Distinguishing among Protein Kinases by Substrate Specificities†

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ABSTRACT: In the previous paper, N-methylated peptides were shown to be sensitive probes of substrate conformation within the adenosine cyclic 3′,5′-phosphate dependent protein kinase (A-kinase) active site. While it has been shown that other protein kinases will catalyze the phosphorylation of the same peptide sequences as A-kinase, there is as yet little information as to whether the protein kinases differentiate between substrates on the basis of conformation. For this reason, the conformationally restricted N-methylated peptides were used to probe the active site of guanosine cyclic 3′,5′-phosphate dependent protein kinase (G-kinase), which is homologous in sequence to [Takio, K., Wade, R. D., Smith, S. B., Krebs, E. G., Walsh, K. A., & Titani, K. (1984) *Biochemistry 23*, 4207–4218] and which has substrate specificities similar to [Lincoln, T. M., & Corbin, J. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3239–3243] those of A-kinase. Although this enzyme appears to bind the peptides in a conformation resembling that of conformation A, it is more able to accommodate backbone methylation than is A-kinase. A peptide substrate at least 700-fold selective for G-kinase over A-kinase was found. Backbone methylation may, therefore, represent a way of making peptide substrates and inhibitors selective for a particular kinase.

Protein kinases are key enzymes in cellular regulation: cAMP[1]-dependent kinases mediate hormone action (Flockhart & Corbin, 1982); several growth factor receptors also possess kinase activity (Stadtmauer & Rosen, 1983); Rous sarcoma virus induced cellular transformation requires a tyrosine protein kinase (Bishop, 1983); and protein kinases are thought to regulate pathways involved in cellular differentiation (Rosoff & Cantley, 1985) as well as memory storage (Dudai, 1985). Specific inhibition of the various protein kinases would facilitate their study and might lead to methods for the control of cellular function. As yet, attempts to synthesize specific inhibitors for protein kinases have met with limited success, in part, because protein kinases have overlapping substrate specificities. For example, the in vitro peptide and protein substrate specificities of A- and G-kinase seem very similar (Lincoln & Corbin, 1977). The overlapping specificity of these and other kinases hampers studies that attempt to determine their biological role and is probably a reflection of the structural similarities of the protein kinases as indicated by their primary sequence homologies (Takio et al., 1984).

Peptide inhibitors highly specific for G-kinase over A-kinase have not yet been reported. One approach to design specific peptide inhibitors for any protein kinase would be to synthesize peptides that reproduce segments of a protein inhibitor that is specific for that protein kinase. For example, Glass et al. (1986) have reported that several synthetic peptides which resemble segments of the heat-stable inhibitor protein of A-kinase are themselves inhibitors that are highly selective for A-kinase over G-kinase. A protein inhibitor specific for G-kinase has yet to be reported, but a strategy to obtain G-kinase peptide inhibitors may be to synthesize peptides that reproduce protein sites that are known to be phosphorylated at a more rapid rate in the presence of G-kinase than A-kinase. If found to be selective, these peptides might be modified to become inhibitors. Glass and Krebs (1979) have used this approach and synthesized a G-kinase preferred histone 2B phosphorylation site. The peptide Arg-Lys-Arg-Ser-Arg-Lys-Glu was phosphorylated 36 times more efficiently in the presence of G-kinase than A-kinase, as judged by the kinetic parameter $k_{cat}/K_{m,peptide}$. The analogous peptide with alanine in place of serine was a 6-fold better inhibitor for G-kinase than for A-kinase, as judged by the parameter K_i (Glass, 1983).

Inhibitors more selective for G-kinase might be found through further studies modeling protein phosphorylation sites. Such modeling depends on the discovery of proteins selectively phosphorylated by G-kinase. For example, the G substrate from mammalian cerebella is a 30-fold better substrate for G- over A-kinase on the basis of the kinetic parameter k_{cat}/K_m (Aswad & Greengard, 1981). Although peptides that model the phosphorylation site of this protein might be selective for G-kinase, it is not likely that a short peptide will accurately reproduce all the specificity elements exhibited by the protein. An alternate, and perhaps simpler, approach to design peptides specific for a single kinase is to take a peptide recognized by both G- and A- kinases and adapt it to become selective for a particular enzyme. While the conformational preference of A-kinase has been clarified (Granot et al., 1981; Rosevear et al., 1984; Bramson et al., 1987), there is no information concerning the relative conformational requirements for the binding of peptide substrates by different protein kinases. The

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

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possibility may be considered that "conformationally restricted" peptides, that is, peptides that cannot assume certain conformations, might be useful to distinguish among protein kinases. In this paper, the results of using the conformationally restricted N-methylated peptides that were described in the two proceding papers (Thomas et al., 1987; Bramson et al., 1987) to probe the active site of G-kinase are described.

