Primary Structural Determinants Essential for Potent Inhibition of cAMP-dependent Protein Kinase by Inhibitory Peptides Corresponding to the Active Portion of the Heat-stable Inhibitor Protein*

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PKI-(5-24)-amide is a 20-residue peptide with the sequence, Thr6-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp24-NH2, that corresponds to the active portion of the heat-stable inhibitor protein of cAMP-dependent protein kinase (Cheng, H.-C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M., and Walsh, D. A. (1986) J. Biol. Chem. 261, 989-992). Amino acid residues in PKI-(5-24)-amide responsible for the potent inhibition (Ki = 2.3 nM) of the catalytic subunit of protein kinase were further investigated using deletion and substitution analogs of the synthetic peptide. Residues 5, 23, and 24 were not required for activity since the 17-residue PKI-(6-22)-amide retained full potency. Sequential removal of the first seven amino acids from the NH2 terminus of PKI-(5-24)-amide caused a progressive 50-fold loss of inhibitory potency. In contrast, substitution of either Thr6, Asp24, or Ile11 with alanine, or Ala6 by leucine, in PKI-(5-22)-amide produced less than 3-fold decreases in potency. Of the 2 aromatic residues in PKI-(5-22)-amide, the individual substitution of Phe7 and Tyr7 by alanine caused, respectively, 90- and 5-fold decreases in inhibitory potency, demonstrating important roles for each. This NH2-terminal portion of the peptide is believed to contain a significant portion of α-helix.

Many recognition or structural determinants are also essential in the COOH-terminal portion of PKI-(5-22)-amide. In addition to the basic subsite provided by the three arginyl residues, several of the residues are critical for full inhibitory potency. Substitution of Ile28 by glycine in either PKI-(5-22)-amide or PKI-(14-22)-amide lowered the inhibitory potency by 150- and 50-fold, respectively. Separate replacement of Gly17 or Asn20, in either PKI-(5-22)-amide or PKI-(14-22)-amide, caused 7-15-fold decreases in potency. Substitution of both Gly17 and Asn20 together (in PKI-(14-22)-amide) produced a synergestic loss of inhibitory activity. [Leu13,Ile24]PKI-(5-22)-amide, a doubly substituted analog exhibited a 42-fold increase in Ki value.

We conclude that Ser13 and/or Gly14, Gly17, Asn20, and Ile24 each contribute important features to the binding of these inhibitory peptides to the protein kinase, either by providing recognition determinants, inducing structure, and/or allowing essential peptide backbone flexibility. Compatible with these conclusions we have also demonstrated that [Ser31]PKI-(14-22)-amide is a highly effective substrate for the protein kinase, considerably more so, for example, than the heptapeptide, Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly).

The heat-stable inhibitor protein of cAMP-dependent protein kinase (PKI) selectively inhibits the free catalytic subunit of that enzyme (1, 2). PKI inhibits the catalytic subunit of the protein kinase with a Ki value in the nanomolar range and acts competitively with respect to phosphoryl-accepting substrate (3-5). This extremely high affinity for catalytic subunit is rivaled only by the regulatory subunit of the enzyme (6, 7). All other known protein or peptide substrates and inhibitors are at least 4 orders of magnitude less potent in their interactions with catalytic subunit (6-11).

The entire 75-amino acid sequence of one of the charge and size isoforms of PKI has been determined (12). An active portion of PKI has been defined by obtaining inhibitory oligopeptides by limited proteolytic digestion with either Staphylococcus aureus V8 protease (13) or mast cell protease II (14). The amino acid sequence of the former peptide (PKI-(5-24))2 is Thr6-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Asp-Ala-Ile-His-Asp4. The inhibition by PKI-(5-24)-amide is competitive with respect to peptide or protein substrate, with a Ki value of 2.3 nM (15). The COOH-terminal half of this peptide contains a pseudosubstrate site in which the multiple arginyl residues are required for potent inhibition of the catalytic subunit of cAMP-dependent protein kinase (15, 16). The pseudosubstrate site contains the sequence Ser1-Thr-Ala-Arg-Arg-Leu-Gly.

