was purified as was peptide 11 (Thomas et al., 1987a). Amino acid analysis: for peptide 14, Ala (1) 1.00, Arg(1) 0.86, Gly(1) 1.03, Leu(1) 0.97, Ser(1) 0.96; for peptide 16, Arg(2) 2.10, Gly(2) 2.00, Leu(2) 1.83. The molecular weight of each peptide was confirmed by fast atom bombardment mass spectrometry.

Synthesis of Leu-Arg-Arg-Ala-Ser-Gly-(N-Me)Gly (Peptide 18). Due to the problems we encountered in syntheses employing N-methylated amino acids esterified to Merrifield resin, peptide 18 was synthesized according to the procedure described in Thomas et al. (1987a) to obtain peptide 11 (Thomas et al., 1987a). Amino acid analysis: Ala(1) 0.93, Arg(2) 1.94, Gly(1) 1.03, Leu(1) 1.00, Ser(1) 0.94. The molecular weight was confirmed by fast atom bombardment mass spectroscopy.

Results

Starting from the two alternative conformations of enzyme-bound peptide 1 consistent with NMR data (Rosevear et al., 1984), the steric effects of *N*-methylamide groups in the peptide backbone were examined. Peptides 7–18, which were used in these studies, are identified in Table I and in Table I of Thomas et al. (1987a). In some cases, steric hindrance due to N-methylation prevents the peptide from assuming a particular conformation. Figure 1 is a computer graphics representation of the A conformation with a N-methylated serine (peptide 9). The van der Waals radius of the *N*-methyl group is highlighted in green, while the van der Waals radii of the groups that comprise alanine are highlighted in red. As can be seen, the overlap between the green and red radii is substantial if the peptide is in conformation A, and a major change in conformation would be required to relieve this interaction. On the other hand, as shown in Figure 2, if the peptide is in conformation B, the N-methylation of the serine nitrogen would not cause major steric problems. In other words, the peptide N-methylated at this position (peptide 9) could come closer to forming the B conformation than the A conformation. Figure 1 illustrates that a serine *N*-methyl group would interact primarily with the β-methyl group of alanine, were the peptide forced into the A conformation. In the B structure shown in Figure 2, the β-methyl group of the alanine is turned away from the *N*-methyl group of the serine. Hence the B conformation could better accommodate an *N*-methyl serine than the A conformation.

Table I: Identities of Peptides 13–18[a]

peptide no.	peptide sequence
1	Leu-Arg-Arg-Ala-Ser-Leu-Gly
13	__ Arg-Arg-Ala-Ser-Leu-Gly[b]
14	Arg-(*N*-Me)Arg-Ala-Ser-Leu-Gly
15	Leu-Arg-Arg-Gly-Ser-Leu-Gly
16	Leu-Arg-Arg-Gly-(*N*-Me)Ser-Leu-Gly
17	Leu-Arg-Arg-Ala-Ser-Gly-Gly
18	Leu-Arg-Arg-Ala-Ser-Gly-(*N*-Me)Gly

[a] The positions at which each peptide differs from peptide 1 are underlined. [b] From Kemp et al. (1977).

Table II: Theoretical Effects of N-Methylation

amino acid N-methylated	van der Waals overlap (Å)	
	structure A	structure B
Leu^1	0	0
Arg^2	0	0
Arg^3	≤0.5	1.5
Ala^4	0	0
Ser^5	2.7	0.8
Leu^6	0.9	1.6
Gly^7	0.6	1.3

Table II summarizes the theoretical effects of N-methylation predicted using molecular models fit to the NMR data for the complex of catalytic subunit, peptide 1, metal ion, and nucleotide. The values used for the overlap of the constituent groups are based on the van der Waals radii given by Pauling (1960). For each position of the heptapeptide backbone that can be N-methylated, the sum of the theoretical van der Waals overlaps of the *N*-methyl group with the neighboring residues that would occur in a peptide locked into an A or B conformation is listed. This is not to say that these overlaps would occur but, rather, that the ability of a given peptide to assume a particular conformation would be reduced as the predicted overlap increases. Sometimes N-methylation is not predicted to distort either peptide conformation as in the cases of peptides N-methylated at Leu^1, Arg^2, or Ala^4. N-Methylation of Arg^3, Leu^6, or Gly^7 is predicted to lead to larger distortions in the B structure, whereas N-methylation of Ser^5 is predicted to be more incompatible with the A than the B conformation.

