

Conformationally constrained analogs of protein kinase inhibitor(6–22)amide: Effect of turn structures in the center of the peptide on inhibition of cAMP-dependent protein kinase

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(RECEIVED September 28, 1994; ACCEPTED December 9, 1994)

Abstract

The high-affinity interaction between protein kinase inhibitor (PKI)(6–22)amide (Thr⁶-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile²²-NH₂) and the catalytic subunit of cAMP-dependent protein kinase requires both the N-terminal Thr⁶ to Ile¹¹ sequence of the inhibitor peptide and its C-terminal pseudosubstrate site comprised of Arg¹⁵ to Ile²². Small angle X-ray scattering data indicate that PKI(6–22)amide has a compact, rather than extended, structure in solution (Reed J et al., 1989, *Biochem J* 264:371–380). CD spectroscopic analysis of the PKI peptide led to the suggestion that a β -turn structure might be located in the -Ala¹²-Ser-Gly-Arg¹⁵- connecting sequence in the middle of the molecule (Reed J, Kinzel V, Cheng HC, Walsh DA, 1987, *Biochemistry* 26:7641–7647). To investigate this possibility further, conformationally constrained and flexible analogs of PKI(6–22)amide were synthesized and used to study the structure–function relationships of this central portion of the inhibitor. (Des12–14)PKI(6–22) amide exhibited over a 200-fold loss in inhibitory activity. Replacement of the omitted -Ala¹²-Ser-Gly¹⁴- sequence with aminocaprylic acid yielded an analog that regained more than 90% of the lost binding energy. The D-alanine¹⁴ PKI analog was as potent as the parent peptide, whereas the β -alanine¹⁴ and the sarcosine¹⁴ analogs were only 10-fold less active. Several peptides that promoted a β -turn structure at residues 12–15 showed about 200-fold decreases in inhibitory activity. Two constrained analogs that could not assume a β -turn conformation were only 30-fold less potent than PKI(6–22)amide. Thus, the structure of the central connecting portion of the PKI peptide, encompassing residues 12–15, greatly influences its ability to effectively bind to and inhibit the catalytic subunit. We conclude, however, that a formal β -turn at this position is not required and is actually detrimental for a high-affinity interaction of PKI(6–22)amide with the enzyme. These results are interpreted in light of the Fourier-transform infrared spectra of the peptide analogs and the crystal structure of the peptide bound at the active site of the protein kinase (Knighton DR et al., 1991b, *Science* 253:414–420).

Keywords: β -turns; CD spectroscopy; conformational constraint; Fourier-transform infrared spectroscopy; inhibitor peptide; peptide synthesis; protein kinase

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Abbreviations: PKI, the α form of the heat-stable inhibitor protein of cAMP-dependent protein kinase (Van Patten et al., 1992); Kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; IC₅₀, the concentration producing 50% inhibition; RP-HPLC, reversed-phase high pressure liquid chromatography; FTIR, Fourier-transform infrared; HOBt, hydroxybenzotriazole; Aib, 2-aminoisobutyric acid; DAla, D-alanine; Acy, aminocaprylic acid (8-aminooctanoic acid); Aba, 4-aminobenzoic acid; Pca, piperidine-4-carboxylic acid; β Ala, beta-alanine; Sar, sarcosine; Boc, butoxycarbonyl; BSA, bovine serum albumin; MES, 2-(N-morpholino)ethanesulfonic acid. All chiral amino acids are the L isomers unless otherwise indicated.

The heat-stable inhibitor protein of the cAMP-dependent protein kinase is a potent and selective inhibitor of the catalytic subunit of that enzyme (Walsh et al., 1990). PKI is a competitive inhibitor with respect to phosphoryl-accepting peptide and protein substrates, although it is not phosphorylated itself. The primary structural determinants within PKI that are responsible for its inhibitory activity have been delineated using analog peptides that correspond to the amino acid sequence of its active portion (Cheng et al., 1986; Scott et al., 1986; Glass et al., 1989a, 1989b). The shortest synthetic PKI peptide that retains a high potency of inhibition is the 17-residue PKI(6–22)amide. This peptide has

Table 1. Amino acid compositions of synthetic PKI peptides following acid hydrolysis^a

Peptide	Residue									
	Asx	Ser ^b	Gly	Thr ^b	Ala	Arg	Pro	Tyr	Ile	Phe
Thr ⁶ -Tyr-Ala-Asp-Phe-Ile-Ala ¹² -Ser ¹³ -Gly ¹⁴ -Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile ²² -NH ₂										
PKI(6-22)NH ₂	2.04	0.97	2.00	1.96	3.02	3.04		1.00	1.98	0.99
(βAla ¹⁴)PKI(6-22)NH ₂ ^c	2.21	1.08	1.06	2.01	2.77	3.14		0.96	1.89	0.88
(des12-14)PKI(6-22)NH ₂	2.31		1.13	2.03	1.91	3.20		0.87	1.76	0.90
(Acy ¹² ,des13,14)PKI(6-22)NH ₂ ^d	2.12		1.11	1.95	2.02	3.14		0.96	1.78	0.97
(DAla ¹⁴)PKI(6-22)NH ₂	2.12	0.94	1.11	2.01	3.95	3.18		0.83	2.02	0.84
(Asn ¹² ,Pro ¹³)PKI(6-22)NH ₂	3.25		1.95	1.98	1.88	3.36	1.01	0.97	1.74	0.88
(Pro ¹³ ,Aib ¹⁴)PKI(6-22)NH ₂ ^d	2.07		1.06	1.98	2.94	3.08	1.21	0.81	1.97	1.11
(Sar ¹⁴)PKI(6-22)NH ₂ ^c	2.17	1.08	1.03	1.97	3.02	3.05		0.96	1.82	0.90
(Aba ¹⁴)PKI(6-22)NH ₂ ^c	2.20	1.15	1.20	2.03	2.81	2.93		0.87	1.89	0.93
(Pca ¹⁴)PKI(6-22)NH ₂ ^c	2.26	1.17	1.24	1.99	2.77	3.11		0.89	1.74	0.82

^a Values are moles of amino acid per mole of peptide and are the mean of duplicate determinations.

^b Corrected for 5-10% destruction during acid hydrolysis.

^c βAla, Sar, Aba, and Pca were detected but not quantitated.

^d Aib and Acy were not fully resolved from reagent peaks in this method of analysis.

tential conformations involving residues -Ala¹²-Ser-Gly-Arg¹⁵- while retaining Arg¹⁵ in their structures.