Experimental Procedures

Materials. cGMP-dependent protein kinase was purified as has been described before (Walter et al., 1980). All other reagents were described in the preceding two papers.

Methods. Enzyme assays were performed as described in Thomas et al. (1987), with the following modifications. The K_m for peptide 1 phosphorylation catalyzed by G-kinase is several hundred micromolar. However, at concentrations of several hundred micromolar, substantial substrate inhibition was observed when some of the peptides employed in these studies were phosphorylated in the presence of cAMP- or cGMP-dependent protein kinases. To simplify the analysis of data, the time courses of single reactions were measured at peptide concentrations far below $K_{m,peptide}$ but at a constant and saturating level of ATP. Under these conditions the reaction is pseudo first order in peptide concentration, permitting the evaluation of $k_{cat}/K_{m,peptide}$ from the reaction progress curve (Thomas et al., 1987). Assays of G-kinase activity included enzyme (0.1–2.5 μM), ATP (100 μM), peptide (10 μM), cGMP (4 μM), and $MgCl_2$ (10 mM). All assays were performed in duplicate.

Results

In search of peptides that are specific for G-kinase and not A-kinase, modified versions of the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (peptide 1), a good substrate for both enzymes, have been tested as enzyme substrates. Utilized in this study were the N-methylated analogues of peptide 1 that were described in Thomas et al. (1987) and Bramson et al. (1987). Each of the N-methylated peptides lacks an amide proton, which conceivably may be important for enzyme–peptide substrate hydrogen bonding. In addition, the presence of a methyl group in place of the smaller hydrogen of an amide can lead to conformational changes in the peptide through steric hindrance. A likely possibility is that a peptide must assume a particular structure to be recognized by G-kinase and that N-methylated amides may influence how well the peptide can assume that conformation. Therefore, the presence of N-methylated amides may alter peptide reactivities with G-kinase either through changes of the peptide's hydrogen-bonding potential or through limiting the conformations that the peptide can assume. In the previous two papers, A-kinase was shown to catalyze the phosphorylation of peptides 7–18 with rates that differ by over 10^7-fold. G-Kinase has substrate selectivities similar to those of A-kinase (Lincoln & Corbin, 1977), and the proteins have extensive sequence homologies (Takio et al., 1984). However, NMR studies investigating the conformation of peptide substrates when bound to G-kinase have not yet been carried out due to the limited amount of enzyme available. While both A- and G-kinase show similar primary sequence specificities, the potential differences in conformational requirements can possibly be exploited through use of peptides 7–18 as G-kinase substrates.

Peptides 7–18 [defined in Table I of Thomas et al. (1987) and Table I of Bramson et al. (1987)], all analogues of Leu-Arg-Arg-Ala-Ser-Leu-Gly (peptide 1), were tested as G-kinase substrates. Included in this study were N-methylated peptides and the equivalent nonmethylated standards. The $k_{cat}/K_{m,peptide}$ for the phosphorylation of each peptide catalyzed by G-kinase is listed in Table I. These values are calculated by using eq 3 as defined in Thomas et al. (1987) under pseudo-first-order conditions where the concentration of peptide is always much less than $K_{m,peptide}$. Only the parameter $k_{cat}/K_{m,peptide}$ is evaluated because this gives the best estimate of enzymatic selectivity (Kezdy & Bender, 1965) and because a high $K_{m,peptide}$ is often encountered in the G-kinase-catalyzed phosphorylation of these peptides. For purposes of standardization, three nonmethylated peptides other than peptide 1 were also assayed in the presence of G-kinase. Peptide 15, which has Gly^4 in place of Ala^4, is phosphorylated in a G-kinase-catalyzed reaction with a $k_{cat}/K_{m,peptide}$ that is 20% of that found for peptide 1. Peptide 17, also used as a standard, contains Gly^6 in place of Leu^6, a change that has more serious consequences for peptide reactivity. The activity of G-kinase for the phosphorylation of this peptide is 4% that for peptide 1 on the basis of the kinetic parameter $k_{cat}/K_{m,peptide}$. Another standard used was peptide 13, which lacks Leu^1, and the data for its phosphorylation by G-kinase are reported in the literature (Lincoln & Corbin, 1977).