If a peptide must assume a particular structure to be recognized by the enzyme, then the reactivity of that peptide should be related to how well the peptide can assume that conformation. How well each peptide can form either conformation A or conformation B should be related to the magnitude of interactions that each *N*-methyl group has with the remainder of the peptide. On the basis of the sums of van der Waals overlaps that are estimated for a peptide that is locked into either conformation, the ability of each peptide N-methylated at the indicated position to assume conforma-

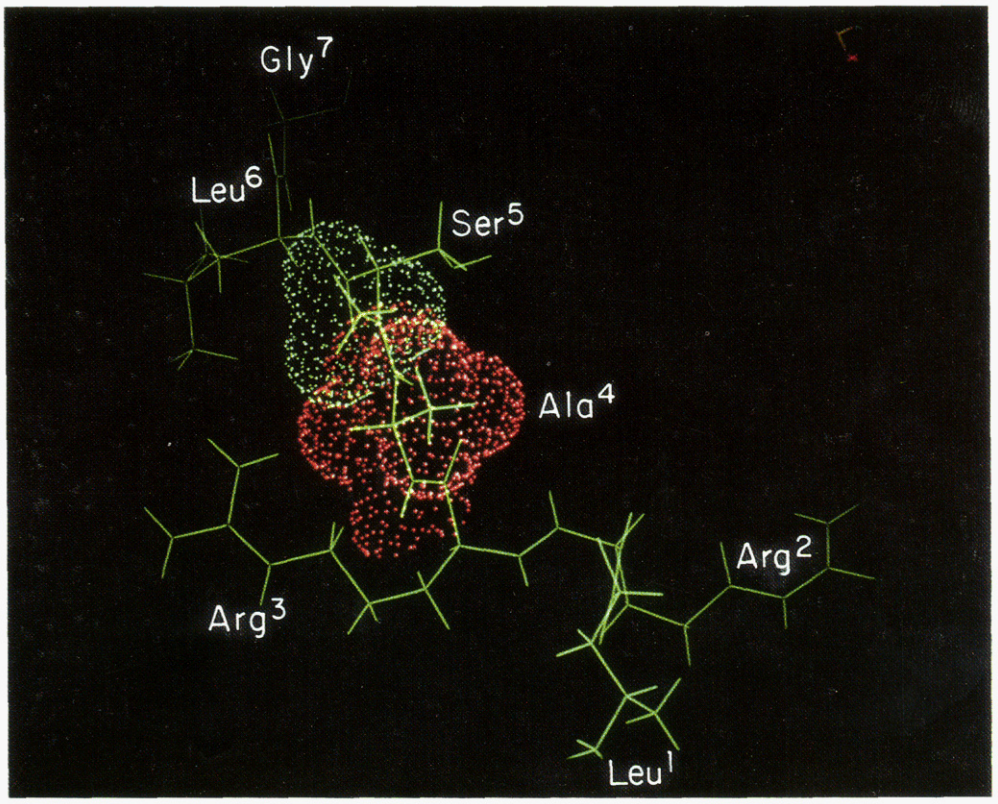

FIGURE 1: Computer graphics simulation of peptide 9 forced into conformation A. The representation, generated by using the FRODO program, was photographed from an Evans and Sutherland PS300 work station. Peptide 9 is N-methylated at Ser 5; the van der Waals radius of this group is highlighted in green, while that for Ala^4 is in red. Note that since the radii of the highlighted atoms have a substantial overlap, this conformation is unfavorable.