1 The abbreviations used are: PKI, heat-stable inhibitor protein of cAMP-dependent protein kinase; PKI-(5-24)-amide, Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Asp-Ala-Ile-His-Asp-NH2; Kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; (Ala)Kemptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly; IC50, the concentration producing 50% inhibition; Boc, tert-butyloxycarbonyl; Sar, sarcoine; Abu, 2-aminobutyric acid; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid.

2 The amino acid residues in the PKI peptides are numbered according to the amino acid sequence of the native inhibitor protein (12).
quence -Arg\(^{11}\)-Thr-Gly-Arg-Arg-Asn-Ala-Ile\(^{22}\), which is similar to the typical -Arg-X-X-Arg-X-Ser-X- phosphorylation site sequence in substrates of CAMP-dependent protein kinase (17). Initial studies with synthetic peptide analogs of PKI hydrolyzed a single bond between Thr and Ala (10-22) and PKI-(11-30) indicated that residues NH\(_2\) terminal to the pseudosubstrate site are also required to confer high affinity toward the CAMP-dependent protein kinase (15, 16). PKI-(5-24) and other analog peptides, but not PKI itself, also inhibit CAMP-dependent protein kinase, but with potencies approximately 4 orders of magnitude less than for the CAMP-dependent enzyme (18). An interaction between PKI peptides and calmodulin has also been reported (19).

In the present study we have (i) investigated the minimal structure of PKI-(5-24)-amide that retains high potency of inhibition and (ii) analyzed the contributions of specific amino acid residues, in addition to the arginines, to this high potency. A series of deletion peptides and analog peptides with amino acid substitutions in both the NH\(_2\) - and COOH-terminal regions have been synthesized and tested as inhibitors of catalytic subunit. These data are correlated with previous (20) and on-going studies directed toward understanding the possible secondary structure of these peptides. Combined, these experiments were designed to investigate the importance to the peptide’s function of the phenolic hydroxyl group of tyrosine 7, the hydroxyl groups of threonine residues, selected hydrophobic and aromatic amino acids, and the residues involved in possible β-turn and α-helical structures.

Also, we have compared some of these PKI peptide analogs to the substantially less potent competitive inhibitor, (Ala)Kemptide (10, 21), in their recognition of the catalytic site of CAMP-dependent protein kinase. Between this and previous studies (15, 16), the role of every residue except one in PKI-(5-22)-amide has now been investigated by synthesis of a single substitution analog.

**EXPERIMENTAL PROCEDURES**

**Synthetic Peptides**—PKI-(5-24)-amide, PKI-(5-22)-amide, and most of their substituted and truncated analogs were synthesized as COOH-terminal amides by solid-phase peptide synthesis on p-methylbenzylaminine resin using the reagents and techniques described previously (15, 22). Peptides were synthesized either manually or on an ABI model 430 synthesizer. Boc-sarcosine and Boc-2-aminoxybutyrate were coupled as preformed symmetrical anhydrides. In the synthesis of many PKI-(5-22)-amide analogs, difficulty was always encountered in the coupling of Boc-Thr(0-benzyl) at positions 5 and 6 as determined by quantitative ninhydrin monitoring of chain elongation (27) which either Henderson or double-reciprocal analyses are suitable, as were the linear relationships of the slope versus substrate concentration replots. The intercepts of the Henderson plots are all the same within experimental error indicating that the peptides interact with the enzyme in a similar fashion although with different potencies. Hill coefficients ranged from 0.85 to 1.15. Representative Henderson plots and double-reciprocal plots for inhibition of catalytic subunit by selected PKI-amide peptides are shown in Figs. 2a and 3, respectively.

The increasing slopes of the lines in the Henderson plots are diagnostic of competitive inhibition versus peptide substrate, as are the linear relationships of the slope versus substrate concentration replots (insets, Fig. 2). The intercepts of the replots yielded the K\(_s\) values. The double-reciprocal plots also indicated competitive inhibition. All synthetic peptides used in this study were competitive inhibitors of the catalytic subunit versus Kemptide as substrate. This is consistent with
previous reports of the parent PKI peptide (13, 15) and the native PKI protein (3, 5).

