

Circular Dichroic Investigations of Secondary Structure in Synthetic Peptide Inhibitors of cAMP-Dependent Protein Kinase: A Model for Inhibitory Potential[†]

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ABSTRACT: The structure of the inhibitory domain of the inhibitor protein of the cAMP-dependent protein kinase has been assessed by circular dichroism studies of synthetic inhibitory peptides. Using the inhibitory peptide PKI(5-22)amide (Thr⁵-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile²²) [Cheng, H.-C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M., & Walsh, D. A. (1986) *J. Biol. Chem.* 261, 989-992] and shorter peptides of this sequence, it has been estimated that this parent peptide is composed of approximately 30% α -helix with the remainder being random coil with one β -turn. The pseudosubstrate arginine cluster (Arg¹⁵-Arg¹⁹) is within the suggested region of random coil and β -turn, representing one critical region of binding recognition by the protein kinase. The α -helix region proposed between Thr⁶ and Ile¹¹ likewise contributes to the full biological potency and specificity of the inhibitor peptide and inhibitor protein. The removal of the two N-terminal threonines, for example, causes both a marked conformational change in the peptide and a diminishment by an order of magnitude of inhibitory activity. It is proposed that this α -helix region could serve one of several possibilities, including that it may provide a suitable constraint on the Tyr⁷ such that the hydroxyl is oriented in a position proximal to the pseudosubstrate domain, and/or may allow the optimal location of other protein kinase recognition signals. These data provide an initial description of some of the structural features of the inhibitor protein that could contribute to its high biological potency.

Circular dichroism and other studies that have examined the interaction between the catalytic subunit of the cAMP-dependent protein kinase and its peptide and protein substrates have suggested that ligand binding takes place in a series of three steps, each of which is dependent on a particular recognition signal offered by these substrates (Reed & Kinzel, 1984a,b; Reed et al., 1985). An initial response, based upon the electrostatic binding of the basic subsite region of the substrate (Zetterquist et al., 1976; Kemp et al., 1977), is followed by one dependent on the substrate's having, or being able to assume, the extended coil form in the binding region (Granot et al., 1981; Rosevear et al., 1984). A third conformational change, involving a possible rotation of a tyrosine residue at the ATP binding site, appears dependent on the presence of a properly oriented hydroxyl group on the target residue for phosphorylation (Reed & Kinzel, 1984b). Kinetic studies of peptide substrates and inhibitors support this conclusion (Whitehouse et al., 1983), and there is evidence that this occurs with the ATP being in or assuming a meta phosphate intermediary state (Granot et al., 1980a,b). A peptide which possesses two of the three signals but not the third, i.e., the Ala-peptide derivative of Kemptide (Leu-Arg-Arg-Ala-Ala-Leu-Gly), functions as a competitive inhibitor. It does not function particularly well in this capacity, having a K_i (0.32 mM; Whitehouse et al., 1983) which is well above the K_m of the equivalent substrate, Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly; K_m = 4.7 μ M; Whitehouse et al., 1983) and several orders of magnitude higher than the K_i for the natural inhibitor, the heat-stable inhibitor protein (PKI) (K_i = 0.5-2.0

nM; Demaille et al., 1977; Whitehouse & Walsh, 1983). It is tempting to consider that one component of the effectiveness of PKI might lie in its ability to mimic the third of the substrate/binding site interactions, namely, the conformational change in the enzyme that is dependent on the presence and correct orientation of a ligand hydroxyl group (Reed & Kinzel, 1984b; Whitehouse et al., 1983). Recently, the full sequence of PKI has been elucidated (Scott et al., 1985a), the domain containing the site of interaction with the protein kinase has been isolated as a 20 amino acid peptide (Cheng et al., 1985; Scott et al., 1985b), and a series of peptide analogues of this domain have been synthesized (Cheng et al., 1986, 1987; Scott et al., 1985b, 1986). There are clearly several of the amino acid constituents which are essential for full inhibitory potency. Notably, it was demonstrated that in addition to containing the basic subsite, PKI also contains other critical recognition or structural features on the N-terminal side of the arginine cluster. It does not, however, contain a strategically located hydroxyl on the C-terminal side of the basic subsite. Kinetic studies have shown that one of these peptides, PKI(5-22)-amide¹ (Thr⁵-Thr-Tyr⁷-Ala-Asp-Phe¹⁰-Ile-Ala-Ser-Gly¹⁴-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile²²), has a K_i (3.1 nM) comparable to that of the original inhibitor protein. Removal of the two threonine residues from the N-terminus results in an increase of nearly an order of magnitude in the K_i (27 nM), and additional deletions degrade the inhibitory capacity even further. This series of peptides has provided a suitable system for studying the structural basis for the unusual inhibitory potency of PKI.

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¹ Since our initial description of the inhibitory peptides derived from the inhibitor protein (Cheng et al., 1985, 1986), the nomenclature of these peptides has been changed to reflect the sequence of the protein deduced by Scott et al. (1985a). The peptides are now named based upon numbered amino acids in the full sequence of the protein and that they were synthesized as the C-terminal amide.