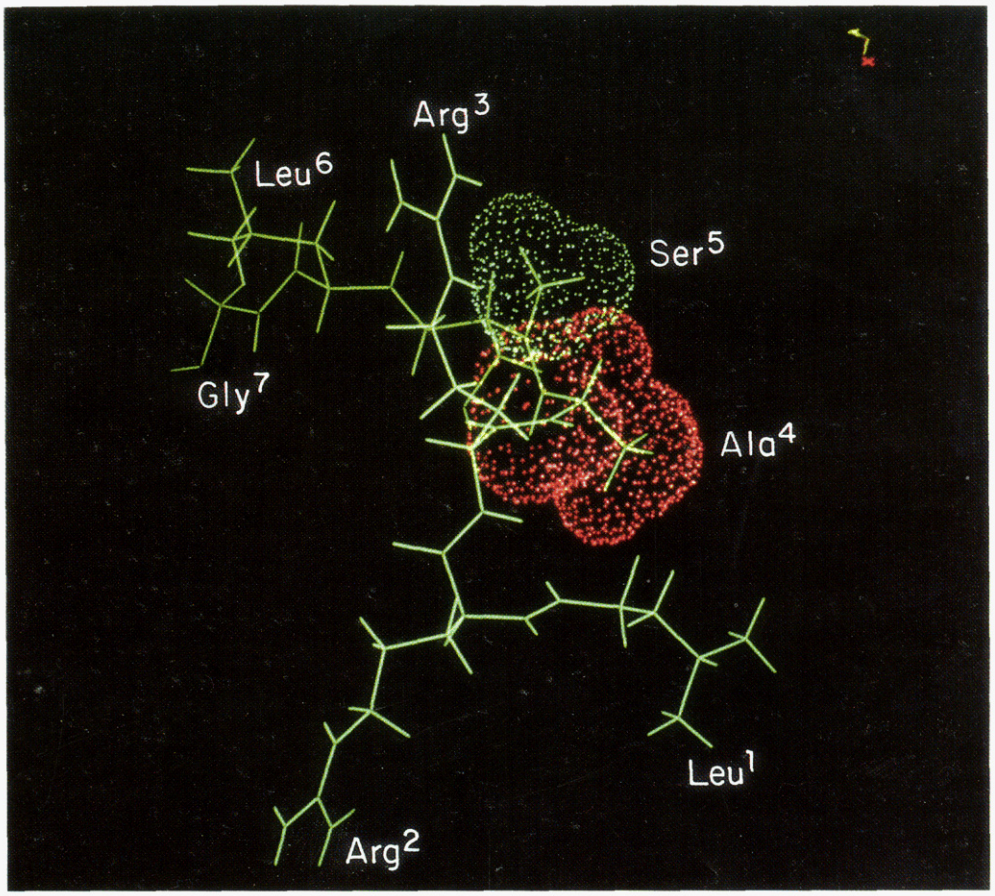

FIGURE 2: Computer graphics simulation of peptide 9 forced into conformation B. In this case the space that the *N*-methyl group electrons occupy (green) is better separated from the space that the Ala^4 electrons occupy (red). Therefore, the negative interactions induced by the *N*-methyl group are not nearly so severe in this case, and peptide 9 can assume a conformation that more closely resembles the B conformation than it can the A.