Interaction of PKI peptide analogs with the catalytic subunit

Amino acid compositions of the synthetic peptides are shown in Table 1, and the structures of nonstandard amino acid residues used therein are depicted in Figure 2. Each of the PKI(6-22) amide peptides tested was able to inhibit completely the protein kinase. Titrations of the phosphotransferase activity to determine IC₅₀ values for the inhibitors are shown in Figure 3. Log-

logit transformations of the titration data gave slopes ranging from -0.90 to -1.11 (mean = -1.03 ± 0.03), indicating that the titration curves were parallel to one another and that the interactions of the peptide inhibitors with the catalytic subunit were noncooperative. Although several of the peptides had only minor sequence alterations compared to the parent PKI(6-22) amide, their inhibitory potencies varied over a range of greater than two orders of magnitude (Fig. 3). Figures 4 and 5 show examples of detailed kinetic analyses of enzyme inhibition by two of the peptides. All of the PKI(6-22)amide analogs, regardless of their structures or affinities, were indeed competitive inhibitors with respect to Kemptide, the peptide substrate of the protein kinase.

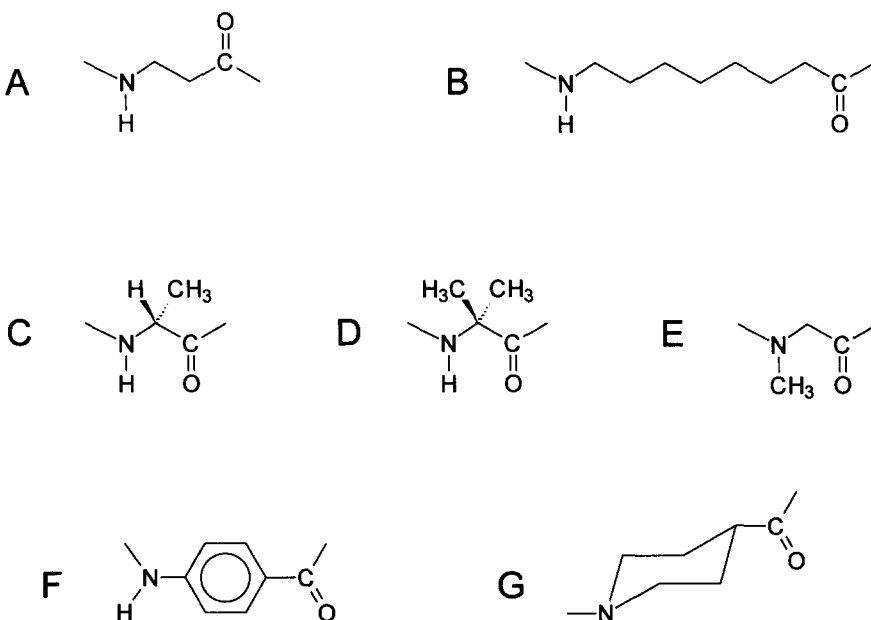


Fig. 2. Structures of the nonstandard amino acids used in the synthetic peptides. Amino acids are drawn as residues in a peptide chain. **A:** β-Alanyl (βAla). **B:** Aminocaprylyl (Acy). **C:** D-Alanyl (DAla). **D:** 2-Aminoisobutyryl (Aib). **E:** Sarcosyl (Sar). **F:** 4-Aminobenzoyl (Aba). **G:** Piperidine-4-carboxylyl (Pca).

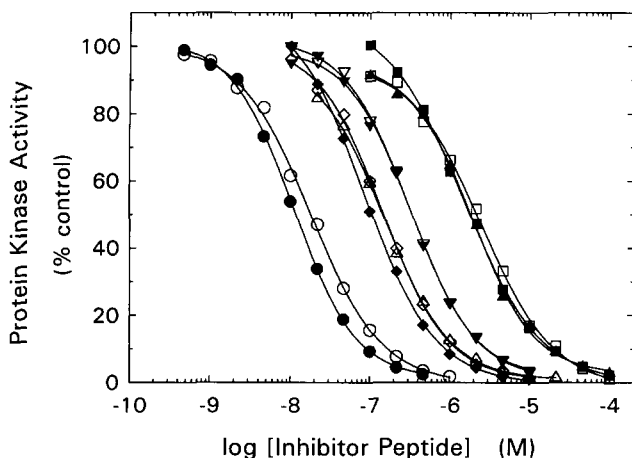


Fig. 3. Titration of protein kinase activity by conformationally constrained and flexible PKI(6-22)amide peptides. Activity of the catalytic subunit was assayed in the absence and presence of various concentrations of inhibitor peptides as described in the Materials and methods. ●, PKI(6-22)amide; ○, (DAla¹⁴)PKI(6-22)amide; ▲, (des12-14)PKI(6-22)amide; △, (Acyl¹²,des13,14)PKI(6-22)amide; ■, (Asn¹²,Pro¹³)PKI(6-22)amide; □, (Pro¹³,Aib¹⁴)PKI(6-22)amide; ▼, (Aba¹⁴)PKI(6-22)amide; ▽, (Pca¹⁴)PKI(6-22)amide; ◆, (βAla¹⁴)PKI(6-22)amide; and ◇, (Sar¹⁴)PKI(6-22)amide.

Spatial relationships in the central connecting region of PKI(6-22)amide

The first structural changes investigated in the peptide were those involving simple intercalation or elision analogs. Insertion of a single methylene group into the peptide backbone next to the C α of Gly¹⁴ yielded the (βAla¹⁴)PKI(6-22)amide analog. As shown in Table 2, this minor alteration, although increasing the flexibility of the connecting region, caused an 11-fold loss in inhibitory activity compared to the parent peptide. The β-alanine

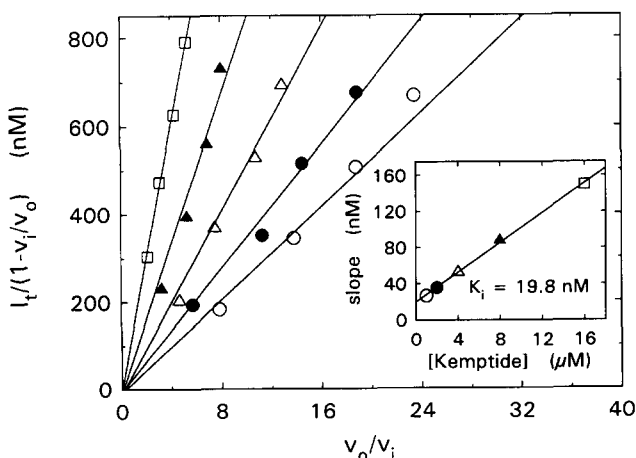


Fig. 4. Henderson analysis of competitive inhibition of protein kinase activity by (βAla¹⁴)PKI(6-22)amide. Enzyme activity was assayed with Kemptide as substrate under the conditions described in the Materials and methods. I_t is total inhibitor peptide concentration and v_0 and v_i are the velocities in the absence and presence of inhibitor, respectively (Henderson, 1972). **Inset** is a replot of the slopes of the Henderson analysis versus Kemptide concentration.

