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Identification of an inhibitory region of the heat-stable protein inhibitor of the cAMP-dependent protein kinase

(phosphorylation)

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ABSTRACT **The present study was undertaken in order to identify the inhibitory site of the heat-stable inhibitor of cAMP-dependent protein kinase (PKI) and to synthesize a peptide that could serve as a useful inhibitor of the enzyme. Digestion of purified PKI by mast cell proteinase II yielded a peptide fragment that retained inhibitory activity. A sequence of 20 amino acids of the peptide, ...Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-Ile-Leu-Val-Ser-Ser-Ala..., revealed the presence of a "pseudosubstrate site" (Arg-Arg-Asn-Ala-Ile) for the cAMP-dependent protein kinase in which alanine replaces the seryl or threonyl residue that is normally phosphorylated. Digestion of PKI with various other proteinases implicated the involvement of arginyl and hydrophobic residues as determinants for the inhibitory activity. The assumption that this region is part of the inhibitory site was confirmed by the synthesis of a corresponding duodecapeptide that displayed strong inhibitory activity. Inhibition by the peptide was competitive with a K_i of 0.8 μM as measured against a number of protein substrates. The sequence of this fragment bears a strong resemblance to the autophosphorylation site in the type II regulatory subunit of cAMP-dependent protein kinase, a region also postulated to interact with the catalytic subunit, and the analogous region of type I regulatory subunit. Neither intact PKI nor the synthetic peptide inhibit the cGMP-dependent protein kinase, phosphorylase kinase, myosin light-chain kinase, casein kinase II, or protein kinase C.**

The heat-stable inhibitor of the cAMP-dependent protein kinase, PKI (1–3), interacts specifically with the enzyme's free catalytic subunit (C) after dissociation of the holoenzyme by cAMP. PKI, which has an M_r of 11,000, contains ≈90 amino acid residues and exists in several isoforms (4–6). It is an extremely potent competitive inhibitor of the kinase with a K_i of 2 nM (7), which is approximately 4 orders of magnitude lower than the K_m for the synthetic peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly (designated Kemptide) commonly used to assay the enzyme (8). K_m values for natural protein substrates of the kinase are generally in the low micromolar range.

Several investigators have established which amino acids are determinants for the recognition and phosphorylation of seryl and threonyl residues by the cAMP-dependent protein kinase (for reviews see refs. 9 and 10). Basic residues, in particular arginine side chains, preceding the serine or threonine appear to be essential (8, 11–14); a minimal structure of -R-R-X-S-X- has been proposed, where X represents essentially any amino acid and R represents arginine (reviewed in ref. 15). The autophosphorylation site in the type II regulatory subunit of the cAMP-dependent protein kinase (RII) contains this structure (16, 17), and it has been postulated that this region of RII is involved in its interaction with C (18). Several lines of evidence were brought forward to support this concept, including the use of arginine-modifying reagents, which destroyed the ability of RII to inhibit C (19). The interaction of PKI with C is similar in that arginyl residues also appear to be essential for its inhibitory action as was shown in 1977 (7). In preliminary work carried out at that time, it was reported that two of the four arginyl residues in PKI were located in the NH_2-terminal portion of the molecule from which an inhibitory peptide could be derived by limited proteolysis (20). Further studies to determine the sequence of this peptide could not be carried out because of the minute amounts of the purified inhibitory fragment that could be obtained and because the analytical methods available were too insensitive.

The objective of this study was to define the inhibitory domain of PKI, taking advantage of the microprocedures for sequence determination that are now at hand. That this particular segment of the molecule is responsible for the inhibitory properties of PKI was confirmed by the chemical synthesis of a peptide of identical structure that proved to be a powerful inhibitor of the cAMP-dependent protein kinase.

EXPERIMENTAL PROCEDURES

Materials. All standard chemicals were purchased from Sigma unless otherwise stated. Submaxillaris proteinase was purchased from Pierce, and endoproteinase-Lys-C was from Boehringer Mannheim. Other proteolytic enzymes were purchased from Worthington. Mast cell proteinase II was a gift from Koiti Titani (Department of Biochemistry, University of Washington, Seattle, WA). All sequenator reagents were purchased from Applied Biosystems (Foster City, CA), and *t*-butyloxycarbonyl derivatives of amino acids were obtained from Peninsula Laboratories (San Carlos, CA) or Vega–Fox (Tucson, AZ). Hepatic pyruvate kinase was a gift from Simon Pilkis (Department of Biochemistry, Vanderbilt University, Nashville, TN), and cardiac troponin I was a gift from Dean Malenchik (Department of Biochemistry, Oregon State University, Corvallis, OR).