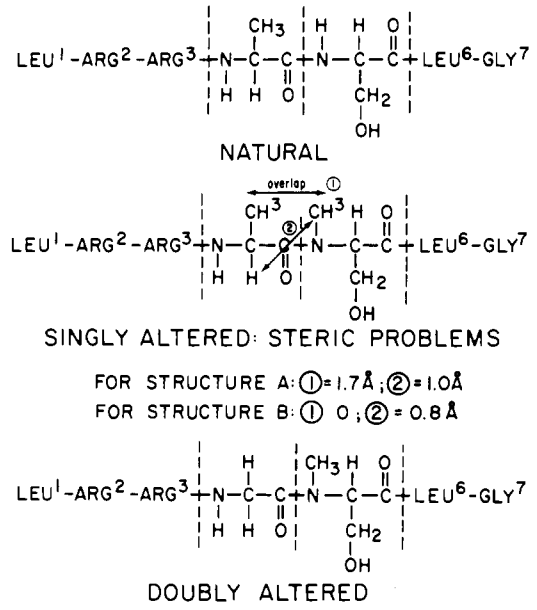

FIGURE 3: Steric problems caused by N-methylation of peptide 1 at the Ser^5 position. An *N*-methyl group on Ser^5 prevents the peptide from adopting an A conformation. If the peptide were forced into this conformation, two interactions would occur, as is illustrated here. An *N*-MeSer[5] containing peptide is better able to adopt a B conformation, since the Ala^4 methyl group would not interact with the *N*-methyl group. The doubly altered peptide should be better able to adopt conformation A than the singly altered peptide, because overlap 1 would be reduced by approximately 1 Å due to the smaller size of a hydrogen vs. a methyl group. The singly and doubly altered peptides should form conformation B equally well, because overlap 1 does not occur.

tions A and B should be $Leu^1 \simeq Arg^2 \simeq Ala^4 > Arg^3 \geq Gly^7 > Leu^6 \gg Ser^5$ and $Leu^1 \simeq Arg^2 \simeq Ala^4 > Ser^5 > Gly^7 \geq Arg^3 \geq Leu^6$, respectively. The reactivities of the peptides should decrease as the overlaps increase; therefore, in these series the peptide reactivities should decrease from left to right. For example, the peptide N-methylated at Ser^5 (peptide 9) in conformation A has the most overlap and, thus, should have the least reactivity. The predicted orders of reactivities are different for structures A and B. The reactivities we have measured [Table II of Thomas et al. (1987a)] are $Gly^7 \geq Ala^4 > Arg^3 > Leu^6 \gg Ser^5$, an order more consistent with that expected for conformation A than for B. The Gly^7 peptide (peptide 11) has a relative reactivity greater than expected for either the A or B conformation. The Gly^7 residue, however, can be removed from peptide 1, leading to a peptide with only 4-fold less reactivity than peptide 1 (Kemp et al., 1977). Having no β carbon and being a carboxy-terminal residue, Gly^7 would be expected to be very flexible, and the above result suggests that it may not bind tightly in the enzymatic active site.

Figure 3 shows a detailed view of the steric interactions that may account for the *N*-methylserine peptide (peptide 9) being such a poor protein kinase substrate. When the natural peptide is N-methylated at Ser^5, potentially two interactions can occur: (1) between the *N*-methyl moiety and the β-methyl group of alanine and (2) between the *N*-methyl and the α proton of alanine. These interactions are quite different for structures A and B. Replacing the alanine methyl group with a proton, in other words replacing Ala with Gly, would decrease overlap 1 by $\sim$1 Å in conformation A, so the Gly^4 variant should be a better substrate for an enzyme that recognizes this structure. Since there is no methyl–methyl interaction in the B conformation, the Gly^4 and Ala^4 *N*-methylserine peptides (the former referred to here as "doubly altered") should show the same

Table III: Comparison of Kinetic Parameters for Phosphorylation of the Altered and Doubly Altered Peptides[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
13[b]	27.5 ± 2.9	716 ± 20	26.0 ± 2	
14	0.494 ± 0.013			
15	87.7 ± 2.6	914 ± 89	10.5 ± 0.4	13.9 ± 1.2
16	(5.5 ± 1.1) × 10^{-4}			
17	0.663 ± 0.025			
18	98.8 ± 1.0			

[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 from 1 to 1500 nM enzyme at 30 °C. [b] Reported by Kemp et al. (1977).