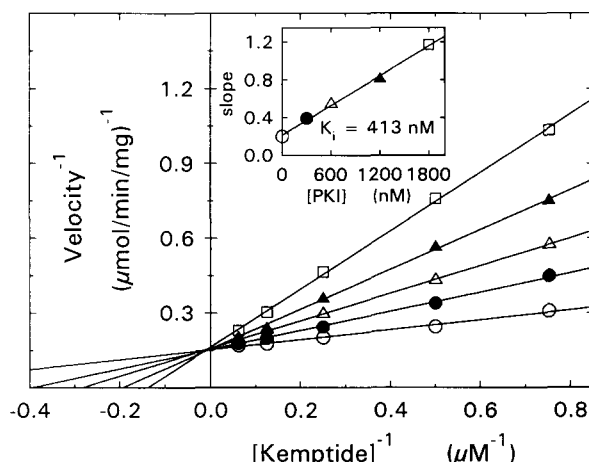


Fig. 5. Double-reciprocal kinetic analysis of competitive inhibition of protein kinase activity by (des12-14)PKI(6-22)amide. Enzyme activity was assayed with Kemptide as substrate under the conditions described in the Materials and methods. **Inset** is a replot of the slopes versus inhibitor peptide concentration.

residue would twist a β-turn slightly, but could still be compatible with such a structure. (Des12-14)PKI(6-22)amide is an analog that omitted most of the residues potentially involved in a β-turn. This elision analog was the least potent of all of the PKI peptides tested, exhibiting a 210-fold higher K_i than PKI(6-22)amide. However, the (Acyl¹²,des13,14)PKI(6-22)amide analog, in which the ω-aminoalkanoic acid aminocaproic acid was substituted in place of multiple amino acid residues, gained back much (93%) of the binding energy that had been lost by removal of the -Ala¹²-Ser-Gly¹⁴- sequence (Table 2). Aminocaproic acid, which is the same length as a tripeptide moiety, would provide nearly identical spacing between the N-terminal α-helix and the C-terminal pseudosubstrate site as in the parent peptide. These results clearly indicate the importance of the normal spacing within the central part of PKI(6-22)amide for its tight binding to the catalytic subunit. Also of note, the aminocaproic acid-substituted peptide cannot contribute a carbonyl in the i position (normally present in Ala¹²) to accept a hydrogen bond for stabilization of a β-turn structure involving parent peptide residues 12-15.

Analogues promoting β-turn structure in PKI(6-22)amide

The possibility that a β-turn in the middle of PKI(6-22)amide might be important for its inhibitory activity was investigated by amino acid substitutions that would promote such a structure. In many peptides, a β-turn (especially a type II β-turn) can be enhanced if an L- followed by a D-amino acid are in sequence in the $i + 1$ and $i + 2$ positions; in this situation, the side chains extend on opposite sides rather than the same side of the polypeptide backbone (Smith & Pease, 1980). Because Gly¹⁴ was present in the $i + 2$ position of a putative β-turn, it was replaced with DAla. This analog also tested whether the Gly¹⁴ residue assumed a pseudo D- or pseudo L-configuration (with respect to φ and ψ angles) when the PKI peptide is bound to the protein kinase. Interestingly, (DAla¹⁴)PKI(6-22)amide was nearly as potent as the parent inhibitor peptide (Table 2). To more effec-

Table 2. Inhibitory activities of peptide models of β -turn structures in PKI(6–22)NH₂^a

Category and peptide	K_i (nM)	n
Thr ⁶ -Tyr-Ala-Asp-Phe-Ile-Ala ¹² -Ser ¹³ -Gly ¹⁴ -Arg ¹⁵ -Thr-Gly-Arg-Arg-Asn-Ala-Ile ²² -NH ₂ ^b <i>i i+1 i+2 i+3</i>		
Parent peptide		
PKI(6–22)amide	1.6 ± 0.1	7
Spatial analogs		
(β Ala ¹⁴)PKI(6–22)amide	18.3 ± 1.3	3
(des12–14)PKI(6–22)amide	336 ± 43.3	3
(Acy ¹² ,des13,14)PKI(6–22)amide	22.9 ± 1.7	3
β -Turn promoters		
(DAla ¹⁴)PKI(6–22)amide	3.4 ± 0.4	3
(Asn ¹² ,Pro ¹³)PKI(6–22)amide	277 ± 10.4	3
(Pro ¹³ ,Aib ¹⁴)PKI(6–22)amide	326 ± 16.4	3
β -Turn breakers		
(Sar ¹⁴)PKI(6–22)amide	22.1 ± 1.9	3
(Aba ¹⁴)PKI(6–22)amide	47.3 ± 3.8	4
(Pca ¹⁴)PKI(6–22)amide	49.9 ± 0.9	3

^a Inhibitory constants were determined by Henderson analysis or double-reciprocal analysis, as appropriate, of initial velocity data as described in the Materials and methods.

^b The i , $i + 1$, $i + 2$, and $i + 3$ residues indicate the four amino acids likely involved in one putative β -turn structure.

tively promote β -turn formation in the inhibitor, two peptides were prepared with a Pro¹³ substitution at the $i + 1$ position. (Asn¹²,Pro¹³)PKI(6–22)amide contains naturally occurring amino acids that impart a high probability of β -turn occurrence at residues 12–15 ($\langle P_i \rangle = 1.40$ and $p_i = 7.83 \times 10^{-4}$). On the other hand, (Pro¹³,Aib¹⁴)PKI(6–22)amide contains α -amino-isobutyric acid, which, because of its geminal methyl groups at C α , imparts considerable conformational rigidity and is known to promote turn formation in acyclic peptides, particularly when following a Pro residue (Crisma et al., 1984; Prasad & Balaram, 1984; Rose et al., 1985). However, as indicated in Table 2, both of these analogs were relatively poor inhibitors of the catalytic subunit, exhibiting K_i values approximately 200-fold higher than that of the parent peptide.