Table I: Ability of G-Kinase To Catalyze Phosphorylation of the N-Methylated Peptides and the Peptide Standards[a]

peptide no.	modification of standard peptide[b]	G-kinase $k_{cat}/K_{m,peptide}$ (min^{-1} μM^{-1})
1	none	10 ± 1
7	*N*-$MeArg^3$	0.99 ± 0.02
8	*N*-$MeAla^4$	3.6 ± 0.1
9	*N*-$MeSer^5$	0.010 ± 0.003
10	*N*-$MeLeu^6$	0.41 ± 0.02
11	*N*-$MeGly^7$	21 ± 1
13	des-Leu^1	1[c]
14	des-Leu^1,*N*-$MeArg^3$	1.0 ± 0.1
15	Gly^4	1.8 ± 0.1
16	Gly^4,*N*-$MeSer^5$	0.061 ± 0.003
17	Gly^6	0.41 ± 0.04
18	Gly^6,*N*-$MeGly^7$	0.88 ± 0.09

[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 20 to 200 nM G-kinase or 1 to 1500 nM A-kinase at 30 °C. [b] The standard peptide is Leu-Arg-Arg-Ala-Ser-Leu-Gly. [c] This is the value (rounded to one digit) reported by Lincoln and Corbin (1977).

Table II: Differential Protein Kinase Specificities[a]

peptide no.[b]	modification of standard peptide[c]	$\frac{k_{cat}/K_{m,peptide}(\text{G-kinase})}{k_{cat}/K_{m,peptide}(\text{A-kinase})}$
1	none	0.1
8	*N*-$MeAla^4$	0.3
14	des-Leu^1,*N*-$MeArg^3$	2.1
7	*N*-$MeArg^3$	2.7
18	Gly^6,*N*-$MeGly^7$	0.9
11	*N*-$MeGly^7$	0.3
10	*N*-$MeLeu^6$	3.8
16	Gly^4,*N*-$MeSer^5$	110
9	*N*-$MeSer^5$	≥690

[a] The phosphorylation conditions were those described in Table I. [b] These peptides are listed according to the magnitude of the van der Waals overlap that has been predicted for each peptide when forced into conformation A [see Table IV in Bramson et al. (1987)]. [c] The standard peptide is Leu-Arg-Arg-Ala-Ser-Leu-Gly.

In Table II, the parameter $k_{cat}/K_{m,peptide}$ for the reaction of each peptide with G-kinase has been divided by the value for A-kinase (Thomas et al., 1987; Bramson et al., 1987). These numbers represent the specificity of G-kinase relative to A-kinase for each peptide and indicate which peptide structural motif might be useful in designing an inhibitor selective for G-kinase. Peptide 1 is a 10-fold better substrate for A-kinase, while at the other extreme peptide 9 is at least a 700-fold better

substrate for G-kinase, a 7000-fold inversion of reactivity. This result is particularly interesting because peptide 9 is virtually unreactive in the presence of A-kinase and MgATP. A-Kinase catalyzes phosphorylation of less than one-tenth of a peptide 9 molecule per enzyme active site per hour.

The observation that G-kinase shows an order of reactivity for peptides 7–18 that is similar to that seen for A-kinase (Thomas et al., 1987; Bramson et al., 1987) suggests that peptide 1 has a similar conformation when bound in the active site of each protein kinase. The results of A-kinase-catalyzed phosphorylations of N-methylated peptides have shown that, of the predictions based on two conformations consistent with the results of NMR experiments (Rosevear et al., 1984), those based on the A conformation correspond better with the kinetic data than those for the B conformation. Therefore, the observation of a similar order of reactivity for the N-methylated peptides toward the G-kinase argues for the formation of the A conformation in the reactive enzyme–substrate complex here as well. *N*-MeAla^4, des-Leu^1,*N*-MeArg^3, *N*-MeArg^3, *N*-MeLeu^6, Gly^4,*N*-MeSer^5, and the *N*-MeSer^5 peptides are phosphorylated at rates 36%, 10%, 9.5%, 4.1%, 3.1%, and 0.1% that of the non-N-methylated standards, respectively. When forced into conformation A, the peptides, listed in order of decreasing reactivity with G-kinase, are predicted to have van der Waals "overlaps" of 0, 0.5, 0.5, 0.9, 1.7, and 2.7 Å, respectively. The same peptides, when forced into conformation B, are expected to have van der Waals overlaps of 0, 0, 1.5, 1.6, 0.8, and 0.8 Å, respectively. As emphasized in the preceding paper, it is not implied that these overlaps would actually occur but, rather, that they provide a measure of the difficulty that a peptide would have in achieving a particular conformation. As for A-kinase, the magnitude of the van der Waals overlaps that are predicted for each peptide if that peptide is forced into conformation B did not correlate with the data for the G-kinase-catalyzed phosphorylations. Both G- and A-kinase catalyze the phosphorylation of the *N*-MeGly^7 peptides as efficiently as the non-N-methylated standards despite the expectation that the *N*-methyl groups should create minor steric problems for a peptide forced into conformation A and major problems for a peptide forced into conformation B. This finding probably indicates that Gly^7, the carboxy-terminal residue, has enough flexibility to avoid the detrimental interactions.

