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Conformational analysis of PKI(5–22)amide, the active inhibitory fragment of the inhibitor protein of the cyclic AMP-dependent protein kinase

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Fourier-transform i.r. spectroscopy, ^{1}H-n.m.r. spectroscopy and X-ray scattering were used to study the conformation and shape of the peptide PKI(5–22)amide, which contains the active site of the inhibitor protein of the cyclic AMP-dependent protein kinase [Cheng, Van Patten, Smith & Walsh (1985) Biochem. J. **231**, 655–661]. The X-ray-scattering solution studies show that the peptide has a compact structure with R_g 0.9 nm (9.0 Å) and a linear maximum dimension of 2.5 nm (25Å). Compatible with this, Fourier-transform i.r. and n.m.r. determinations indicate that the peptide contains approx. 26% α-helix located in the *N*-terminal one-third of the molecule. This region contains the phenylalanine residue that is one essential recognition determinant for high-affinity binding to the protein kinase catalytic site.

INTRODUCTION

The heat- and acid-stable inhibitor protein (PKI) specifically inhibits the catalytic subunit of cyclic AMP-dependent protein kinase (Walsh *et al.*, 1971; Ashby & Walsh, 1972). Its affinity for this enzyme is extremely high (Demaille *et al.*, 1977; Cheng *et al.*, 1985), yet it is totally ineffective against even such a closely homologous enzyme as the cyclic GMP-dependent protein kinase (Gill *et al.*, 1976; Glass *et al.*, 1986). A 20-amino acid-residue fragment of PKI has been demonstrated to show a comparable inhibitory potency (Cheng *et al.*, 1985; Scott *et al.* 1985*b*). Synthesis of this fragment and various truncated or substituted analogues has allowed systematic studies to elucidate what amino acid residues contribute to the high specificity and affinity of the inhibitor (Scott *et al.* 1985*b*, 1986; Cheng *et al.* 1986; Glass *et al.*, 1989*a*,*b*). Maximal inhibitory activity is retained in the peptide PKI(5–22)amide (Thr^5-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile^{22}-NH_2). From the studies of a range of modified peptides it is clear that there are several major contributors to the high potency of inhibition, which include the arginine residues 15, 18 and 19, the phenylalanine residue at position 10, the hydrophobic residue isoleucine-22 and the *N*-terminal domain of threonine-6 to alanine-12. Previous c.d. data suggested the possibility that PKI(5–22)amide may be structured (Reed *et al.*, 1987), which likewise may contribute to inhibitory potency. In the present study the possible structure was investigated by Fourier-transform i.r. spectroscopy, ^{1}H-n.m.r. spectroscopy and X-ray scattering. These show that the peptide's average conformation in solution is compact and contains an α-helical segment in the *N*-terminal domain. This latter domain contains the recognition determinant phenylalanine-10.

EXPERIMENTAL

Synthetic peptides

Synthesis and purification of PKI(5–22)amide and various analogues were carried out as described previously (Cheng *et al.*, 1986; Glass *et al.*, 1989*a*). Table 1 gives the primary sequence of the parent peptide and truncated and substituted derivatives used in this study, as well as citation to the procedures for synthesis and the K_i values for inhibition of the cyclic AMP-dependent protein kinase. The names given to the peptides are those used in the text and are based upon the sequence of the native PKI. [Before the knowledge of the full sequence of PKI (Scott *et al.*, 1985*a*), PKI(5–22)amide was designated 'Thr^1–Ile^{18} amide' (Cheng *et al.*, 1986).]

I.r. spectroscopy

I.r. spectra were measured at 4 cm^{-1} resolution by using a Perkin–Elmer solution cell with CaF_2 windows and a Mattson (Alpha Centuri) Fourier-transform i.r. spectrometer. Details of data acquisition are described in Trewhella *et al.* (1989). Peptide solutions for i.r measurement were prepared from purified peptide by repeated freeze-drying from ^2H_2O, and after the last freeze-drying the peptide was taken up in deuterated 10 mM-phosphate buffer, pD 7.0 (uncorrected meter reading). The deuterated phosphate buffer was prepared from sodium salts that had been repeatedly freeze-dried from ^2H_2O. The final solutions were prepared in 99.96% ^2H_2O at peptide concentrations in the range 5–15 mg/ml. The Fourier-transform i.r. spectra were analysed by using spectral subtraction, and resolution-enhancement techniques of Fourier deconvolution and second-derivative analysis, all of which are described in detail in Trewhella *et al.* (1989) and references cited therein.

Abbreviations used: PKI, protein kinase inhibitor protein; PKI(5–22)amide, inhibitory fragment of PKI.