Analogues preventing β -turn structure in PKI(6–22)amide

The *N*-methylated residue at position $i + 2$ in the (Sar¹⁴)PKI(6–22)amide analog would clash with the side chain of the Ser¹³ $i + 1$ residue and thus greatly disfavor a type I β -turn (although a type II turn would be allowed) (Rose et al., 1985). The sarcosine substitution would also preclude hydrogen bonding of the Gly¹⁴ amide proton of the peptide to an acceptor in the enzyme. Although the (Sar¹⁴) analog was 13-fold less potent than the parent inhibitor, it retained reasonable inhibitory activity toward the protein kinase (Table 2). Finally, two PKI peptides were designed that would definitively break a putative β -turn located at residues 12–15. In analogs containing the constrained ring structures of either Aba¹⁴ or Pca¹⁴ substitutions in position $i + 2$, the amide proton of Arg¹⁵ ($i + 3$) cannot reach to hydrogen bond with the carbonyl oxygen of Ala¹² (position i). Both of these analogs were approximately 30-fold less active than the parent peptide, but were still appreciably more potent than ei-

ther (Asn¹²,Pro¹³)PKI(6–22)amide or (Pro¹³,Aib¹⁴)PKI(6–22)amide (Table 2).

CD spectroscopy of PKI peptide analogs in solution

As indicated in Figure 6, the CD spectrum of PKI(6–22)amide at 222 nm confirms the presence of some α -helix as originally reported by Reed et al. (1987) for PKI(5–22)amide. The spectra of these two peptides were similar, indicating that the shortening of PKI(5–22)amide by one residue to yield the 17-residue peptide used in this study did not greatly alter the conformations that it populates in solution. In addition to potentially affecting β -turn formation or random coil structure of the peptides, the amino acid substitutions made in the center of the PKI analogs might also have caused the loss, or alternatively the actual extension, of the N-terminal amphiphilic α -helical structure. However, as is shown by the representative CD spectra in Figure 6, the spectra of the analog peptides were generally quite comparable to that of the parent peptide PKI(6–22)amide, indicating that they retained a similar degree of α -helix.

FTIR spectroscopy of PKI peptides in solution

The buffer-subtracted FTIR spectra of two representative PKI(6–22)amide analogs and their corresponding Fourier deconvolved spectra are shown in Figure 7A, B, and C, respectively. Frequency assignments and peak area data for all of the peptides are presented in Table 3. These spectra were collected at higher resolution (2 cm⁻¹ compared to 4 cm⁻¹) than those previously published for PKI(5–22)amide (Reed et al., 1989), allowing a more complete analysis of the components in the amide I' region of the spectrum. The higher resolution FTIR spectra facilitated discrimination of four peaks at frequencies of 1,628–

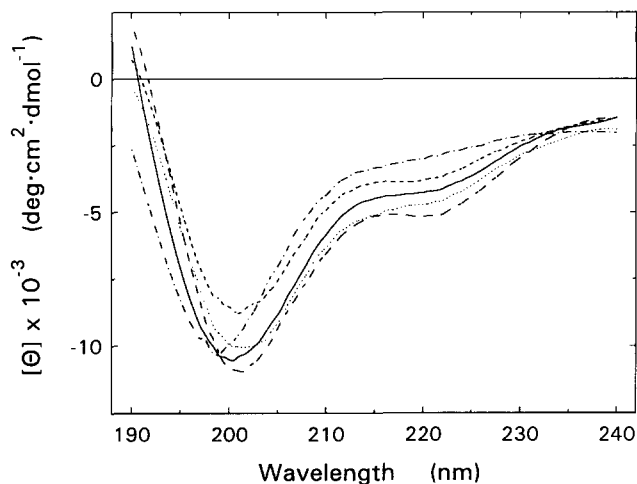


Fig. 6. CD spectra of PKI(6-22)amide and selected PKI peptides. —, PKI(6-22)amide; ---, PKI(5-22)amide; ·····, (DAla¹⁴)PKI(6-22)amide; ·····, (des12-14)PKI(6-22)amide; and - - - - , (Pca¹⁴)PKI(6-22)amide. All spectra were obtained as described in the Materials and methods.

1,630, 1,640-1,643, 1,654-1,657, and 1,668-1,673 cm^{-1} in PKI(6-22)amide and in all of its substituted analogs (Table 3). The percentage contributions of the $\sim 1,629 \text{ cm}^{-1}$ and $\sim 1,655 \text{ cm}^{-1}$ peaks, with average relative intensities of 11% and 25%, respectively, were quite similar in the spectra among all peptides. In contrast, the $\sim 1,641$ - and $\sim 1,673 \text{ cm}^{-1}$ bands showed quite large and apparently complementary variations among the peptides (Table 3). Based on these FTIR spectral differences, PKI(6-22)amide analogs could be divided into two distinct groups, termed for convenience class I and class II. An example of the spectrum for one peptide from each group is shown in Figure 7 showing the clear distinction between them. Class I spectra, which included that of the parent peptide PKI(6-22)amide, had less intensity (20-28%) in the $\sim 1,641 \text{ cm}^{-1}$ band and more intensity (36-40%) in the $\sim 1,673 \text{ cm}^{-1}$ band (Table 3). Conversely, class II spectra had more intensity (31-39%) in the $\sim 1,641 \text{ cm}^{-1}$ band and less (19-27%) in the $\sim 1,673 \text{ cm}^{-1}$ band. Additionally, a minor peak (5%) at $1,689\text{-}1,691 \text{ cm}^{-1}$ was resolved in the spectra of all the class I peptides, but was absent from those of class II peptides except for (Acy¹²,des13,14)PKI(6-22)amide, which exhibited a possible minor contribution at the somewhat lower frequency of $1,685 \text{ cm}^{-1}$.