Table I: Secondary Structure Probabilities of the Active Domain of the cAMP-Dependent Protein Kinase Inhibitor Protein^a

	5	7	8	10	12	14	21	22	24	
	----Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp----									
α -Helix	i	i	b	H	I	h	h	H	i	B
β -Sheet	h	h	H	i	B	h	H	i	b	b
β -Turn						+	+			

^a The sequence of PKI is from the data of Scott et al. (1985a) with amino acids numbered from the N-terminus of the native protein. The peptide nomenclature refers to these numbered amino acids. Predictions of structure conformation from Chou and Fasman (1978) with H = strong α -helix or β -sheet former, h = moderate α -helix or β -sheet former, I = weak α -helix or β -sheet former, i = indifferent, b = moderate α -helix or β -sheet blocker, B = strong α -helix or β -sheet blocker, and + = favored β -turn.

MATERIALS AND METHODS

The peptides PKI(5-22)amide, PKI(7-22)amide, PKI(10-24)amide (FIASGRTGRRNAIHD), and PKI(14-24)amide, whose sequences are based upon the active domain of PKI (Scott et al., 1985a,b; Cheng et al., 1985, 1986), were synthesized by solid-phase synthesis as COOH-terminal amides and donated by Dr. B. E. Kemp of the University of Melbourne, Australia. (See also Table I for the sequences of these peptides.) The peptides were purified as described by Cheng et al. (1986), and their purity was confirmed by high-performance liquid chromatography (HPLC), amino acid analysis, and sequencing (Cheng et al., 1985, 1986). The K_i values of these peptides for the cAMP-dependent protein kinase, as determined by Henderson analysis (Henderson, 1972) and as reported previously (Cheng et al., 1986, 1987), are as follows: PKI(5-22)amide, 3.1 nM; PKI(7-22)amide, 27.5 nM; PKI(10-24)amide, 73 nM; PKI(14-24)amide, 57 nM.

Circular dichroic (CD) spectra were obtained by using a Jasco J-500 automatic recording spectropolarimeter coupled to a Jasco J-DPY data processor. A constant dispersion of 1.0 nm was maintained by automatic slit width control. Far-UV spectra were measured from 190 to 240 nm with a sensitivity of 2.0 mdeg/cm, a time constant of 1.0 s, a scan speed of 5 nm/min, and a wavelength expansion of 5 nm/cm and were signal-averaged 8 times. The spectra were measured at a sample concentration of 0.5 mg/mL in a 0.2-mm cuvette. Near-UV CD spectra were taken from 250 to 300 nm with a sensitivity of 0.5 mdeg/cm, a time constant of 2.0 s, a scan speed of 10 nm/min, and a wavelength expansion of 5 nm/cm and were signal-averaged 32 times. A 1.0-cm cuvette was used for near-UV measurements. All spectra were digitized and displayed with a similarly measured and signal-averaged base line subtracted. Comparison standard CD spectra for the proportional composition of α -helix, β -sheet, and random coil were generated as described by Greenfield and Fasman (1969). Included with these were the β -bend structures as proposed by Crisma et al. (1984) from studies of model peptides, described as type I (Z-Aib-L-Pro-Aib-L-Pro-OMe in cyclohexane), type II (Piv-L-Pro-Aib-NH-Me in methanol), type III/I (Z-Aib-L-Pro-Aib-L-Pro-OMe in methanol or methanol/H₂O), type V (Piv-L-Pro-Aib-NH-Me in cyclohexane), "mixed" type I (Piv-L-Pro-L-Val-NH-Me in cyclohexane), and "heterogeneous" (Piv-L-Pro-L-Val-NH-Me in methanol).

RESULTS

Far-UV CD. The far-UV CD spectra of the four synthetic inhibitor peptides are shown in Figure 1. The spectrum of PKI(5-22)amide (Panel A) is not typical of a peptide adopting extended coil conformation in solution (Greenfield & Fasman, 1969; Aebersold & Pysh, 1970; Alder et al., 1973). It differs from a standard extended coil spectrum at a number of points, notably the following:

(1) The CD curve of model peptides known to exist as extended coil (Aebersold & Pysh, 1970) (and also of Kemp-

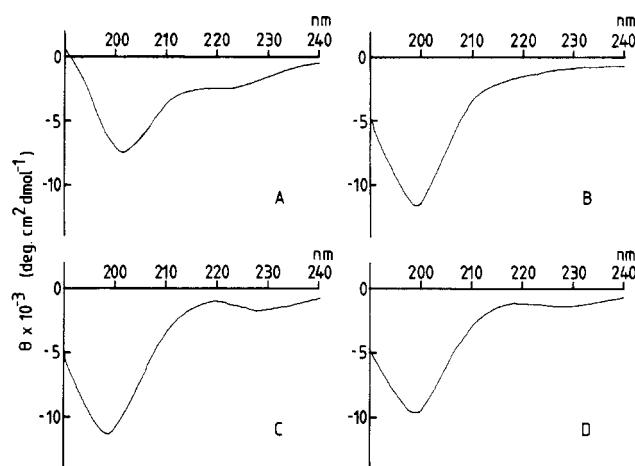


FIGURE 1: Far-UV CD of synthetic inhibitory peptides. (A) PKI(5-22)amide; (B) PKI(7-22)amide; (C) PKI(10-24)amide; (D) PKI(14-24)amide.