The similarity of the preferences that G-kinase and A-kinase exhibit for peptides 7–18 is suggestive of the fact that the same factors influence substrate recognition by each kinase. G-Kinase, like A-kinase, is proposed to differentiate among these peptides on the basis of each peptide's ability to form conformation A. Each *N*-methyl group seems to affect G-kinase's ability to catalyze peptide phosphorylation by causing intrapeptide steric hindrance. This proposal was tested with peptides that by modification of amino acid side chains improve the ability of the N-methylated peptide to assume the conformation in question (Bramson et al., 1987). In particular, the alanine side chain in peptide 9 is predicted from the model of structure A to interact with the *N*-methyl of Ser^5, as shown in Figure 3 of the paper by Bramson et al. (1987). A similar peptide (peptide 16), which contains not only *N*-MeSer^5 but also a Gly^4 in place of Ala^4, is 30-fold more reactive than peptide 9 in the presence of G-kinase (Table I). This result is more consistent with the peptide being bound in conformation A than in B within the enzymatic active sites of both G-kinase and A-kinase. In a different case removal of Leu^1 from peptide 7, in which Arg^3 is methylated, to form a des-Leu,*N*-MeArg^3 peptide (peptide 14), is expected to result in a better substrate only if the peptide is bound in the enzymatic active site while in the B conformation. G-Kinase was found to interact with both the *N*-MeArg^3-containing peptides equally well, a result most consistent with peptide 1 being bound in the A conformation.

Discussion

G-Kinase appears to bind peptide 1 in a conformation resembling that of conformation A but is more able to accommodate methylation at the amide linkages in the peptide chain. The different abilities of A-kinase and G-kinase to accommodate N-methylation in peptides may lead to substrates and inhibitors selective for G-kinase. A 700-fold selectivity of a peptide for G-kinase over A-kinase was achieved by N-methylation of the phosphorylatable serine residue of peptide 1. This compares favorably with the 36-fold specificity of a peptide substrate for G-kinase relative to A-kinase reported by Glass and Krebs (1979). However, the studies reported here were begun in a disadvantageous position toward the goal of making a peptide substrate selective for G-kinase. They utilized as a starting structure a peptide 10-fold selective for A-kinase over G-kinase. While the absolute rate of phosphorylation of peptide 9 catalyzed by G-kinase is low, this peptide is at least 700-fold selective for G-kinase over A-kinase, and this might be improved upon by incorporation of the *N*-methylserine residue into peptides already selective for G-kinase. An interesting possibility is that the *N*-MeSer^5 peptide might not be recognized at all by A-kinase. Less than one-tenth of a turnover per enzyme active site per hour occurs in the presence of A-kinase, and this level of phosphorylation could be due to the modification of the catalytic subunit itself. Perhaps any G-kinase substrate will become G-kinase specific if *N*-MeSer is incorporated at the phosphorylatable site.

To reiterate, both A-kinase and G-kinase follow a similar order in their ability to catalyze the phosphorylation of peptides 7–18, an order more consistent with the peptides being bound in the enzymic active sites in conformation A than in B. However, the magnitudes of decreases in relative peptide reactivity seen upon N-methylation at certain positions are much greater for A-kinase than G-kinase. The fact that the magnitudes of the reactivity changes observed upon methylation of the peptide amide groups are less for G-kinase might suggest that this enzyme has an active site that is more flexible than that of A-kinase. Overall, the studies indicate the possible utility of N-methylation of amide residues in the development of selective inhibitors. N-Methylation of peptides might provide a method for targeting peptide-based inhibitors for selected protein kinases in vivo.