According to the FTIR band assignments of Byler and Susi (1986), the peaks at $1,628\text{-}1,630$ and $1,668\text{-}1,673 \text{ cm}^{-1}$ are attributable to extended chain structures, with the latter band possibly also including a contribution from turns or bends. The bands around $1,641 \text{ cm}^{-1}$ are due to random coil structures, the $1,655\text{-}1,657 \text{ cm}^{-1}$ bands are attributable to α -helix, and the small peak at $\sim 1,690 \text{ cm}^{-1}$ is assigned to a turn or bend conformation. The FTIR spectrum of PKI(5-22)amide (Table 3) agreed well with that observed previously for this peptide (Reed et al., 1989) and was very similar to that of the shorter PKI(6-22)amide, the parent peptide in the present study, with only a small redistribution of intensities between bands at $1,629$, $1,641$, and $\sim 1,655 \text{ cm}^{-1}$. The spectra of all the peptides showed a band at $\sim 1,655 \text{ cm}^{-1}$ due to α -helix content, and the average relative intensity of this band was approximately 25%. Only (Pro¹³, Aib¹⁴)PKI(6-22)amide with 19% intensity and (Sar¹⁴)PKI

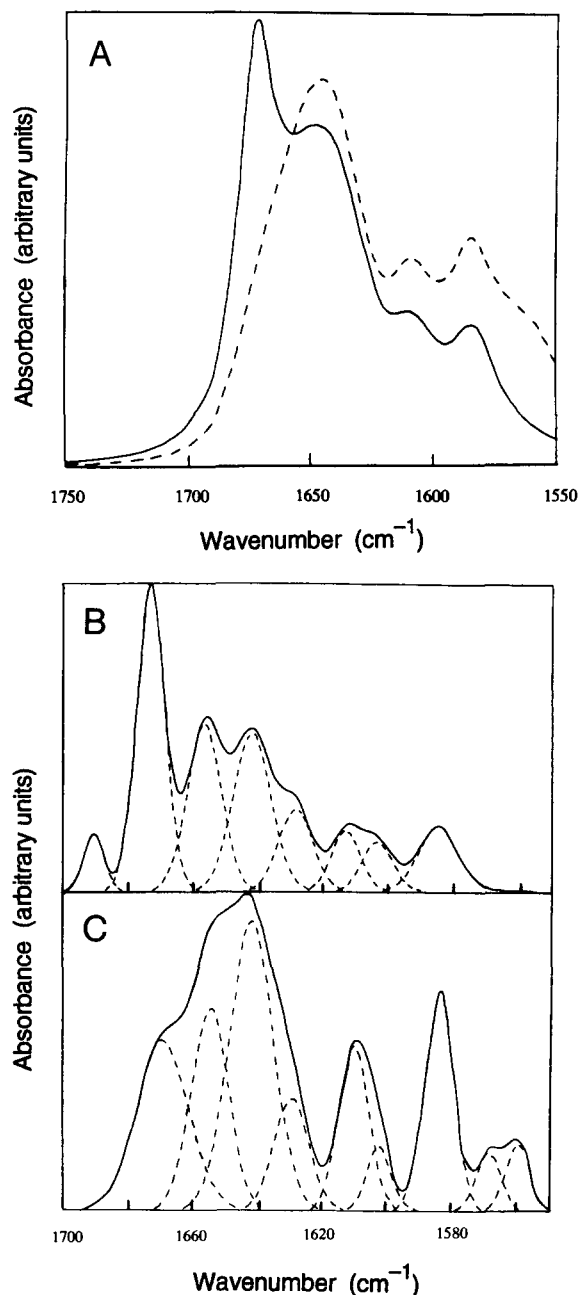


Fig. 7. A: Buffer-subtracted FTIR spectra of selected PKI peptides. —, (β Ala¹⁴)PKI(6-22)amide and ---, (des12-14)PKI(6-22)amide peptides exhibiting class I and class II spectra, respectively. Spectra were classified as defined in Table 3 (see text for details). B: (β Ala¹⁴)PKI(6-22)amide. C: (des12-14)PKI(6-22)amide. Shown in B and C are —, Fourier deconvolutions and ·····, individual Gaussian components for the two spectra shown in (A) analyzed as described in the Materials and methods.

(6-22)amide with 32% intensity deviated more than a few percent from this average value. Of note, this degree of α -helix was maintained in the spectrum of (des12-14)PKI(6-22)amide, a finding compatible with the assignment of the helix to the first six N-terminal amino acids.

The redistribution of intensity between FTIR bands for the class I and class II spectra indicates structural differences in these

Table 3. Frequency assignments and percent areas for absorption peaks in the amide I' region of the deconvolved FTIR spectra of PKI(6-22)amide and analog peptides

Peptide	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Class I spectra ^a					
PKI(5-22)NH ₂	1,629 ^b (12% ^c)	1,641 (20%)	1,655 (28%)	1,673 (36%)	1,689 (5%)
PKI(6-22)NH ₂	1,629 (7%)	1,641 (28%)	1,657 (24%)	1,673 (36%)	1,690 (5%)
(βAla ¹⁴)PKI(6-22)NH ₂	1,629 (12%)	1,642 (20%)	1,656 (28%)	1,673 (36%)	1,690 (5%)
(DAla ¹⁴)PKI(6-22)NH ₂	1,630 (10%)	1,643 (23%)	1,657 (24%)	1,673 (37%)	1,690 (5%)
(Pro ¹³ ,Aib ¹⁴)PKI(6-22)NH ₂	1,628 (11%)	1,642 (25%)	1,657 (19%)	1,673 (40%)	1,691 (5%)
Class II spectra					
(des12-14)PKI(6-22)NH ₂	1,630 (12%)	1,643 (39%)	1,655 (23%)	1,669 (27%)	ND ^d
(Acy ¹² ,des13,14)PKI(6-22)NH ₂	1,628 (11%)	1,640 (37%)	1,654 (25%)	1,670 (26%)	1,685 (2%)
(Asn ¹² ,Pro ¹³)PKI(6-22)NH ₂	1,629 (15%)	1,641 (31%)	1,654 (27%)	1,668 (27%)	ND
(Sar ¹⁴)PKI(6-22)NH ₂	1,629 (11%)	1,641 (38%)	1,656 (32%)	1,671 (19%)	ND
(Pca ¹⁴)PKI(6-22)NH ₂	1,628 (14%)	1,641 (37%)	1,654 (25%)	1,670 (24%)	ND

^a Peptide spectra were classified based on major differences in the areas of bands at 1,641, 1,673, and 1,690 cm⁻¹ as illustrated in Figure 7.

^b Frequency in cm⁻¹.

^c Relative band intensity as percent of the total peak area in the amide I' region.

^d ND, not detectable.

peptides involving an exchange of turn and/or bend structure (and possibly some extended structure) in PKI(6-22)amide and other peptides that gave class I spectra for more random coil conformation in those that gave class II spectra (Table 3). These observations were generally consistent with the anticipated effects of each of the amino acid substitutions. For example, two of the peptide analogs designed to be unfavorable for β-turn structures [i.e., (Sar¹⁴)PKI(6-22)amide and (Pca¹⁴)PKI(6-22)amide] gave rise to class II spectra. In addition, (des12-14)PKI(6-22)amide and (Acy¹²,des13,14)PKI(6-22)amide, which omitted most of the residues potentially involved in the β-turn, also yielded class II spectra. In contrast, (DAla¹⁴)PKI(6-22)amide and (Pro¹³,Aib¹⁴)PKI(6-22)amide, two of the substituted peptides designed to stabilize a putative β-turn structure, gave rise to class I spectra similar to that of the parent peptide. The spatial peptide analog (βAla¹⁴)PKI(6-22)amide, expected to be compatible with a turn structure, also gave a class I spectrum. The only peptide that appeared contrary to expectation was (Asn¹²,Pro¹³)PKI(6-22)amide, which was designed to contain a β-turn promoting sequence, but gave a class II FTIR spectrum.