tide, the model substrate of the cAMP-dependent protein kinase; Reed et al., 1985) displays no ellipticity at 240 nm and rises slowly to a positive maximum at around 215 nm. The spectrum of PKI(5-22)amide is slightly negative at 240 nm and becomes progressively more so at shorter wavelengths.

(2) A typical extended coil spectrum has a positive maximum between 215 and 220 nm. Although the curve for PKI(5-22)amide has a shoulder which might indicate a weak positive Cotton effect at this wavelength, the spectrum never enters the positive region.

(3) The negative ellipticity maximum in an extended coil spectrum is typically at 198 nm. For PKI(5-22)amide, the corresponding negative band is at 202 nm.

(4) Although an extended coil spectrum may rise after the 198-nm negative, the absolute ellipticity at 190 nm is still negative. The spectrum of PKI(5-22)amide rises rapidly after the 202-nm minimum and is actually slightly positive by 190 nm.

CD spectroscopy in the far-UV region has been shown to provide a reliable method for the estimate of the α -helix content of peptides and proteins (Siegel et al., 1980; Hennessey & Johnson, 1981; Gusev & Barskaya, 1984). If the CD spectrum of PKI(5-22)amide is compared with the curves calculated for mixtures of secondary structure (Greenfield & Fasman, 1969), it corresponds quite closely to that expected from a theoretical content of 30% α -helix plus 70% extended coil (Figure 2A). The inclusion of this much α -helical structure can reproduce certain features of the spectrum, such as the absolute negative ellipticity from 240 to 193 nm and the rise toward the positive at 190 nm as well as some of the red shift of the negative band. The presence of aromatic side chains can result in Cotton effects in the 190-240-nm region affecting the interpretation of secondary structure contribution. These, in general, result in too low an estimate of the amount of structure present (Alder et al., 1973). As discussed in more

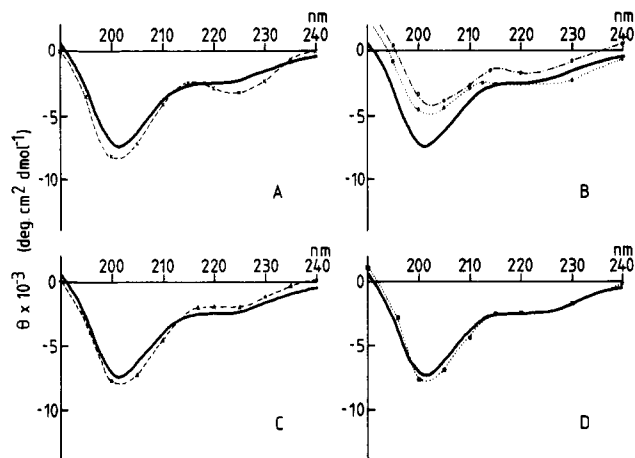


FIGURE 2: Curve fitting of PKI(5-22)amide far-UV CD. In all panels, the experimental CD spectrum of PKI(5-22)amide is denoted by (—). (A) (X) 70% random coil/30% α -helix. (B) (O---O) 70% random coil/30% α -helix, one type II β -turn; (●---●) 70% random coil/30% α -helix, one type V β -turn. (C) (▲) 70% random coil/30% α -helix, one type I β -turn. (D) (□) 70% random coil/30% α -helix, one type III/I β -turn.

detail below, however, the estimate of the amount of α -helix present in PKI(5-22)amide corresponds quite well with the maximum number of likely α -helix-forming residues. Some differences between the CD data of PKI(5-22)amide and the calculated spectra in Figure 2A can, however, be noted. The calculated 30%/70% curve does not actually enter the positive region at 192 nm as does that of PKI(5-22)amide. Further, as illustrated in the data of Greenfield and Fasman (1969), there is a palpable positive deflection at 215–220 nm in all the calculated curves generated by a mixture of α -helix plus extended coil, even in those containing a high level of α -helix. Thus, the comparatively weak influence in the PKI(5-22)-amide spectrum of the 215–220-nm positive band normally associated with extended coil structure cannot entirely be accounted for by a model containing α -helix and random coil alone.