reactivity with the enzyme if the enzyme recognizes structure B. Therefore, an increase in reactivity with Gly at position 4 is more consistent with structure A and also implies that low reactivity of the *N*-MeSer peptide is due to deleterious intrapeptide and not peptide–enzyme steric interactions. The Gly^4,*N*-MeSer[5] peptide (peptide 16) is at least 37-fold better as a substrate for the protein kinase than the Ala^4,*N*-MeSer[5] peptide (peptide 9), when the kinetics are analyzed according to a pseudo-first-order rate law (Thomas et al., 1987a). Peptides containing glycine at position 4 are not intrinsically better substrates than those containing alanine since the Gly^4 analogue (peptide 15) of Leu-Arg-Arg-Ala-Ser-Leu-Gly (peptide 1) is only 1.3-fold more reactive than peptide 1, the parent Ala^4 peptide (Table III). The $k_{cat}/K_{m,peptide}$ for the Gly^4,*N*-MeSer[5] peptide (peptide 16), along with the data for the Gly^4 analogue (peptide 15) as a standard for comparison, is listed in Table III.

N-Methylation of the amino group of Arg^3 causes different steric interactions depending upon whether the peptide is presumed to be bound in an A or B conformation. In an A conformation, the prediction is that a minor (0.5-Å) interaction between the *N*-methyl group and the β-methylene group of the Arg^2 side chain will occur. If a peptide is in conformation B, however, a major (1.5-Å) interaction between the *N*-methyl group and the carbonyl of Leu^1 is expected. Leu^1 can be eliminated, which would relieve the strain entirely if the peptide is in conformation B. This would have no effect on the peptide's ability to assume conformation A. No substantial difference in reactivity is found between the N-methylated Arg^3 peptide with Leu^1 and that without Leu^1 (peptides 7 and 14, respectively). The $k_{cat}/K_{m,peptide}$ for the *N*-MeArg[3],des-Leu[1] peptide (peptide 14), along with data for the des-Leu^1 analogue (peptide 13) as a standard for comparison, is listed in Table II of Thomas et al. (1987a) and Table III of this paper. Conformation A is again more consistent with the data.

In Table IV the predicted overlaps for each N-methylated peptide in both conformation A and conformation B have been summed and are tabulated along with the relative ability of A-kinase to catalyze phosphorylation of each peptide, as judged by the kinetic parameter $k_{cat}/K_{m,peptide}$. The reactivity of the corresponding unmethylated heptapeptide substrate is set in each case at 100%. The two peptides N-methylated at Gly^7 (peptides 11 and 18) have reactivities of 97% and 149%, respectively, values higher than predicted, as discussed above. Except in the case of the *N*-MeGly^7 peptides, the reactivity of each peptide decreases according to the order of the increasing van der Waals overlaps that are predicted to occur in conformation A. The *N*-MeAla^4 peptide (peptide 8) has no detectable overlap, and it has 17% reactivity relative to the parent heptapeptide. The des-Leu^1,*N*-MeArg[3] peptide (peptide 14) would have a 0.5-Å overlap with the side chain of Arg^2 in structure A and has a reactivity of 0.72%. The *N*-MeArg[3]

Table IV: Structure–Activity Relationships[a,b]

peptide	predicted overlap (Å) conformation A[c]	conformation B[c]	obsd rel reactivity[b] (%)
parent[a]	0	0	100
N-MeAla[4]	0	0	17
des-Leu[1],*N*-MeArg[3]	0.5	0	0.72
N-MeArg[3]	≤0.5	1.5	0.54
Gly[6],*N*-MeGly[7]	0.6	0.2	149
N-MeGly[7]	0.6	1.3	97
N-MeLeu[6]	0.9	1.6	0.16
Gly[4],*N*-MeSer[5]	1.7	0.8	0.00081
N-MeSer[5]	2.7	0.8	0.000022

[a] The parent peptide, on which the sequences of all the peptides are based, is Leu[1]-Arg[2]-Arg[3]-Ala[4]-Ser[5]-Leu[6]-Gly[7]. [b] Relative reactivity is defined in terms of the $k_{cat}/K_{M,peptide}$ that describes A-kinase-catalyzed phosphorylation of the peptide. To correct for the effect that an amino acid substitution alone has upon the reactivity of a peptide, reactivity is expressed relative to A-kinase's ability to catalyze phosphorylation of an equivalent nonmethylated peptide. [c] Overlaps are those predicted between the *N*-methyl group electron density and electron densities from sterically interacting groups in the remainder of the peptide (defined further in the text).