Peptide Length and NH2-terminal Deletion Peptides—In previous studies, we showed that histidine 23 and aspartic acid 24 in PKI-(5-24)-amide are not required for potent inhibition of catalytic subunit (15). PKI-(5-24)-amide and PKI-(5-22)-amide had nearly identical K, values of 2 -3 nM. It was also demonstrated that the omission of the NH2-terminal 6 residues from PKI-(5-24)-amide caused a 50-fold increase in the K, of the resulting PKI-(11-24)-amide (15). However, the minimal PKI peptide structure that still retained high potency was not known. Therefore, both PKI-(5-24)-amide and PKI-(5-22)-amide were used as parent sequences in a series of analogs in which a variable number of residues at the NH2 termini were deleted. The sequences and K, values of these peptides are listed in Table I.

As noted previously, the deletion of residues 5-9 caused a 30-fold decrease in inhibitory activity and the further deletion of phenylalanine 10 from PKI-(10-24)-amide caused an additional 50% loss of potency. Omission of isoleucine 11 from PKI-(11-24)-amide caused no further loss of inhibitory activity, but removal of alanine 12 and serine 13 resulted in the peptide PKI-(14-22)-amide, which regained some degree of inhibitory potency. Further, shortening of the peptide by removal of glycine 14, and especially of arginine 15, produced relatively weak inhibitors. This result confirmed the functional importance of arginine 15 established in previous studies (15, 16).

Omission of threonine 5 from PKI-(5-22)-amide resulted in a peptide with a slightly greater potency of inhibition than the parent peptide (Table I) and the N-acetylated PKI-(6-22)-amide was also highly potent. The combined removal of both threonine 5 and threonine 6 caused a loss in potency of 1 order of magnitude. This increase in the K, value was not simply due to a free NH2 terminus in PKI-(7-22)-amide because its N-acetylated analog was slightly less potent. Further removal of amino acid residues in this series of peptides caused additional losses in inhibitory activity. Interestingly, removal of aspartic acid 9 through serine 13, which generated the shortened analog PKI-(14-22)-amide, caused some recovery of potency. This is similar to that seen and discussed above with PKI-(14-22)-amide, caused some recovery of potency. This is similar to that seen and discussed above with PKI-(14-22)-amide, caused some recovery of potency. This is similar to that seen and discussed above with PKI-(14-22)-amide, caused some recovery of potency. This is similar to that seen and discussed above with PKI-(14-22)-amide, caused some recovery of potency. This is similar to that seen and discussed above with PKI-(14-22)-amide, caused some recovery of potency.

NH2-terminal Substitution Peptides—Because the NH2-terminal portion of PKI-(5-22)-amide was required for its high affinity interaction with catalytic subunit, we investigated the potential roles of individual amino acids in this area of the peptide. A series of analogs of PKI-(5-22)-amide was synthe-
peptides.

and alanine 21 in the COOH-terminal pseudosubstrate site of the PKI peptides have been previously investigated in analog procedures. The structure-function of protein kinase inhibitor peptides. The plots of inhibition of catalytic subunit activity by inhibitor peptides. The insets are replots of $K_{\text{m(app)}}$ versus PKI inhibitor peptide concentrations.

tested the effects of single substitutions for the other four amino acids in that portion of PKI peptides, because these nonspecific residues in the typical -Arg-X-X-Arg-X-Ser-X-substrate phosphorylation site sequence have not been studied by the synthetic peptide approach. These data on the substitution of threonine 16, glycine 17, asparagine 20, and isoleucine 22 are presented in Tables III and IV. Substitution of either glycine 17 or asparagine 20 with leucine or alanine, respectively, had detrimental effects in either PKI-(5-22)-amide or PKI-(14-22)-amide causing between 7- and 15-fold increases in the $K_i$ values (Table III). Tested with PKI-(14-22)-amide, the combined substitutions for both glycine 17 and asparagine 20 caused a synergistic decrease in inhibitory activity when compared to either substitution alone.

Replacement of threonine 16, with the threonine analog 2-amino butyric acid, had no effect on the $K_i$ value of PKI-(5-22)-amide (Table III). However, substitution of threonine 16 by alanine in PKI-(14-22)-amide caused a 4-fold decrease in potency. We do not yet know whether this difference was due to the type of residue substituted or the lengths of the parent peptides that were tested. The structural alteration having the most dramatic effect on inhibitory activity of PKI-(5-22)-amide was to remove the hydrophobic side chain at position 22 by substituting isoleucine 22 by glycine. As indicated by the data of Table IV, maximum inhibitory activity required the presence of a large hydrophobic residue at this position. In either PKI-(5-22)-amide or PKI-(14-22)-amide, substitution of isoleucine 22, by leucine, caused rather modest increases in the $K_i$ values whereas substitution by glycine caused 150- and 40-fold increases, respectively, in the $K_i$ values of the two peptides. Note that the full-length [Gly22]PKI-(5-22)-amide peptide, even though it contained the important NH$_2$-terminal region, was less active than were both of the short PKI-(14-22)-amide peptides that had bulky side chains at position 22.