To help account for these differences, and given that in a peptide of so short a length as PKI(5-22)amide there can be a maximum of only a limited number of secondary structure possibilities, we have explored the inclusion of a single additional feature of structure in addition to the α -helical content. Since negative CD bands in the region from 201 to 220 nm are a feature of a number of β -bend conformations (Woody, 1974; Crisma et al., 1984), it was considered that the structure of PKI(5-22)amide in solution may contain a β -bend. This possibility would be supported by the application of folding algorithms to the primary structure of PKI(5-22)amide (Table I). Given that several apparent types of β -bend structure exist (Woody, 1974) with differing features of CD spectra (Crisma et al., 1984), these were explored to examine the best fit. Figure 2, panels B–D, gives examples of the types of curves generated if one includes a single four-residue β -bend in the calculated CD spectrum. Of these, the CD spectra with the inclusion of an estimated type II or V bend definitely do not improve the fit; however, when that of either a type I or a type III/I β -bend is included, the pure 30% α -helix/70% extended coil curve shifts to a very close approximation of the CD spectrum of PKI(5-22)amide. (Minor differences in the absolute intensity of the peaks would be expected in comparing model peptides to one with different side chains.) The curve generated by addition of a single type III/I bend even reproduces the slight rise into the positive at 190 nm exhibited by PKI(5-22)amide, and both curves result in a “flattening”

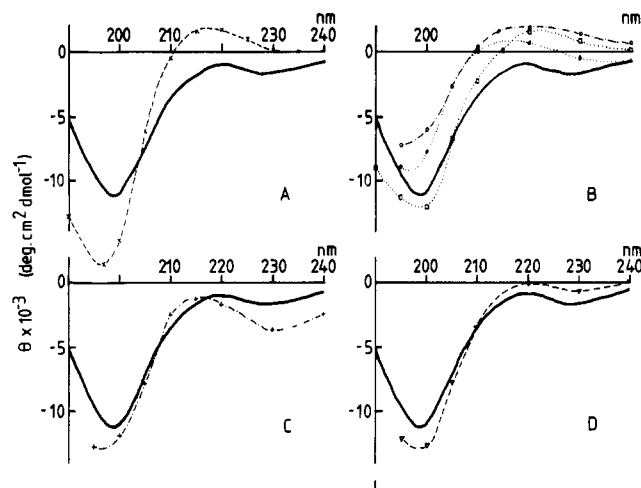


FIGURE 3: Curve fitting of PKI(10-24)amide far-UV CD. In all panels, the experimental CD spectrum of PKI(10-24)amide is denoted by (—). (A) (X) 100% random coil. (B) (□) Random coil plus one type I/III β -turn; (O---O) random coil plus one type II β -turn; (●---●) random coil plus one type V β -turn. (C) (+) Random coil plus one “mixed” type I β -turn. (D) (▽) Random coil plus one “heterogeneous” β -turn.

of the effect of the 215-nm positive band generated by the extended coil component. The inclusion of a single β -bend within the predicted structure thus appears reasonable both from the CD spectra and as predicted by the sequence; which of all of the possible types of β -bend is present still needs to be elaborated. There was no combination of β -sheet structure, extended coil, and β -turn that gave anything approximating a fit to the PKI(5-22)amide CD spectrum.

The CD spectra of PKI(10-24)amide (Figure 1C) and PKI(14-24)amide (Figure 1D) are very similar and more nearly approximate a pure extended coil curve (Greenfield & Fasman, 1969; Aebersold & Pysh, 1970) than either of the other two inhibitory peptides. The tendency of the spectra of both to remain negative at longer wavelengths, however, suggests that some structural feature is present. The spectra in panels C and D of Figure 1 are, in fact, quite close to those calculated by Greenfield and Fasman (1969) for 80% extended coil plus 20% α -helix, but this is probably misleading. As seen in Table I, the primary sequence to the C-terminal side of Phe¹⁰ does not tend to favor the formation of either α -helix or β -sheet, and the probability strongly favors an extended coil conformation. The possibility of a β -bend in the sequence of either PKI(10-24)amide or PKI(14-24)amide does exist, and Figure 3 shows attempts to fit the CD spectrum of PKI(10-24)amide by including a single β -bend in an otherwise totally extended coil peptide. The dichroism associated with bends identified as type II, type V, and type III/I did not correct for the departure of the PKI(10-24)amide curve from an extended coil spectrum (Figure 3B). The type of β -bend spectrum exhibited in the studies of Crisma et al. (1984) by the model peptide Piv-L-Pro-L-Val-NH-Me in cyclohexane and especially in methanol, when included with that of the random coil (panels C and D, respectively, of Figure 3), does result in a fair correspondence to the spectra obtained for PKI(10-24)amide [and also for PKI(14-24)amide, see Figure 1D]. The spectrum of Piv-L-Pro-L-Val-NH-Me was interpreted as arising from a mixed population of β -bends. The CD data would thus be compatible with these two peptides containing a single β -bend.