Discussion

The -Ala¹²-Ser-Gly¹⁴- connecting sequence in the central region of PKI(6-22)amide plays a crucial role in allowing the peptide to exert maximal inhibitory activity toward the protein kinase. These residues must either directly provide specific recognition determinants for binding sites on the protein kinase or contribute to an essential overall conformation of the PKI peptide that promotes a high-affinity interaction, or both. The (des12-14)PKI(6-22)amide elision analog changes the spatial orientation of the two ends of the PKI molecule relative to one another, functionally producing a "frame shift" in the concerted binding of these regions to the protein kinase. The 210-fold increase in the *K_i* of the des12-14 analog relative to the parent peptide suggests that binding of the N-terminal Phe¹⁰ determinant to the enzyme has been prevented, although the α-helix of this region

appears to have been maintained. This loss of potency is, in fact, quite similar to that observed (266-fold increase) when Phe¹⁰ is simply replaced by alanine in the full length PKI(6-22)amide (Glass et al., 1989b). Both the inhibitory potency and the relative spacing of the N- and C-terminal binding determinants of the inhibitor peptide were rather well restored in the (Acy¹²,des13,14)PKI(6-22)amide analog. Further, the extreme similarity of the FTIR spectra of (des12-14)PKI(6-22)amide and of (Acy¹²,des13,14)-PKI(6-22)amide specifically demonstrates that the difference in inhibitory activities between these two analogs is not due to a variation in the overall conformations of these peptides. The aminocaprylic acid-containing peptide, which has few main-chain and no side-chain functional groups that correspond to residues 12-15, and both the βAla¹⁴ and Sar¹⁴ analogs, which contain nearly all of the functional groups of the parent inhibitor, exhibited quite similar potencies (all *K_i* values ~20 nM). This suggests that the center of the peptide is more important for correct spacing than for providing functional groups for specific intermolecular bonding to the catalytic subunit. Thus, the major role of -Ala¹²-Ser-Gly¹⁴- appears to be providing the proper spatial orientation of the N- and C-terminal segments of PKI(6-22)amide relative to one another to allow an optimal interaction of both of these regions with their recognition sites on the protein kinase.

This conclusion of a highly fixed topography between the Phe¹⁰ side chain and the binding determinants within the C-terminal pseudosubstrate region is consistent with the enzyme-bound conformation of the PKI peptide seen in the crystal structure of the catalytic subunit · inhibitor peptide complex (Knighton et al., 1991b, 1993; Bossemeyer et al., 1993). As pointed out by these authors, the peptide inhibitor is bound in a rather extended conformation and covers a large portion of the enzyme surface. Figure 8 depicts the bound form of PKI(6-22)amide. The N-terminal amphiphilic α-helix that contains Phe¹⁰ is recognized by a hydrophobic pocket on the surface of the enzyme, whereas the pseudosubstrate portion of the inhibitor is bound primarily by specific electrostatic contacts involving the Arg¹⁵,

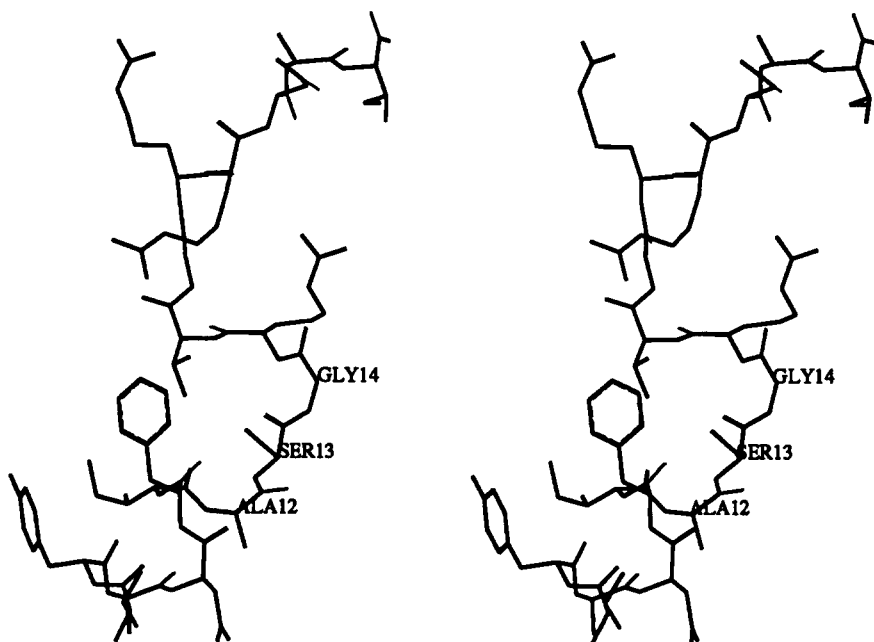


Fig. 8. Stereo diagram of the enzyme-bound structure of PKI(6-22)amide with the central connecting sequence -Ala¹²-Ser-Gly¹⁴- highlighted. Atomic coordinates are from Knighton et al. (1993) (Protein Data Bank entry 1APM). View is from solvent with catalytic subunit (not shown) located essentially behind the peptide. Residues 5, 23, and 24 were removed from the structure of PKI(5-24) in the PDB file.

Arg¹⁸, and Arg¹⁹ guanido groups (Gibbs & Zoller, 1991), which are located approximately 10.2, 15.4, and 7.3 Å, respectively, away from the phenyl ring determinant of Phe¹⁰ (center of Phe ring to Arg C ζ). The essential conformational relationship between the Phe¹⁰ side chain and catalytic subunit has also been evident from our previous studies, which altered the structure of this residue in the PKI peptide by replacement with nonstandard amino acids (Glass et al., 1989b). Clearly, a critical spatial relationship exists among the recognition determinants in PKI(6-22)amide in order to allow optimal binding to the catalytic subunit. This required topography explains why many of the PKI peptide analogs explored in this study exhibit decreased inhibitory potencies.