Previous CD spectral data (20) have suggested that PKI-(5-22)-amide might contain a $\beta$-turn. Two potential regions of probable $\beta$-turn conformation are suggested from the secondary structure parameters of the sequence as described by Chou and Fasman (38). These are in the middle of the peptide between alanine 12 and arginine 15 (or serine 13 and threonine 16) and in the pseudosubstrate region between glycine 17 and asparagine 20. The detrimental effects of the substitution of leucine for glycine 17 and alanine for asparagine 20 (Table III) could possibly be related to perturbation of this structure at the latter position. In an attempt to evaluate the importance of the other potential $\beta$-turn location for inhibition of catalytic activity, the peptide analog [Leu$_{17}$,Ile$_{21}$]PKI-(5-22)-amide was synthesized and tested. The replacement of serine 13 and glycine 14 by large hydrophobic residues, substantially reduces the potential of that segment of the peptide to assume a $\beta$-turn structure (38). As indicated in Table III, [Leu$_{17}$,Ile$_{21}$]PKI-(5-22)-amide was a poor inhibitor of the protein kinase, having a $K_i$ value that was 42-fold higher than that of the parent PKI-(5-22)-amide. Although from these substitution experiments no distinction of the possible $\beta$-turn location can be made, these data do indicate that residues in both potential $\beta$-turn locations make important contributions to the inhibitory activity of PKI-(5-22)-amide.

Comparison of PKI Peptides with Kemptide—As illustrated in Table III, when both glycine 17 and asparagine 20 were replaced by leucine and alanine, respectively, both the sequence and resulting loss of inhibitory potency were reminiscent of (Ala)$\text{Kemptide}$, the competitive inhibitor of the enzyme first described by Feramisco and Krebs (21). Even with the loss of activity provoked by these changes in [Leu$_{17}$,Ala$_{20}$]

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FIG. 3. Kinetic analysis of inhibition of cAMP-dependent protein kinase activity by inhibitor peptides. Double-reciprocal plots of inhibition of catalytic subunit activity by A, PKI-(15-24)-amide, and B, [Ala$_{10}$,Leu$_{18}$]PKI-(14-22)-amide. Inhibitor peptide concentrations were as indicated. Protein kinase activity was assayed with Kemptide as variable substrate as described under “Experimental Procedures.” The insets are replots of $K_{\text{m(app)}}$ versus PKI inhibitor peptide concentrations.

sized in which a single residue in positions 5–11 was substituted, usually with alanine. This approach allowed identification of functionally important amino acids in the NH$_2$-terminal region without the confounding factor of varying chain length of the peptides. The capabilities of these peptides to inhibit catalytic subunit are shown in Table II. As expected, substitution of threonine 5, in this case by sarcosine, had little effect. Substitution of threonine 6, aspartic acid 9, and isoleucine 11 with alanine, and of alanine 8 by leucine, caused less than 3-fold increases in the $K_i$ values of the resulting peptides. Substitution of tyrosine 7 by alanine produced a somewhat greater loss of inhibitory activity. The peptide [Ala$_{10}$]PKI-(5-22)-amide, in which the aromatic ring of phenylalanine 10 was absent, exhibited approximately a 2-order of magnitude loss of activity. This was the most significant alteration in potency caused by any of the NH$_2$-terminal substitutions and suggests that phenylalanine 10 plays a critical role in the inhibition of protein kinase by the PKI peptides.