The spectrum of PKI(7-22)amide (Figure 1B) requires special attention. Although differing from PKI(5-22)amide by only the removal of the two N-terminal threonines, as

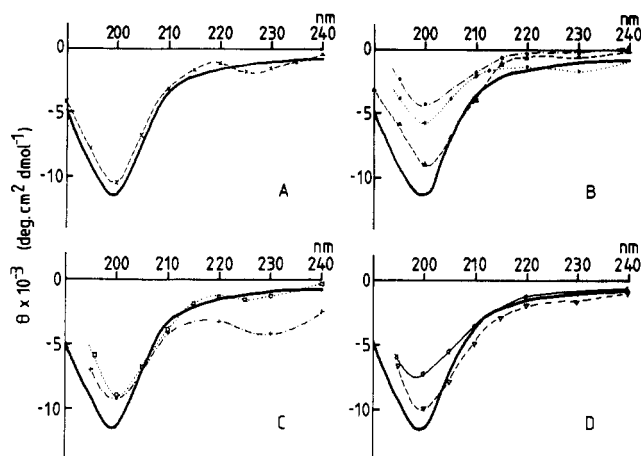


FIGURE 4: Curve fitting of PKI(7-22)amide far-UV CD. In all panels, the experimental CD spectrum of PKI(7-22)amide is denoted by (—). (A) (×) 80% random coil/20% α -helix. (B) (▲) 80% random coil/20% α -helix, one type I β -turn; (○---○) 80% random coil/20% α -helix, one type II β -turn; (●---●) 80% random coil/20% α -helix, one type V β -turn. (C) (+) 80% random coil/20% α -helix, one "mixed" type I β -turn; (□) 80% random coil/20% α -helix, one type III/I β -turn. (D) (▽) 80% random coil/20% α -helix, one "heterogeneous" β -turn; (□) 90% random coil/10% α -helix, one "heterogeneous" β -turn.

reflected by the far-UV CD spectrum, there is a noticeable change in conformation. Since the N-terminal region contains a proportion of helix builders (Table I), fitting was attempted by using a theoretical 80% coil plus 20% α -helix curve modified by the addition of a single β -bend of various types (Figure 4). As shown, the inclusion of either the type I (Figure 4B) or the "heterogeneous" β -bend of the type present in the model peptide Piv-L-Val-NH-Me in methanol (Figure 4D) gives a fairly good qualitative fit. Superimposition of a single such bend on a 90% extended coil/10% helix base curve did not materially improve the fit (Figure 4D).

Near-UV CD. The CD spectra in the aromatic region (250–300 nm) of the four synthetic inhibitory peptides are given in Figure 5. With two of these, PKI(5-22)amide (panel A) and PKI(7-22)amide (panel B), there are contributions from both the single phenylalanine and tyrosine residues; PKI(10-24)amide (panel C) only contains a single phenylalanine, and PKI(14-24)amide (panel D) has no aromatic amino acids. Given these aromatic amino acid compositional differences, the interpretation of the spectra becomes relatively straightforward, and tyrosine- and phenylalanine-associated bands are readily distinguished.

(A) Phenylalanine. The negative minima at 268 and 262 nm in PKI(5-22)amide and PKI(7-22)amide are attributable to the 0-0 and 0 + 930 phenylalanine progression bands (Horwitz et al., 1969). These peaks either in phenylalanine itself or in *N*-acetyl-L-phenylalanine amide (which is more closely analogous to phenylalanine in a peptide) are strong and negative (Horwitz et al., 1969; measurements at 25 °C in methanol/glycerol, 9:1). Also, with either of these compounds, the 0 + 520 progression gives rise to weaker positive bands at 264.4 and 258.6 nm (Phe) and at 263.7 and 259 nm (*N*-Ac-Phe-amide), and equivalent positive peaks are evident in the spectra of the phenylalanine-containing peptides [PKI(5-22)amide, 265 and 258.5 nm; PKI(7-22)amide, 266 and 259.5 nm]. In the spectra of PKI(7-22)amide, although the position and sign of all of these phenylalanine bands are virtually the same as in PKI(5-22)amide, the entire area is more positive. This shift seems unlikely to be due to scattering since in the spectrum of PKI(14-24)amide the scattering never goes positive; the peptides also do not contain disulfide bonds which might cause a broad shift of this nature. It is, therefore, most

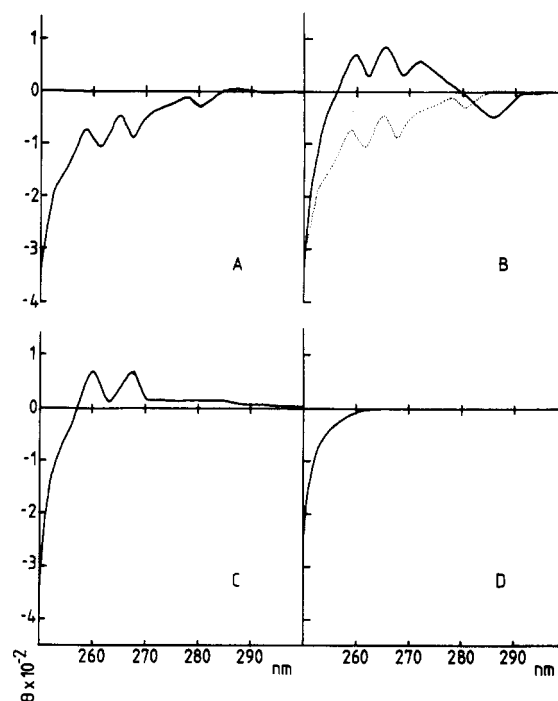


FIGURE 5: Aromatic region CD of synthetic inhibitory peptides. (A) PKI(5-22)amide; (B) PKI(7-22)amide; (C) PKI(10-24)amide; (D) PKI(14-24)amide. In (B), (---) is the spectrum of PKI(5-22)amide for comparison.