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Extended Binding Inhibitors of Chymotrypsin That Interact with Leaving Group Subsites S_1'-S_3' †

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ABSTRACT: We have synthesized inhibitors of chymotrypsin, based on fluoromethyl ketones, that bind at S and S′ subsites. "Small" inhibitors of serine proteases, which have previously been synthesized, only interact with S subsites. The parent compound is Ac-Leu-*ambo*-Phe-CF_2H (**1**) ($K_i = 25 \times 10^{-6}$ M). This inhibitor was modified by successively replacing H of the $-CF_2H$ group by $-CH_2CH_2CONHCH_3$ (**4**), $-CH_2CH_2CONH$-Leu-NHMe (**5**), $-CH_2CH_2CONH$-Leu-Val-OEt (**6**), and $-CH_2CH_2CONH$-Leu-Arg-OMe (**7**). Corresponding K_i values are 7.8 (**4**), 0.23 (**5**), 0.21 (**6**), and 0.014 (**7**) μM. Extending **5** to **6** by addition of Val-OEt at P_3' does not decrease K_i. In contrast, extension of **5** to **7** by incorporating Arg-OMe at P_3' decreases K_i approximately 15-fold, suggesting interaction between Arg and the S_3' subsite but no corresponding interaction at that subsite with Val. These results are in accordance with results obtained with the homologous family of avian ovomucoid third domain proteins. Proteins with Arg at the P_3' position show highly favorable interactions with the protease at the S_3' subsite [Park, S. J. (1985) Ph.D. Thesis, Purdue University; M. Laskowski, Jr., personal communication]. These results establish that incorporation of residues which interact with S′ subsites significantly increases the efficacy of inhibitors and that valuable information concerning the most effective amino acid composition of small inhibitors can be obtained from the amino acid sequence of protein inhibitors.

Effective inhibitors of the serine proteases chymotrypsin and porcine pancreatic elastase can be obtained by incorporating a difluoro- or trifluoromethyl ketone moiety into substrate analogues (Imperiali & Abeles, 1986a). The fluoromethyl ketones appear to be acting as transition-state analogue inhibitors (Wolfenden, 1976). The inhibitors described to date occupy maximally the S_1-S_4 binding subsites[1] of the proteolytic enzyme. There is reason to believe that K_i could be further reduced by utilizing interactions with binding subsites on the leaving group side of the cleaved peptide (the S_1'-S_3' subsites). Small synthetic inhibitors of serine proteases that interact in this extended manner have not to date been available due to the chemical limitations of the incorporated functional groups (e.g., aldehydes, boronic acid derivatives, etc.) (Westerik & Wolfenden, 1972; Kettner & Shenvi, 1984).

The work of Fersht et al. (1973) clearly establishes the importance of leaving group effects on peptide hydrolysis. It was observed that with the specific acyl-enzyme (acetylphenylalanyl)chymotrypsin the rate constants for attack on the acyl-enzyme vary greatly with different nucleophiles. For example, the ratio of reactivity of alaninamide:glycinamide:hydrazine:water (55 M) is 44:11.5:2:1. Although hydrazine is the best nucleophile of the series of compounds, alaninamide reacted over 20 times faster. It is interesting to note that when the acyl-enzyme is not specific (such as with the furoylchymotrypsin) the reactivity toward nucleophile then parallels the chemical reactivity of the incipient nucleophile. Thus specificity on the leaving group side does influence chymotrypsin-catalyzed peptide hydrolysis. Fersht also examined effects on k_{cat}/K_m and observed that the specificity for better substrates is expressed in the k_{cat} and not in K_m. The binding energy on the leaving group side is used to lower the activation energy of the rate-limiting chemical steps. It is, therefore, reasonable to assume that these interactions will also lower K_i of a transition-state analogue. Further evidence

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[1] The terminology used to describe the residues was originally proposed by Schecter and Berger (1967). The amino acid residues of substrate (or in this case, inhibitor) are designated P_1, P_2, etc., numbering from the carbonyl of the scissile amide bond in the direction of the amino terminal. The corresponding subsites are termed S_1, S_2, etc. The residues in the direction of the carboxyl group from the scissile bond are designated P_1', P_2', etc., and the corresponding subsites S_1', S_2', etc. We shall refer to inhibitors that interact with S and S′ subsites as extended inhibitors.

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