COOH-terminal Substitution Peptides—The three arginines and alanine 21 in the COOH-terminal pseudosubstrate site of the PKI peptides have been previously investigated in analog studies (15, 16), which notably demonstrated the importance of the cluster of arginine residues. We elected, therefore, to
TABLE I

Effect of length of PKI amide peptides on inhibitory potency

Inhibitory constants were obtained from initial velocity data either by Henderson analysis or double-reciprocal plots as described under "Experimental Procedures." The "parent" peptides in each series of analogs are PKI-(5-24)-amide (top series) and PKI-(5-22)-amide (bottom series).

| Peptide sequence | Ki (nM) |
|------------------|---------|
| Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-His-Asp-NH₂ | 2.3* |
| Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-His-Asp-NH₂ | 73* |
| Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-His-Asp-NH₂ | 115* |
| Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-His-Asp-NH₂ | 119* |
| Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-His-Asp-NH₂ | 57* |
| Arg-Thr-Gly-Arg-Asn-Ala-Ile-His-Asp-NH₂ | 250 |
| Thr-Gly-Arg-Asn-Ala-Ile-His-Asp-NH₂ | 1250 |

* Data from Cheng et al. (15).

TABLE II

Effect of substitutions in the NH₂-terminal domain of PKI-(5-22)-amide on inhibitory potency

Inhibitory constants were obtained from initial velocity data either by Henderson analysis or double-reciprocal plots as described under "Experimental Procedures."

| Peptide sequence | Ki (nM) |
|------------------|---------|
| Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂ | 3.1* |
| Thr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂ | 1.7 |
| Thr-Thr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂ | 7.1 |
| Thr-Thr-A1a-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂ | 14 |
| Thr-Thr-Leu-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂ | 8.9 |
| Thr-Thr-Ala-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂ | 8.2 |
| Thr-Thr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂ | 270 |
| Thr-Thr-Ala-Asp-Phe-Ile Ala Ser Gly Arg Thr Gly Arg Asn Alae NH₂ | 8.1 |

* Data from Cheng et al. (15).

TABLE III

Effect of substitutions in the COOH-terminal domains of PKI-(5-22)-amide and PKI-(14-22)-amide on inhibitory potency

Inhibitory constants were obtained from initial velocity data either by Henderson analysis or double-reciprocal plots as described under "Experimental Procedures."

| Residues tested | Peptide sequence | Ki (nM) |
|-----------------|-----------------|---------|
| Parent peptides | Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂ | 3.1* |
| Thr-Gly-Arg-Asn-Ala-Ile-NH₂ | 36* |
| Thr-Thr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Ala-Ala-Ile-NH₂ | 21 |
| Thr-Thr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Leu-Arg-Asn-Ala-Ile-NH₂ | 26 |
| Gly-Thr-Gly-Arg-Ala-Ala-Ile-NH₂ | 550 |
| Gly-Thr-Gly-Arg-Ala-Ala-Ile-NH₂ | 110 |
| Gly-Thr-Leu-Arg-Asn-Ala-Ile-NH₂ | 390 |
| Gly-Thr-Leu-Arg-Asn-Ala-Ile-NH₂ | 400* |
| Thr-Thr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Ala-Ala-Ile-NH₂ | 2.3 |
| Thr-Thr-Ala-Asp-Phe-Ile-Ala-Leu-Ile-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂ | 140 |
| Gly-Thr-Ala-Gly-Arg-Asn-Ala-Ile-NH₂ | 130 |

* Data from Cheng et al. (15).

* Data from Table I.
PKI-(14–22)-amide, it was approximately 100-fold more potent than the quite similarly sequenced (Ala)Kemptide. This comparison was further explored using the additional synthetic peptides whose characteristics are presented in Table V. The additional substitution of isoleucine 22 by leucine in the parental peptide (Table IV), this has been reinvestigated. In considerable distinction to that resulting from this same substitution in the parent peptides (Table IV), [Ala\(^{14,16,18}\),Leu\(^{22}\)]PKI-(14–22)-amide, the resultant peptide, differed from (Ala)Kemptide by the NH\(_{2}\)-terminal extension of Gly-Arg-Thr and the COOH-terminal replacement of an amide by glycine, but each contained the identical central core sequence of -Leu-Arg-Arg-Ala-Leu-. This PKI peptide was still 30-fold more potent an inhibitor than was (Ala)Kemptide. As an alternative approach, replacement of glycine 14, arginine 15, and threonine 16 in [Leu\(^{22}\)]PKI-(14–22)-amide by alanine (to give peptide [Ala\(^{14,16,18}\),Leu\(^{22}\)]PKI-(14–22)-amide) increased the \(K_i\) value from 180 to 3800 nM (Table V). Again, however, this peptide, differed from (Ala)Kemptide by the NH\(_{2}\)-terminal extension of Gly-Arg-Thr and the COOH-terminal replacement of an amide by glycine, but each contained the identical central core sequence of -Leu-Arg-Arg-Ala-Leu-. The minimal length PKI peptide analog that inhibited CAMP-dependent protein kinase with high potency was the 17-residue PKI-(6–22)-amide which has the sequence Thr\(^{6–17}\). Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NHz.