Methods. *Purification of proteins.* Rabbit skeletal muscle PKI was initially purified to homogenity by the method of Demaille *et al.* (7) and subsequently by a procedure involving reversed-phase HPLC (unpublished method). The catalytic subunit of cAMP-dependent protein kinase was isolated from bovine heart (21); the cGMP-dependent protein kinase, from bovine lung (22); and phosphorylase kinase, from rabbit skeletal muscle (23). Bovine liver casein kinase II was partially purified as described (24), and rat brain protein

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Abbreviations: PKI, the heat-stable inhibitor of the cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; RI and RII, types I and II regulatory subunits of the cAMP-dependent protein kinase.

kinase C was partially purified through gel filtration by the method of Le Peuch *et al.* (25). Myosin light chain and myosin light chain kinase were purified from rabbit skeletal muscle as described by Blumenthal and Stull (26).

Assays. The inhibitory activity of PKI or related peptides was determined by the extent of inhibition of C. All components were dissolved or diluted in 50 mM 3(*N*-morpholino)propanesulfonic acid/50 mM NaCl/2 mM $MgCl_2$/1 mM dithiothreitol, pH 6.8 (buffer A), except for the enzyme, which was diluted in buffer B, (buffer A containing bovine serum albumin at 0.5 mg/ml). Aliquots of PKI or inhibitory peptide were incubated with 50 nM C subunit and 1 mM Kemptide for 5 min at 30°C. The reaction was started by the addition of 2 mM [γ-^{32}P]ATP (200 cpm/pmol) and was continued for 15 min at 30°C. After incubation, 30 μl of the reaction mixture was absorbed onto Whatman P 81 phosphocellulose paper (27), and the samples were washed four times with a total of 500 ml of 75 mM H_3PO_4 (2-min washes) followed by ethanol (2 min) prior to drying. Radioactivity associated with filter papers was determined by liquid scintillation spectrometry. One unit of inhibitor was defined as the amount required to reduce phosphorylation of a substrate by 50% when incubated with 50 nM C. Testing for inhibition of protein kinases other than the cAMP-dependent protein kinase followed the above procedure except for myosin light chain kinase; in this instance the phosphorylated light chains were precipitated onto Whatman 3 MM filter paper by three 15-min washes in 5% (wt/vol) trichloroacetic acid.

Proteinase digestion and peptide isolation. To test the effect of proteolytic digestion on the inhibitory activity of PKI, the latter protein in buffer A was incubated with various proteinases (enzyme-to-substrate molar ratio of 1:50) at 37°C. At specified times, aliquots (20 μl) were removed and immediately frozen prior to assay for inhibitory activity.

Peptide sequencing. Sequence determinations were performed with an Applied Biosystems AB 50 gas-phase protein sequenator by the method of Hewick *et al.* (28).

Solid-phase peptide synthesis. Peptides were synthesized on a Beckman 990B automated solid-phase peptide synthesizer as described (29). Cleavage from the resin and deprotection were achieved by incubation in 75% HF/25% anisole for 30 min at 0°C (30). The amount and composition of each peptide were confirmed by amino acid analysis and sequence determination.

RESULTS

Digestion of PKI with Various Proteinases. Experiments were carried out to determine which proteinases would be best suited to generate inhibitory fragments from PKI. To this end the protein was subjected to proteolytic attack by a variety of proteinases, and the inhibitory activities of the digests were tested at intervals. Trypsin, which cleaves at lysine and arginine residues, caused an almost immediate loss of inhibitory activity that was essentially complete within 10 min (Fig. 1). The submaxillaris proteinase, which favors cleavage at arginine but not at lysine residues, also caused rapid loss of activity. By contrast, there was no loss of activity when PKI was digested with endopeptidase Lys-C, which catalyzes lysine-specific cleavages. Thermolysin, which is relatively specific for hydrophobic residues, caused a rapid loss of activity as did chymotrypsin, though at a somewhat slower rate. Staphylococcal V8 proteinase, which cleaves at glutamic and aspartic acid residues, also brought about a definite loss of activity under the experimental conditions used in Fig. 1. This result was surprising in view of the fact that the enzyme had been used earlier to generate an inhibitory fragment (20). Mast cell proteinase II, which did not cause any loss of inhibitory capability, was finally chosen for digestion on PKI on a preparative scale as described below.