Promotion of a formal β -turn structure at residues 12-15 in the central connecting region of PKI(6-22)amide actually appears to be detrimental to tight binding of the peptide to the protein kinase, as evidenced by the poor inhibitory activities of both the (Asn¹²,Pro¹³)PKI(6-22)amide and (Pro¹³,Aib¹⁴)PKI(6-22)amide analogs. Indeed, such a turn conformation is inconsistent with the structure of the enzyme-bound peptide, as the -Ala¹²-Ser-Gly-Arg¹⁵- sequence terminates the α -helix in an extended structure and does not assume a β -turn (Knighton et al., 1991b, 1993; Bossemeyer et al., 1993). However, as shown in Figure 8, the central connecting region of bound PKI(6-22)amide does contain one turn of a distorted right-handed 3_{10} -helix (or type III reverse turn) that is located in the PKI peptide slightly C-terminal to the area investigated in the present experiments. This turn of distorted 3_{10} -helix involves the sequence -Gly¹⁴-Arg-Thr-Gly¹⁷-, which is extended such that the amide hydrogen of Gly¹⁷ does not formally hydrogen bond to the carbonyl oxygen of Gly¹⁴. None of the amino acid substitutions in the PKI peptide analogs, including the aminocaproic acid derivative, removed the carbonyl oxygen corresponding to that of Gly¹⁴, so all the of the peptides in this study still had the potential (based on this criteria only) to form this turn of 3_{10} -helix. However, the analogs containing Aib, Pca, or Aba at position-

14 may present different steric or electronic constraints to the facile formation of this structure. A probable cause of the altered binding affinities of some peptide analogs is that the β -turn promoting or disrupting structures substituted into the -Ala¹²-Ser-Gly¹⁴- sequence adversely influence the orientation of the side chain of the adjacent Arg¹⁵ residue (within the distorted 3_{10} -helix), which is known to be an important determinant of high-affinity interaction with the catalytic subunit (Cheng et al., 1986; Scott et al., 1986). In this regard, the hydrogen bond between the γ oxygen of Ser¹³ and the amide proton of Arg¹⁵ in PKI(6-22)amide would not be possible in several of the PKI analogs.

The potential consequences of several amino acid substitutions in PKI(6-22)amide can be deduced from the crystal structure of the enzyme-bound inhibitor (Knighton et al., 1991b, 1993; Bossemeyer et al., 1993). In general, the -Ala¹²-Ser-Gly¹⁴- portion of the peptide inhibitor makes few direct contacts with the protein kinase (Fig. 8). The conversion of Gly¹⁴ to DAla in the potent analog, (DAla¹⁴)PKI(6-22)amide, is easily accommodated because the methyl group of DAla¹⁴ that replaces the pro-R C α proton projects out into the solvent without any steric hindrance. Likewise, the *N*-methyl substitution in the (Sar¹⁴)PKI(6-22)amide analog, which retained reasonable inhibitory activity, also points toward solvent (if rotation of the side chain of Asp²⁴¹ on the enzyme surface is allowed). On the other hand, two of the poorest inhibitors were (Asn¹²,Pro¹³)PKI(6-22)amide and (Pro¹³,Aib¹⁴)PKI(6-22)amide, which both contain a proline substitution for Ser¹³. Inspection of the bound conformation of the PKI peptide suggests a possible reason for the low activities of these proline-containing analogs. The amide proton of Ser¹³ is hydrogen bonded to the carbonyl oxygen of Asp⁹ as well as, across the top of the distorted α -helix, to that of Phe¹⁰. These stabilizing hydrogen bonds would be prevented by proline, which cannot donate a proton. However, the ϕ angle of Ser¹³ of the PKI peptide in the crystal structure is -73° , which is close to the restricted ϕ an-

gle of a prolyl residue of about -60° . Also, the aminoisobutyric acid residue in (Pro¹³,Aib¹⁴)PKI(6-22)amide is constrained to ϕ and ψ angles in the range of -50° and -50° (or $+50^\circ$ and $+50^\circ$), respectively (Prasad & Balaram, 1984), whereas the corresponding angles for Gly¹⁴ in the enzyme-bound parent peptide are -81° and $+1^\circ$. Additionally, the pro-S methyl group of Aib¹⁴, which replaces Gly¹⁴ in this PKI peptide analog, would collide with the main-chain and side-chain atoms of Asp²⁴¹ in the catalytic subunit.

The FTIR spectra indicate that PKI(6-22)amide and other peptide analogs classified as exhibiting type I spectra (Table 3) contain significant extended chain conformation, turn or bend structure, and α -helix, as well as some random coil. The removal of turn structure(s) in PKI(6-22)amide by various amino acid substitutions significantly increases the random coil content of the resulting analogs, giving rise to the class II spectra. FTIR spectroscopy measures only the time and ensemble average structure of a peptide, and these hydrated peptides are likely to show great flexibility, giving rise to the random coil component. This flexibility would be expected to increase upon destabilization of any β -turn structure that otherwise tended to promote more ordered peptide conformations. Thus, the FTIR data indicate that the attempts to promote, retard, or remove β -turn structure from PKI(6-22)amide in solution by making specific sequence substitutions in the connecting region were generally successful. The apparent exception to this was (Asn¹²,Pro¹³)PKI(6-22)amide, which for unknown reasons seemed to exhibit less, rather than more, turn structure than the parent peptide.

There appears to be little correlation between the solution structures of the PKI peptides, as characterized by FTIR spectroscopy (class I or II spectra), and their K_i values. The apparent α -helical content changed little for any of the substituted peptides, hence large variations in K_i cannot be correlated to α -helical structure itself. The presence or absence of spectral features characteristic of turn or bend components appears to be equally unrelated to the potency of inhibition. Hence, it is probable that each of the PKI peptides only spends part of its time in a conformation that is optimal for recognition by the protein kinase. Thus, the weaker binding constants of some of the PKI peptides, particularly those that might populate β -turn conformations, probably reflect the additional energy needed to convert their solution structures to more extended forms required for interaction with the enzyme. The unfavorable entropy change for initially binding the peptide to the protein kinase will be larger for a constrained peptide whose conformation must be altered in the binding process (Page, 1977). Alternately, the initial affinity of the enzyme for an inhibitor peptide might be dominated by a few critical side-chain interactions (probably electrostatic attractions) that can be accommodated by multiple peptide conformations. The subsequent rearrangement of PKI(6-22)amide on the catalytic subunit of the protein kinase would then stabilize the final bound peptide conformation relative to that of its solution structures.