Scott et al. (16) have reported upon the potential role of the residues of PKI peptides to contribute to binding affinity of a protein kinase substrate but observed that the peptide [Ser\(^{21}\)]PKI-(11–30) was indeed only a rather poor substrate \((K_i = 240 \text{ nM})\) than is either PKI-(5–22)-amide or even PKI-(14–22)-amide. Accordingly, given the major difference in inhibitory potency between PKI-(6–22)-amide and (Ala)Kemptide, and the critical role of several of the non-arginine residues to this difference in potency (Tables III and IV), this has been reinvestigated. In considerable distinction to that observed with [Ser\(^{21}\)]PKI-(11–30), [Ser\(^{21}\)]PKI-(14–22)-amide has been found to be an exceptionally good substrate for the CAMP-dependent protein kinase exhibiting a \(K_i\) 40-fold lower than (Table V), and an apparent \(V_{\text{max}}\) twice as great as (Fig. 4), that observed with Kemptide.

\[ K_i = \frac{[S]}{V_{\text{max}}} = \frac{1}{K_m} \]

Incubation at an elevated enzyme concentration resulted in an increase in inhibitory potency by glycine 17, asparagine 20, and part or parts of the triplet Gly\(^{14–16}\)-Arg-Thr\(^{20}\) in the recognition of PKI peptides by the catalytic site of the protein kinase.

**DISCUSSION**

The minimal length PKI peptide analog that inhibited cAMP-dependent protein kinase with high potency was the 17-residue PKI-(6–22)-amide which has the sequence Thr\(^{6–17}\). Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NHz.
Asa-Ala-Ile22-NH2. This inhibitor interacted at the peptide/protein binding portion of the active site of the enzyme in a competitive manner versus peptide substrate and had a low nanomolar $K_m$ value. The fact that PKI-(5-22)-amide, [Ser2]$^-$PKI-(5-22)-amide, and Acetyl-PKI-(6-22)-amide had potencies similar to that of PKI-(6-22)-amide indicates that the $K_m$ term in this inhibitor peptide can be modified without loss of activity. This property may be useful in developing inhibitors with increased resistance to intracellular degradation as compared to the native peptide.

To inhibit cAMP-dependent protein kinase with high potency, PKI-(6-22)-amide required both the pseudosubstrate and the NH2-terminal region of the peptide. These results are consistent with the observed lower activity of Ala$^-$PKI-(5-22)-amide compared with the parent peptide. We have suggested previously that the phenolic hydroxyl group of tyrosine 7 in the PKI peptide could possibly contribute to inhibition by assuming the position in the active site of the enzyme normally occupied by the phospho-accepting seryl hydroxyl group of a substrate protein (13, 20), thus, serving as an additional recognition determinant as proposed for the seryl hydroxyl in Kemp tide (41). However, this now seems unlikely or, at most, only plays a minor role since the $K_m$ of [Ala]$^-$PKI-(6-22)-amide, which lacks the tyrosine, is still 14 nM.

Circular dichroism spectra (20) have suggested that the secondary structure of PKI-(5-22)-amide in solution consists of approximately 30% $\alpha$-helix and possibly one $\beta$-turn. Predictions by computer algorithms (20, 38), would indicate that the most likely location of the $\alpha$-helical domain would be in the NH2-terminal region of the peptide; if so, it is probably an amphiphilic $\alpha$-helix. It should be noted that none of the amino acid substitutions that were made in the NH2-terminal part of the PKI peptides, including alanine at position 10, would tend to break any $\alpha$-helical structure that was present (38). This suggests that the importance of phenylalanine 10 for inhibitory activity may not be because it maintains the peptide in the proper secondary structure but, instead, because it may directly interact with a residue(s) in or near the active site of the protein kinase. We are now further investigating the specific location of the $\alpha$-helix in the peptide and the importance of phenylalanine 10; a series of analogs of PKI-(6-22)-amide with nonstandard amino acid substitutions at position 10 is being synthesized that will address phenylalanine's steric, aromatic, and electronic properties.