peptide (peptide 7), which also would have the same overlap (0.5 Å) in structure A, has a reactivity of 0.54%. The *N*-MeLeu[6] peptide (peptide 10) would have an overlap of 0.9 Å with the side chain of Ser[5] in the A structure and was found to have a reactivity of 0.16%. The doubly altered Gly[4],*N*-MeSer[5] peptide (peptide 16) would have an interaction of 1.7 Å between the *N*-methyl group and C_α hydrogen of Gly[4] in the A conformation and was found to have a correspondingly low reactivity of 0.00081%. The N-methylated Ser[5] peptide (peptide 9) would have an overlap of 2.7 Å in the A conformation and is virtually unreactive. Thus, the results are consistent with the postulate that structure A is the conformation of the peptide in the active site of the protein kinase. As shown in Table IV, the peptide reactivities that have been measured do not follow a pattern consistent with the predictions made for a peptide in the B conformation. These results illustrate that conformation B is unlikely to be the conformation of the peptide bound in the enzymic active site.

Discussion

The data presented demonstrate how *N*-methyl amino acids can be used to probe the conformation of peptides bound within protein kinase active sites. As an initial target, the active site of A-kinase was probed because considerable conformational information was already available from NMR studies of the conformation of the enzyme-bound peptide. The kinetic data presented here are consistent with the postulate that this enzyme recognizes the peptide in conformation A, one of the two alternative structures proposed from the NMR experiments (Rosevear et al., 1984). The assumptions that were made during the analysis of the results are as follows: A-kinase recognizes and binds peptide or protein substrates while in a particular conformation, and conformations A and B are considered to be relatively rigid when the effects of including *N*-methyl amino acids at each position in the peptide are predicted. The close correspondence between the predictions made for the N-methylated peptides and the kinetic results obtained supports the validity of these assumptions.

If, as is suggested by the data, the effects of the *N*-methyl groups are due to intrapeptide steric interactions, then changes in the primary sequence of an *N*-methyl peptide might change the magnitude of these interactions. The relative reactivities of the doubly altered peptides 14 and 16 were predicted from examination of the A conformation but not from examination of the B conformation. The close correspondence of the predicted and measured reactivities of these peptides supports the accuracy of conformation A in representing the structure of the peptide bound in the enzymatic active site. To test further the hypothesis that structure A corresponds to the reactive conformation of the heptapeptide substrates bound to the catalytic subunit, it would be desirable to measure the reactivity of other doubly altered peptides. For example, the *N*-methyl group at Leu[6] is predicted to interact with the side group of the phosphorylatable serine residue if the peptide is in conformation A. Unfortunately, this could not be studied further because the serine residue and the arginine residues are essential for substrate reactivity and cannot be exchanged for glycines.

The use of the N-methylated peptides appears to permit discrimination between whether the A or B conformation is the conformation in which the peptide binds to the enzyme. Since conformations A and B have similar features, this indicates the potential sensitivity of *N*-methyl amino acid residues as probes of peptide conformation. Overall, this method is attractive because it complements NMR studies, is sensitive, and can be utilized while requiring relatively small amounts of enzyme. Therefore, *N*-methyl peptides should be attractive reagents for probing the conformation of substrates bound within the active sites of less abundant protein kinases. The elucidation of these conformations would facilitate the design of artificial inhibitors targeted for specific protein kinases. Additionally, since the *N*-methyl peptides are themselves conformationally restricted, they can be used to study whether conformation is a determinant of protein kinase specificities. In the following paper, the differences between the conformational preferences of two protein kinases with similarly broad specificities are investigated (Thomas et al., 1987b). Ways to exploit these differences for the design of selective substrates and inhibitors shall be proposed.

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