The watershed marked by the determination of the crystal structure of the catalytic subunit (Knighton et al., 1991a, 1991b) in conjunction with other structure-function studies of the PKI peptide (Glass et al., 1989a, 1989b; Gibbs & Zoller, 1991) and the present investigations have provided a detailed understanding of the essential recognition domains and their geometries that must exist at the active site and extended regions of the cAMP-dependent protein kinase for optimal binding of peptide and

protein substrates and inhibitors. This information should now allow design of improved peptide and even peptide mimetic inhibitors. Some of the synthetic peptides employed in this study, particularly the (dAla¹⁴)PKI(6-22)amide derivative and the analog containing the hydrophobic aminocaprylic acid residue substituted for -Ala¹²-Ser-Gly¹⁴-, will serve as starting points for development of PKI peptides that are more resistant to enzymatic degradation than the parent peptide, yet retain a high degree of inhibitory activity and selectivity toward the catalytic subunit. Such compounds will be most useful for experimental analysis of physiological functions mediated by the cAMP-dependent protein kinase (Walsh & Glass, 1991).

Materials and methods

Peptide synthesis and characterization

PKI(6-22)amide and its analogs were synthesized as C-terminal amides on *p*-methylbenzhydrylamine resins using an ABI 430A synthesizer (Glass et al., 1989a, 1989b, 1991). Boc-derivatives of dAla, Sar, and β Ala were from Peninsula Laboratories, and those of Aib, Acy, Aba, and Pca were from OmniBiochem. Boc-derivatives of Acy, Aba, and Pca were coupled as their HOBt active esters, whereas the other unusual Boc-amino acids were coupled as preformed symmetrical anhydrides. Couplings were conducted with custom-written synthesizer cycles appropriate for the solubilities and activation rates of each nonstandard Boc-amino acid. Completed peptidyl-resins were cleaved and deprotected by standard anhydrous HF:anisole (10:1) treatment at 0° . Crude peptides were desalted and purified to 97-99% apparent purity by semipreparative RP-HPLC (Glass et al., 1991). All peptides except the (Pro¹³,Aib¹⁴), (Aba¹⁴), and (Acy¹²,des13,14) analogs eluted with similar retention times to that of the parent PKI(6-22)amide, which was 20.1 min in the analytical HPLC gradient described by Glass et al. (1991). Those three peptides eluted 0.5, 0.7, and 2.1 min, respectively, later than the parent inhibitor, reflecting their greater degrees of hydrophobicity. The UV spectrum of (Aba¹⁴)PKI(6-22)amide was characteristic for *p*-aminobenzoic acid. Concentrations of stock solutions of each peptide were determined by quantitative amino acid analysis (Heinrikson & Meredith, 1984).

Enzyme purification

Homogeneous catalytic subunit of cAMP-dependent protein kinase (type II) was prepared from bovine heart (Bechtel et al., 1977). This preparation is predominantly composed of the C α isoform of the enzyme (Showers & Maurer, 1986). Enzyme activity in the absence of inhibitor peptide averaged $17.3 \pm 0.9 \mu\text{mol}/\text{min}/\text{mg}$ in different experiments under assay conditions described below.

Phosphotransferase assays

Protein kinase activity was titrated with increasing concentrations of PKI(6-22)amide analogs. IC₅₀ values of the inhibitor peptides were used to calculate their K_i values by the method of Cheng and Prusoff (1973). Assays were conducted for 10 min at 30° in 30 mM MES, pH 6.9, 5 mM Mg(acetate)₂, 125 μM [γ -³²P]ATP (~150 cpm/pmol), 22.5 μM Kemptide, 0.03 nM to 100 μM of inhibitor peptide as indicated, 3.75 mM 2-mercapto-

ethanol, 0.4 mg/mL BSA, and 0.05 to 0.1 $\mu\text{g/mL}$ (1.2–2.5 nM) catalytic subunit. Phosphorylated Kemptide was isolated and quantitated on phosphocellulose papers (Roskoski, 1983).

For direct determination of K_i values, the above conditions were changed to 8 μM [γ - ^{32}P]ATP (450–1,000 cpm/pmol) as fixed substrate, with 1, 2, 4, 8, and 16 μM concentrations of Kemptide, and inhibitor peptide concentrations of either 1–6-fold or 10–40-fold greater than their estimated K_i values, depending on the type of kinetic analysis performed. These assays were conducted for 2 min and were also terminated by the phosphocellulose paper method.

Inhibition constants

Inhibition constants of PKI peptides were determined by one of two procedures depending on their potencies. For peptides with lower potencies ($K_i \geq 50$ –100 nM), kinetics were determined by standard double-reciprocal plots of initial velocity data. For inhibitor peptides with high affinities ($K_i < 50$ nM), inhibition constants were analyzed according to Henderson (1972). K_i values were determined from suitable replots of each analysis. These K_i values for the peptides agreed quite well with inhibitory constants calculated from the independently determined IC_{50} data of titration assays.

CD spectroscopy

CD spectra of PKI(6–22)amide and its analogs were collected on an AVIV model 62DS spectrometer at 25° under an N_2 atmosphere in 1.0-mm-pathlength fused quartz cuvettes. Peptides were dissolved in water at a final concentration of 75 μM (~0.14 mg/mL). Spectra were measured from 240 to 190 nm in 0.5-nm scan steps with a time constant of 1.0 s, a constant bandwidth of 1.0 nm, and were signal averaged at least five times. A signal-averaged baseline of water was subtracted from all digitalized spectra, which were then smoothed by a least-squares polynomial fitting algorithm supplied by the instrument manufacturer. Data are expressed as mean residue ellipticity in $\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. (Acyl¹²,des13,14)PKI(6–22)amide was considered to contain 15 residues in calculating its mean residue ellipticity.

FTIR spectroscopy

FTIR spectra were measured using a Perkin-Elmer solution cell with CaF_2 windows and a Mattson (Alpha Centauri) FTIR spectrometer. Details of the data acquisition are described in Trehwella et al. (1989). Spectra were recorded at 2 cm^{-1} resolution with 1,024 scans. Peptide solutions were prepared in D_2O at a concentration of 10 mg/mL as described by Reed et al. (1989). The FTIR spectra were analyzed using spectral subtraction, Fourier deconvolution, and second-derivative analysis as described by Trehwella et al. (1989).

Acknowledgments

This work was supported by National Institutes of Health grants GM28144 and RR05364 (to D.B.G.), AM21019 (to D.A.W.), and GM40528 (to J.T.). We thank Drs. Daniel Knighton, Susan Taylor, and Janusz Sowadski for providing coordinate files, and we gratefully acknowledge the technical assistance of Lucy Lundquist and Kevin Liddle.

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