Aspartic acid 9 in the NH2-terminal region was not required for activity; so an ionic interaction of this residue with one or more of the arginines in PKI-(5-22)-amide itself or with a basic residue in catalytic subunit is not obligatory for inhibitory activity. This result is consistent with that of Scott et al. (16) in which deletion of aspartic acid 9 from a PKI peptide caused a modest increase in potency. The loss of inhibitory activity on substitution of serine 13 and glycine 14 by the potential $\beta$-turn breakers leucine and isoleucine suggests that a $\beta$-turn conformation in this area of the PKI peptide could be a requirement for high potency. The data, however, would be equally compatible with the possibilities that the side chain of serine 13 is directly interacting with the protein kinase, that the flexibility of the backbone of the peptide itself in the region of glycine 14 makes an important contribution to inhibitory activity, and/or that when leucine and isoleucine are in these positions they are simply sterically or conformationally hindering. However, it is unlikely that serine 13 undergoes a required hydrogen bonding to the enzyme because substitution of this residue by alanine in PKI-(11-30) had no effect on the $K_m$ value (16). The fact that inhibitory potency again increases in PKI-(14-22 or -24)-amide after deletion of residues including alanine 12 and serine 13 from truncated peptides suggests the influence of some structure in this region of the peptide. Additional PKI peptide analogs of constrained or modified conformation are being synthesized to further investigate these various possibilities.

As indicated by the high potency of [Abu$^+$]PKI-(5-22)-amide, the hydroxyl group of threonine 16 is not required for inhibitory activity. Scott et al. (16) had previously used 2-aminobutyric acid in this position in a substituted PKI-(7-30) peptide, but multiple substitutions in their sequence made it difficult to interpret the influence of threonine 16 alone. They concluded that the 2-aminobutyric acid for threonine substitution was responsible for the 5-fold increase in $K_m$ but the present results using a singly substituted PKI-(5-22)-amide indicate that hydrogen bonding of the hydroxyl group of threonine 16 with the enzyme probably does not contribute to the binding affinity. Substitution of threonine 16 by alanine in PKI-(14-22)-amide did modestly diminish inhibitory potency in accord with the data of Scott et al. (16). The reason...
for these differences upon the substitution of this residue in the longer and shorter peptides is not known but may relate to either the replacement residue that was tested and/or to different structures of the PKI peptides imposed by the truncation.

Isoleucine 22 immediately COOH-terminal to the pseudosubstrate site was an important requirement for activity of the PKI peptide. This is consistent with the studies of Meggio et al. (42) using synthetic proline peptides as substrates for cAMP-dependent protein kinase which indicated the importance of a hydrophobic residue immediately COOH-terminal to the phosphorylation site. A hydrophobic amino acid is conserved at this position in known phosphorylation sites in a great majority of physiologically relevant protein substrates of cAMP-dependent protein kinase (reviewed in Refs. 43-45). Notable exceptions to this are the phosphorylation sites in histone H1 (46), protamines (47), cardiac troponin-I (48), and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (11). However, in most of these cases, hydrophobic amino acids occur as the 2nd and 3rd residues COOH-terminal to the phosphorylatable serine and/or there are multiple basic amino acids NH2-terminal to the phosphorylation site. It is also possible that the influence of isoleucine 22 on activity is due more to its property to restrict flexibility than on hydrophobicity.

Glycine 17 and asparagine 20 were also important determinants for activity in the pseudosubstrate region of the PKI peptide. The 4 orders of magnitude greater potency of PKI-(14-22)-amide compared to the similar (Ala)Kemptide is due, in great part, to the presence of these residues as well as to the third arginine and probably to the lack of a free COOH terminus in the former peptide. The importance of the third arginine NH2-terminal to the pseudophosphorylation site has been discussed previously (13, 15, 49). PKI-(16-24)-amide and [Ala14,15,16,Leu17]PKI-(14-22)-amide, which contain only the pair of arginine residues, were also appreciably more potent than was (Ala)Kemptide (Tables I and V). This further underscores the importance of glycine 17 and asparagine 20 for tight binding to the cAMP-dependent protein kinase. Glycine 17 may not be a determinant per se, but it may simply be that a bulky side chain in this position, as with the leucine in Kemptide, is a negative determinant or that the flexibility provided by glycine is required for optimal binding. Asparagine 20 has the potential to hydrogen bond with a residue in the active site of catalytic subunit, whereas an alanyl residue, which is in this position in Kemptide, does not.