likely that the changes in this region reflect a change in the environment of the phenylalanine in the two peptides, prompted by the removal of the two N-terminal threonines, even though the phenylalanine is several amino acids removed from the N-terminus.

(B) Tyrosine. The only well-resolved tyrosine band in PKI(5-22)amide (Figure 5A) is a negative ellipticity at 281 nm corresponding to the initial member of the 0-0 1L_b progression. Its position at 281 nm is characteristic of tyrosine in a hydrophilic environment (Horwitz et al., 1970). Removal of the two N-terminal threonines, so that tyrosine is now the N-terminal residue, might have been expected to lead to the tyrosine being even more exposed, causing the position of this tyrosine peak in PKI(7-22)amide either to be lower than 281 nm or, since this is already at the extreme blue-shift for a hydrophilic environment, to be unchanged. Instead, the peak is red-shifted to 285.5 nm. Loss of the N-terminal threonines thus appears to cause the tyrosine actually to be in a more hydrophobic environment, even though it is at the N-terminus.

DISCUSSION

The pitfalls inherent in using the CD spectra of peptides as a basis for the estimation of secondary structure in globular proteins are well-known and have been described in detail (Chang et al., 1978). In studying the structure of small peptides, however, use of the model peptide curves for α -helix, β -sheet, and random coil may offer an advantage over more sophisticated methods such as the CONTIN program (Provencher & Glockner, 1981) because corrections for large protein influences (interior vs. exterior location, domain interaction, heterogeneity of segment length, etc.) are unnecessary. Although in the case of proteins model peptide reference curves correlate less well with disordered than with some highly ordered structures, far-UV CD-derived estimates of peptide secondary structure can agree quite well with results from other physicochemical methods and have been used successfully in a number of cases (Blundell et al., 1982;

Holladay & Puett, 1976; Martenson et al., 1985; Stone et al., 1985). Prediction of α -helix from CD data, as is a primary result in this study, has appeared quite reliable (Siegel et al., 1980; Hennessey & Johnson, 1981).

Use of peptide models for estimation of the CD contribution from β -turns is more questionable and requires some discussion. A long history of attempts to calculate the CD spectra expected from various types of β -turn (Woody, 1974; Chang et al., 1978) and to derive model peptides embodying the characteristics of these turns to serve as standards (Urry et al., 1974; Zimmerman et al., 1982) has led to conflicting results (Toniolo, 1980; Smith & Pease, 1980; Rose et al., 1985). One of the most recent studies (Crisma et al., 1984) has concluded that while their reference spectra for type I and type II bends are fairly reliable, those for type III and V are tentative at this point and that some interconversion between forms may occur. Despite this, the type of CD exhibited by β -turns in general does differ from that of the other classes of structure, and we have taken the reference spectra provided by Crisma et al. (1984) as the most reliable and most suitable for the simple peptides studied here where most likely only a single β -bend would occur. With this in mind, we have used these spectra in curve fitting to fully correct the spectra obtained in designating the contribution made by the α -helical portion. While some information as to the type of β -bend is also suggested by these studies, these type identifications must be regarded with caution. Overall, however, the CD spectra do imply that each of these inhibitory peptides contains a β -bend structure of some type.

Application of theoretical folding algorithms (Chou & Fasman, 1978) to the primary structure of the inhibitory peptide(s) (Table I) indicates that, while the N-terminal half of PKI(5-22)amide, notably from Ala⁸ to Ser¹³, has areas that could form α -helix, the majority of the peptide, in particular the C-terminal half, most probably exists as random coil. In addition, there are two possible places where a β -turn is favored. This would tie in well with the structure deduced by fitting of the CD curve for PKI(5-22)amide. The five to six residues potentially involved in the α -helix make up 27%-33% of the peptide, and the CD curve is most closely approximated by a 70% random coil/30% α -helix curve containing a single β -bend. It is worth noting that the basic subsite of the inhibitory peptide that corresponds to the primary substrate recognition sequence (Arg-Arg-Asn-Ala, with Ala at the position Ser would occupy in a substrate) occurs in the C-terminal random coil half; a random or extended coil structure has been implicated in the second step of the substrate/binding site interaction (Granot et al., 1981; Rosevear et al., 1984; Reed et al., 1985). In this regard, a possible β -bend in this pseudosubstrate region of the inhibitory peptides appears to be in contrast to the work of Granot et al. (1981) and Rosevear et al. (1984). From their studies, it was deduced by NMR measurements that, when peptide substrates occupy the protein kinase catalytic site, they are present in an extended structure incompatible with the presence of a β -bend. Two possibilities could readily account for the apparent differences between those observations and the conclusions from the CD spectra that the free inhibitory peptides contain a β -bend in the region of the arginine cluster pseudosubstrate region. The first of these would be that the inhibitory peptides, upon binding to the protein kinase, are then induced into an extended conformation and occupy the catalytic site in a manner identical with peptide substrates (i.e., the β -bend upon peptide binding is no longer present because a modified conformation has been induced by interaction with the protein kinase). The second