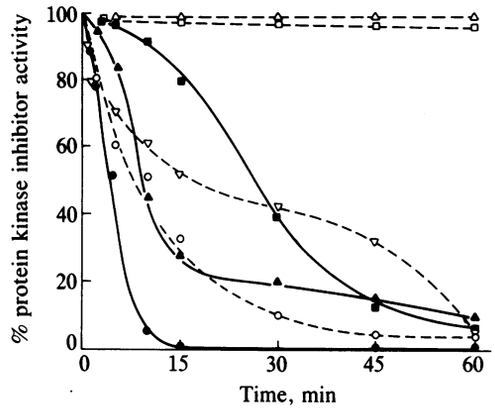

FIG. 1. The effect of proteolysis on PKI activity. PKI was incubated at 37°C with trypsin (●), submaxillaris proteinase (○), endoproteinase-Lys-C (△), chymotrypsin (■), thermolysin (▲), mast cell proteinase II (□), and staphylococcal V8 proteinase (▽) at an enzyme-to-substrate ratio of 1–50 as described. Samples were removed at intervals of 1, 2, 5, 10, 15, 30, 45, and 60 min prior to assay for inhibitory activity toward C. These assays were carried out at an initial (before digestion) ratio of PKI to C of 100 to 1 on a molar basis.

Isolation and Structure of an Inhibitory Fragment of PKI. Purified PKI (50 μg, 4.5 nmol) in 1 ml of 0.1 M NH_4HCO_3 was incubated with 1 μg of mast cell proteinase II for 4.5 hr at 37°C. The reaction mixture was freeze-dried and dissolved in 0.1% trifluoroacetic acid, and the pH was adjusted to 2.5. The resulting peptides were fractionated on a Varian 5000 HPLC system using a Synchropack RPP C_{18} column preequilibrated in 0.1% trifluoroacetic acid. Elution was carried out with a linear gradient of up to 40% acetonitrile. Four major fragments and a number of minor peptides were obtained; these were freeze-dried and then assayed for inhibitory activity (Fig. 2). Of these, only one major peptide, which eluted at 32% acetonitrile, was found to be inhibitory. Because of the scarcity of material, only subnanomolar quantities of this peptide could be isolated. While this precluded any detailed quantitative analysis of its structure, it was sufficient nonetheless to allow for a partial determination of its sequence by gas-phase sequencing microdetermination. The amino acid

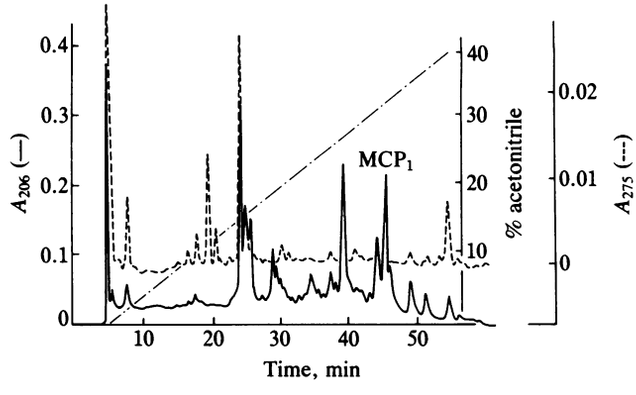

FIG. 2. Separation of PKI fragments digested with mast cell proteinase II. Fractionation was carried out on a Syncropack RPP-C_{18} reversed-phase HPLC column equilibrated in trifluoroacetic acid; elution was by a linear gradient of acetonitrile (–·–). Elution of peptides was monitored by absorbance at 206 nm and 275 nM. All peptides were assayed for inhibition of C as described in the text. MCP$_1$ denotes the peptide that retained inhibitory activity.

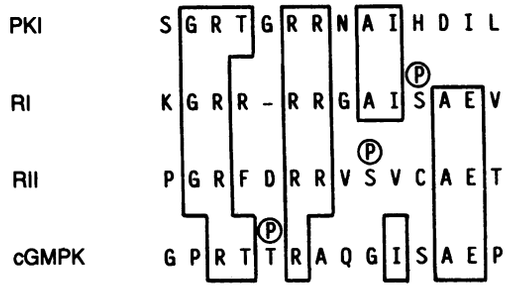

FIG. 3. The alignment of amino acid sequences to show maximum homology between rabbit skeletal muscle PKI, RI, RII, and cGMP-dependent protein kinase (cGMPK). Identical residues are enclosed in boxes. The dash indicates a gap placed to optimize homology. Circled P indicates the site of phosphorylation of RI by cGMP-dependent protein kinase (32), the autophosphorylation site in RII (16), and the autophosphorylation site (33) in the cGMP-dependent protein kinase.

sequence of 20 residues of this peptide was determined to be: ...Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-Ile-Leu-Val-Ser-Ser-Ala... . The cleavage product itself was >20 residues, but sequence analysis to the COOH-terminus is not included.