The high efficacy of [Ser21]PKI-(14-22)-amide as a substrate emphasizes the similarities between protein kinase substrate binding to the catalytic site and that by PKI peptides, particularly with the finding that a peptide whose sequence was based solely on that of the active site of the inhibitor protein exhibited both a high affinity $K_a$ and a high $V_{max}$. Although Scott et al. (16) reported that, as a substrate, [Ser21]PKI-(11-30) had a $K_a$ of only 280 $\mu$M, two reasons now explain such a low substrate efficacy. Firstly, [Ser21]PKI-(11-30) contains at its NH2 terminus Ile11-Ala12-Ser13 and in similarly truncated PKI inhibitory peptides this region appears to diminish inhibitory potency (i.e. PKI-(11-24)-amide and PKI-(12-24)-amide are less active than PKI-(14-24)-amide). In addition, in the parent peptide PKI-(11-30) residues 25-30 also seem to diminish binding to the protein kinase since its $K_a$ (800 nM; Refs. 14 and 16) is markedly higher than that of the shorter peptide PKI-(11-24)-amide, where residues 25-30 are absent. Similarly the $K_i$ for PKI-(14-30)-amide is also much higher than that of PKI-(14-24)-amide (75 $\mu$M versus 57 nM, respectively; Refs. 14, 16, and Table I). With such results, it thus appears likely that both the first 3 NH2-terminal and the last 6 COOH-terminal residues of [Ser21]PKI-(11-30) negatively influence its binding to the protein kinase, accounting for its very poor efficacy as a substrate. In contrast, [Ser21]PKI-(14-22)-amide is the best peptide substrate for the cAMP-dependent protein kinase, so far encountered, and clearly it is so because of several of its constituent residues in addition to the pair of arginines.

From the range of peptides examined in this and previous studies (13-16), many of the important determinants that endow PKI-(6-22)-amide (and probably PKI itself) with high potency are now apparent. PKI-(6-22)-amide clearly mimics protein and peptide substrates in interacting with the substrate binding region of the active site of catalytic subunit particularly in the COOH-terminal pseudosubstrate basic domain. The importance of the arginine residues in this region has been well documented (8, 15, 16, 21) and they probably bind ionically to essential aspartic or glutamic acids (or a phosphothreonine) in the active site of the enzyme (50-52). Additional evidence that the PKI peptide interacts with the substrate binding site now comes from the finding that [Ser21]PKI-(14-22)-amide is a very effective substrate; [phosphoasparaginyl]PKI-(11-30) has been previously shown to be a product inhibitor with a $K_i$ value of 96 $\mu$M (16). Residues other than the arginines in the pseudosubstrate site, most notably isoleucine 22, also contribute to the binding affinity. These interactions between the pseudosubstrate site and the enzyme are supplemented by additional hydrophobic interactions of the NH2-terminal domain, particularly phenylalanine 10, with a proposed hydrophobic cleft or aromatic residue near the active site. The secondary structure of PKI-(6-22)-amide may be important in properly aligning its determinants for binding to the corresponding amino acid residues in the active site of cAMP-dependent protein kinase (20). The overall affinity of PKI-(6-22)-amide is undoubtedly due to the number of individual hydrogen bonds and other bonding interactions it has with the active site of the cAMP-dependent protein kinase. Apparently, the heat-stable inhibitor protein has evolved to maximize the number of these bonds. Substrate proteins, on the other hand, are usually present in the cell at higher concentrations than is PKI and must also leave the enzyme rapidly as phosphorylated products so that the catalytic cycle can continue. Therefore, substrate proteins need not interact as avidly with the enzyme as does PKI although, interestingly, they would have the potential to do so as judged from the results seen with [Ser21]PKI-(14-22)-amide. The full structural correlates of binding of substrates or PKI to the cAMP-dependent protein kinase will likely only be completely understood, however, when the crystal structure of the catalytic subunit of the enzyme is solved (53).

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