possibility, and an attractive alternative, would be that the inhibitory peptides are in fact inhibitory because the β -bend causes them to bind to the protein kinase catalytic site in a manner that is different from that of substrates and to dissociate at a slower rate.

As has been emphasized by previous studies of inhibitory peptides of various lengths (Scott et al., 1985b, 1986; Cheng et al., 1986, 1987), PKI(5-22)amide contains at least two regions important for its potency, the first being the arginine cluster pseudosubstrate domain and the second being the N-terminal region between Thr⁶ and Ile¹¹. It is this latter region which is suggested by these studies to form an α -helix. As previously mentioned, the inhibitory ability of the PKI-(5-22)amide drops by an order of magnitude when the two N-terminal threonine residues are removed (Cheng et al., 1986, 1987). The CD spectra indicate that the loss of these two residues is accompanied by definite conformational changes:

- (1) The tyrosine residue appears to move into a more hydrophobic environment. The simultaneous change in the intensity of the phenylalanine CD bands suggests that this may come about through rotation of the tyrosine so as to align itself more closely with the phenylalanine.

- (2) The amount of helix in the peptide apparently drops although the main helix-forming residues are still present.

- (3) The CD spectrum of PKI(7-22)amide is also best fitted by including a β -turn, but in PKI(5-22)amide this bend may be fairly well-defined, and in PKI(7-22)amide the results are best interpreted by assuming a mixture of types is present.

PKI(10-24)amide and PKI(14-24)amide seem to have lost any remaining helix content, as indicated by the CD spectra and consistent with the folding algorithm, and contain a single, probably heterogeneous β -turn. Their K_i is increased 2-3-fold over PKI(7-22)amide. Overall there appears clearly to be a close association between the inhibitory potential of peptides as measured kinetically and their conformational properties as reflected by changes in the near- and far-UV CD spectra.

The location of an α -helical region at the N-terminal portion of the fully inhibitory peptides is of additional interest since from comparative studies of the effects of these peptides on the cAMP- and cGMP-dependent protein kinase (Glass et al., 1986), this region is one that dictates the high degree of specificity of PKI for the cAMP-dependent enzyme. Although all four of the inhibitory peptides studied here are orders of magnitude less inhibitory with the cGMP-dependent protein kinase, the shorter peptides were the most inhibitory, and those containing the α -helical region were less potent.

We conclude from these studies that a large part of the inhibitory potency of PKI(5-22)amide (and the inhibitor protein itself) lies in their secondary structure. The α -helical portion of the N-terminal end may well serve to maintain a particular orientation of the tyrosine residue, since as the estimated helical content drops, the tyrosine appears to follow a natural tendency to occupy a more nonpolar environment. Using molecular models, one can see that if only the region from Ala⁸ to Ala¹² is coiled in an α -helix, the tyrosine is held somewhat away from the phenylalanine but the flexibility allowed by the free N-terminal residues still allows it to approach the phenylalanine. If the N-terminal threonines participate in helix-type hydrogen bonding with backbone NH groups, the tyrosine is firmly fixed away from the phenylalanine. The attractive potential energy of aromatic/aromatic interactions has been estimated at -1 to -2 kcal/mol (Burley & Petsko, 1985). The binding energy of ca. 2-2.5 kcal/mol contributed by intrachain bonding of each of the two threonines (Nozaki & Tanford, 1971; Schultz & Schirmer, 1979) is