The partial sequence of the inhibitory peptide is of interest for several reasons. First, it contains a "pseudosubstrate site" whose sequence, Arg-Arg-Asn-Ala, is identical to the classical cAMP-dependent protein kinase-catalyzed phosphorylation sites except that an alanine replaces a serine. In confirmation of the earlier conclusions (7), arginine residues are prominent in the inhibitory region of PKI, which is in keeping with the rapid loss in activity that resulted from treatment with trypsin or the submaxillaris proteinase (Fig. 1). No lysine residues are present, so the failure of endopeptidase Lys-C to destroy activity (Fig. 1) is not surprising. The sequence contains several sites at which thermolysin or chymotrypsin might catalyze cleavages. It should be noted that no glutamic acid residues are present to explain the loss of activity due to the action of staphylococcal V8 proteinase. This point will be discussed later. Second, it is homologous to the autophosphorylation site in RII (17, 31) and to the corresponding regions in RI (32) and in the cGMP-dependent protein kinase, though to a lesser extent (33) (Fig. 3).

Synthesis and Inhibitory Activity of Peptides Related to PKI Based on the Sequence of the Inhibitory Site of PKI. Since it was not possible to isolate a sufficient amount of the inhibitory mast cell proteinase II fragment to characterize more fully its inhibitory properties, it was important to make a synthetic peptide of corresponding structure to: (*i*) confirm that it possessed inhibitory activity, (*ii*) identify the structural determinants that might be involved, and (*iii*) try to establish what might be a minimal structure for inhibition. Accordingly, a series of peptides were prepared that range in length from 8 to 20 residues and contain identical COOH-terminal sequences but extend towards the NH_2-terminus to include the "pseudosubstrate site" and beyond (Table 1). The

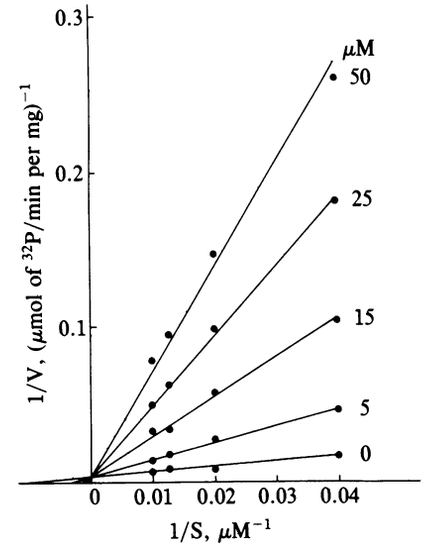

FIG. 4. Determinations of inhibition constants for peptide 1. Inhibition constant values were determined by double reciprocal plots. Peptide 1 concentrations were 50, 25, 15, and 5 μM. The substrate (S) was Kemptide at concentrations of 25, 50, 75, and 100 μM. Assay conditions are given within the text.

inhibitory properties of each was assessed by the degree of inhibition of the C subunit acting on Kemptide as substrate; inhibition constants were determined graphically by Lineweaver–Burk plots (Fig. 4). The parent peptide in this series (peptide 1) proved to be a potent competitive inhibitor of C with a K_i of 0.8 μM. Peptide 2, in which the first three amino acids are deleted, was still a reasonably strong inhibitor with a K_i of 75 μM. With deletion of four more amino acids (peptide 3), the K_i value rose to 1.5 mM. Peptides 4 and 5, in which the pseudosubstrate site was incomplete or missing, were noninhibitory at concentrations up to 2.4 mM.

To determine whether the potency of peptide 1 was influenced by the nature of the substrate for the protein kinase, inhibition constants were determined in the presence of four different substrates for C, namely Kemptide, hepatic pyruvate kinase (the parent protein from which the Kemptide sequence was derived), cardiac troponin I, and histone IIa (Table 2). The values obtained with these substrates did not vary markedly, suggesting that inhibition is minimally affected by the type of substrate, at least within the group tested. However, in view of the fact that the synthetic peptide is a competitive inhibitor, it would seem probable that K_i values would vary with substrates that showed a greater diversity in their affinities for C. Similar results were obtained with native PKI, although, in this instance, the inhibition constants were 2–3 orders of magnitude lower than with peptide 1. Under these conditions the competitive nature of the inhibition was not immediately evident.

Specificities of Inhibitory Peptide 1 and PKI. The only protein kinase known to be inhibited by PKI is the cAMP-dependent enzyme. To establish if this also applies to peptide 1, the latter was tested at a concentration of 2.5 mM on the

Table 1. Inhibitory properties of synthetic peptides patterned after the mast cell proteinase fragment

Peptide	Structure	K_i, μM
1	Ile–Ala–Ser–Gly–Arg–Thr–Gly–Arg–Arg–Asn–Ala–Ile–His–Asp–Ile–Leu–Val–Ser–Ser–Ala	0.8
2	Gly–Arg–Thr–Gly–Arg–Arg–Asn–Ala–Ile–His–Asp–Ile–Leu–Val–Ser–Ser–Ala	75
3	Arg–Arg–Asn–Ala–Ile–His–Asp–Ile–Leu–Val–Ser–Ser–Ala	1500
4	Asn–Ala–Ile–His–Asp–Ile–Leu–Val–Ser–Ser–Ala	ND
5	His–Asp–Ile–Leu–Val–Ser–Ser–Ala	ND

Peptides 1–5 were synthesized, characterized, and assayed for inhibition of the cAMP-dependent protein kinase as described in the text. ND, not determined.

Table 2. Inhibition constants for peptide 1 and PKI with different substrates for the cAMP-dependent protein kinase

Substrate	K_m, μM	Peptide 1 K_i, μM	PKI K_i, nM
Kemptide	16*	0.80 ± 0.04	5.4 ± 1.8
Hepatic pyruvate kinase	17†	1.67 ± 0.15	12.4 ± 2.5
Cardiac troponin I	21.5‡	0.78 ± 0.08	7.7 ± 1.4
Histone IIa	71.6§	1.11 ± 0.18	3.0 ± 1.1

*From ref. 12.
†From ref. 34.
‡From ref. 35.
§Determined in this study.

following kinases, with their protein substrates indicated in parentheses: phosphorylase kinase (phosphorylase *b*), skeletal muscle myosin light chain kinase (skeletal muscle myosin light chains), protein kinase C (histone H1), casein kinase II [synthetic peptide substrate (24)], and cGMP-dependent protein kinase (mixed histones). No inhibition was seen with any of these enzymes. The same negative result was obtained with PKI added at a final concentration of 10 μM.

DISCUSSION

The results described herein indicate that most of the determinants essential for inhibition of the cAMP-dependent protein kinase by PKI are located within a linear sequence of ≈20 amino acids. This region, representing 25% of the native molecule, is homologous to the "hinge regions" (17, 31) of the regulatory subunits types I and II (RI and RII), which also modulate kinase activity (Fig. 3). These regulatory subunits, like PKI, competitively inhibit C, but contrary to PKI, their inhibition is abolished by cAMP, thereby providing for the hormonal modulation of enzyme activity.

Neither PKI nor RI is phosphorylated by C, but both contain a "pseudosubstrate site" of sequence Arg-Arg-(X)-Ala-Ile-. The difference between such a site and a typical substrate (R-R-X-S-) is subtle, and involves solely the replacement of alanine by serine. These two amino acids differ only at the β carbon atom, where the hydroxyl group in serine that can be phosphorylated is replaced by hydrogen in alanine. Such a small difference in structure could allow the correct orientation of the inhibitor at the active site of C. It should be noted, however, that phosphorylation of the seryl residue at the hinge region of RII does not bring about the dissociation of the R_2C_2 inactive complex, indicating that some structural leeway must exist at that site.

Results of experiments in which PKI was degraded by various proteinases suggest that an arginine-containing segment of ≈20 amino acids covering the "pseudosubstrate site" is required for inhibition. Demaille *et al.* (20) showed that chemical modification of the guanidino groups of PKI abolished inhibition, a finding that is consistent with the rapid loss of activity observed upon proteolytic cleavage at arginines (Fig. 1).

The extent of involvement of arginines 8 and 9 can be evaluated by considering the properties of synthetic peptide 3 listed in Table 1. Its low affinity (K_i = 1.5 mM) suggests that the Arg-Arg grouping by itself is not sufficient to account for the high-affinity displayed by PKI for C. A 20-fold increase in affinity is observed in peptide 2 (K_i = 75 μM), which contains all three arginyl residues (Arg-5, -8, and -9) and a close to 100-fold increase in affinity results from the further addition of four residues as seen in peptide 1.