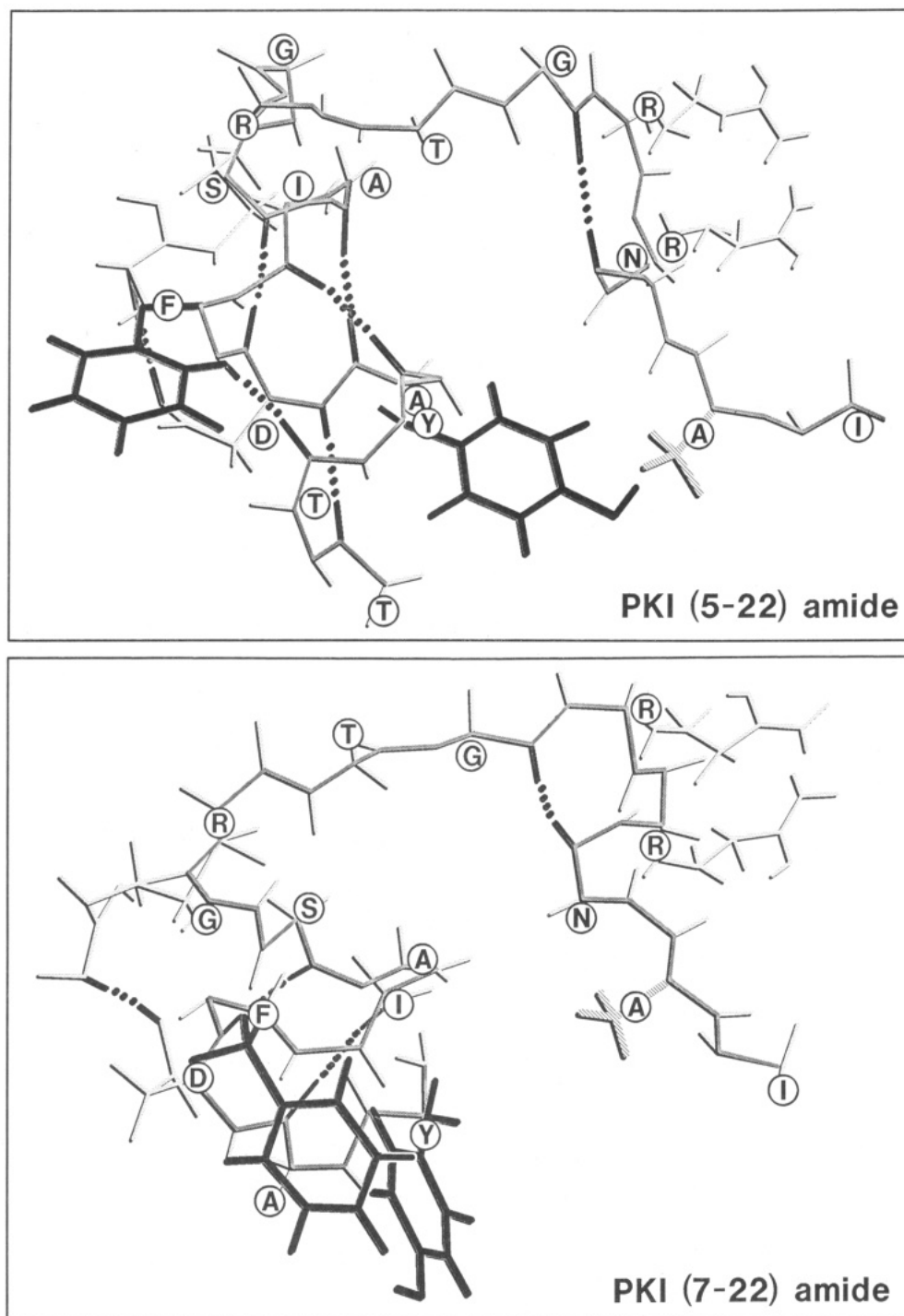


FIGURE 6: Molecular models proposed for PKI(5-22)amide and PKI(7-22)amide inhibitory peptides.

enough to offset this. Without the added stabilization of the N-terminal residues, the tendency for a tyrosine/phenylalanine interaction will dominate.

Models consistent with the above features of PKI(5-22)-amide and PKI(7-22)amide are shown in Figure 6. With PKI(5-22)amide in the indicated configuration, the tyrosine hydroxyl group can take up a position in the same space that would be occupied by the hydroxyl group of a target serine at Ala²¹. It is thus conceivable that it could trigger the third, substrate hydroxyl group dependent step in the enzyme's conformational response as suggested by our past studies (Reed & Kinzel, 1984b; Whitehouse et al., 1983). This may explain the close association between tyrosine orientation and K_i . Certainly, the tyrosine hydroxyl group per se (and/or its lo-

cation and/or its absence of charge) is important to the inhibitory process, since phosphorylation of this tyrosine in the native inhibitor protein by EGF receptor (Van Patten et al., 1987) increases the K_i by about an order of magnitude. If, when bound to the cAMP-dependent protein kinase, the inhibitor can "fool" the enzyme into accepting it as a bona fide substrate without offering a suitable phosphorylatable residue to trigger the release mechanism, the remarkable efficacy of the natural inhibitor would be at least partly explained. The tyrosine is not necessarily fixed in this position, and rotation around the C_α-C' bond would remove it from the critical area, consistent with the ultimate reversibility of PKI inhibition. Studies testing this model are in the process of being carried out. Suitably locating the tyrosine residue is one possible role

for an α -helical region of the inhibitory peptides and PKI. This does not, however, exclude that the structure of the α -helix might serve to correctly orient other protein kinase recognition sites and/or that the bulk of the helix itself prohibits conformational changes in the protein kinase that promulgate the release of peptide and protein substrates.

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