The importance of the Arg-Arg cluster one residue removed from the serine or threonine undergoing phosphorylation has been well documented (13, 15). Also the location of a third basic group, preferably arginine, six residues removed is important for substrate recognition in some cases (14). The positioning of these three arginines in PKI satisfies both criteria: the residues are conserved in RII, while RI contains four arginine side chains within its hinge region (Fig. 3). The presence of multiple arginyl residues in that particular location at sites implicated in C interaction for each of these modulator proteins must be of special significance. They might be responsible for the high affinity displayed by these proteins for the kinase. In contrast to most substrates of the cAMP-dependent protein kinase, the inhibitory region of PKI is rich in hydrophobic residues, as seen by its susceptibility to thermolysin digestion.

In the present study, staphylococcal V8 proteinase digestion resulted in a gradual loss of PKI activity. These data are in contrast to an earlier report in which an inhibitory fragment thought to be 15 amino acids long was obtained after V8 proteinase attack (20). Since this fragment was refractory to Edman degradation, it was assumed that it originated from the NH_2-terminal portion of PKI, which is blocked. Within peptide 1, the only bond susceptible to V8 proteinase is at Asp-14, three residues removed from the pseudosubstrate Ala-11, and cleavage at this site may effect the inhibitory activity.

Peptide 1 (K_i = 0.8 μM) was the most potent synthetic inhibitor thus far obtained with an affinity at least 100-fold greater than peptide 2 or any other synthetic inhibitor previously described (29). The considerable difference in affinity between peptides 1 and 2 suggests that the three additional NH_2-terminal residues greatly enhance the inhibitor–C subunit interaction.

Inhibition of C by peptide 1 was studied in the presence of four different substrates. Hepatic pyruvate kinase was chosen mainly because it is regulated by the cAMP-dependent protein kinase (34), and the primary structure of its site of phosphorylation served as a model for the synthesis of Kemptide. Even though cAMP-dependent protein kinase displayed the same K_m when pyruvate kinase and Kemptide were used as substrates, the affinities measured for both peptide 1 and PKI were lower by a factor of 2 in the presence of pyruvate kinase than in the presence of Kemptide. The difference obtained might be due to certain extrastructural interferences provided by the native enzyme molecule. Other substrates were histone IIa and cardiac troponin I with K_m values also in the micromolar range (Table 2). But in all instances, the inhibition constants determined in the presence of each of these substrates were very similar, indicating that peptide 1 was able to compete effectively for the enzyme quite independently of the nature of the substrate.

For all enzyme substrates, the K_i for peptide 1 is close to 1 μM. This is ≈200 times greater than the inhibition constant for native PKI. Thus, while our data indicate that the pseudosubstrate region is the primary site of inhibition, other groups or regions of the molecule must contribute to the stability of the enzyme–inhibitor complex. Demaille *et al.* (20) reported that proteolysis of PKI toward its COOH-terminus decreased its affinity and proposed two sites of attachment to the enzyme involving both the NH_2- and COOH-terminal regions. In a detailed kinetic study on the mode of action of PKI, Whitehouse and Walsh (36) proposed that the enzyme–inhibitor complex was mediated by formation of a tertiary complex with the second substrate ATP and suggested that other groups may interact directly with the enzyme surface.

It is interesting that neither PKI nor peptide 1 will inhibit the cGMP-dependent protein kinase. The substrate specificity of this enzyme is similar, albeit not identical, to that of the cAMP-dependent enzyme when peptides are used (37). Both require two or three arginyl or lysyl side chains NH_2-terminal to the residue to be phosphorylated. These are the same determinants one finds at the inhibitory site of PKI. It is not understood why PKI or any of its peptide fragments that

interact so effectively with the cAMP-dependent kinase would be without effect on the cGMP-dependent enzyme.

Because of the low concentration at which the inhibitor exists in muscle tissue, the possibility was considered that it might arise as a breakdown product of R. This hypothesis was discounted when it was found that the inhibitory activity of R was destroyed by CNBr treatment, whereas PKI is resistant since it lacks methionyl residues (7). The data presented here provide the first direct evidence that PKI is a different gene product from those of RI and RII.

Note Added in Proof. A preliminary report of this work has been presented (38). After this manuscript was submitted, the complete amino acid sequence of PKI was completed, and the inhibitory site represents residues 11 to 30 (39).

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