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Table 1. Amino acid sequences of PKI(5–22)amide and truncated or mono-substituted analogues

Peptide	Sequence	K_i (nM)	Synthesis source
Parent peptide			
PKI(5–22)amide	T T Y A D F I A S G R T G R R N A I-NH_2	3.1	Cheng *et al.* (1986)
Mono-substituted analogues			
[Ala^6]PKI(5–22)amide	T A Y A D F I A S G R T G R R N A I-NH_2	7.1	Glass *et al.* (1989*a*)
[Ala^7]PKI(5–22)amide	T T A A D F I A S G R T G R R N A I-NH_2	14	Glass *et al.* (1989*a*)
[Leu^8]PKI(5–22)amide	T T Y L D F I A S G R T G R R N A I-NH_2	8.9	Glass *et al.* (1989*a*)
[Ala^9]PKI(5–22)amide	T T Y A A F I A S G R T G R R N A I-NH_2	8.2	Glass *et al.* (1989*a*)
[Ala^{10}]PKI(5–22)amide	T T Y A D A I A S G R T G R R N A I-NH_2	270	Glass *et al.* (1989*a*)
[Ala^{11}]PKI(5–22)amide	T T Y A D F A A S G R T G R R N A I-NH_2	8.1	Glass *et al.* (1989*a*)
Truncated peptide			
PKI(14–22)amide	G R T G R R N A I-NH_2	36	Glass *et al.* (1989*a*)

^{1}H-n.m.r. spectroscopy

^{1}H-n.m.r. spectra at 500 MHz were obtained on a Nicolet NM-500 spectrometer. Typical spectrometer conditions for experiments conducted in ^2H_2O were 16k data points over an 8 kHz bandwidth with an approx. 60° observation pulse and a repetition time of 2 s. The number of transients was generally 500, with 0.5 Hz line-broadening introduced through exponential apodization. The residual proton signal of the solvent was suppressed with a selective low-power pulse from the decoupler before the observation pulse. For experiments conducted in ^1H_2O the 1–3–3–1 pulse sequence (Hore, 1983) was used without any solvent saturation in order to observe exchangeable protons. Peak shifts were referenced to the water signal, which in turn was calibrated against internal 4,4-dimethyl-4-silapentane-1-sulphonate. The probe temperature was actively regulated to ±0.1 °C. Data were recorded at 25.0 °C unless stated otherwise.

Peptides used for ^{1}H-n.m.r. spectroscopy of non-exchangeable protons were freeze-dried repeatedly from 99.8% ^2H_2O and finally taken up at a concentration of 1–2 mg/ml in either, as noted, 99.96% ^2H_2O or 99.8% ^2H_2O in 50 mM-potassium phosphate buffer, pH 7.0, for measurement. Samples used for n.m.r. spectroscopy of exchangeable protons were dissolved in $^1H_2O/^2H_2O$ (9:1, v/v). pH titration was carried out on samples in 50 mM-phosphate buffer in 99.8% ^2H_2O, with 1% NaO^2H and ^2HCl. $^1H/^2H$ exchange rates were measured on fully hydrogen-saturated PKI(5–22)amide. The peptide was freeze-dried from ^1H_2O and dissolved in 99.8% ^2H_2O in 50 mM-potassium phosphate buffer, pH 7.0, immediately before measurement. Dead time from initial exposure to ^2H_2O to the beginning of signal accumulation was approx. 3.5 min.

X-ray scattering

Samples for X-ray scattering were made by dissolving freeze-dried peptide in ^1H_2O, concentrations were determined from amino acid analysis, and dilutions were done gravimetrically. X-ray data were collected on a small-angle scattering instrument at Los Alamos that has previously been described in detail (Heidorn & Trewhella, 1988). Samples were centrifuged into a 1 mm-diam. glass capillary maintained at 23 °C. Data were analysed by using the Guinier (1939) approximation as well as the indirect Fourier-transform analysis described by Moore (1980). The details of application of these methods together with a description of the controls employed and the methods of analysis are given in detail in Heidorn & Trewhella (1988).

RESULTS

I.r. spectroscopy of PKI(5–22)amide

Fig. 1. presents the buffer-subtracted spectra of the parent peptide PKI(5–22)amide (Fig. 1*a*), the alanine-10-substituted peptide (Fig. 1*b*), and the truncated analogue PKI(14–22)amide (Fig. 1*c*). The Fourier-deconvolved spectrum of PKI(5–22)amide gives five partially resolved peaks with frequency assignments of 1585, 1610, 1639 1653 and 1674 cm^{-1}. Similar frequency assignments were resolved by the second-derivative spectra (not shown), with the one exception that the single band at 1639 cm^{-1} was consistently resolved into two bands at 1634 and 1645 cm^{-1}. The remaining assignments derived from the second-derivative spectra were 1585, 1606, 1650 and 1674 cm^{-1}.

The Amide I′ region (1620–1720 cm^{-1}) contains bands arising from the conformationally sensitive stretching frequencies of carbonyl groups in the peptide backbone (Byler & Susi, 1986); the intensity of each band is proportional to the number of contributing carbonyl groups. From the assignments given by Byler & Susi (1986) the band at 1653 cm^{-1} in PKI(5–22)amide is diagnostic of α-helix and those at 1674 and 1639 cm^{-1} most probably arise from extended chain structures. Bands due to turns or bends are reported by Byler & Susi (1986) with frequencies at 1663(±4), 1670(±2), 1688(±2) and 1694(±2) cm^{-1}. The 1674 cm^{-1} band in the PKI(5–22) amide spectrum lies very near the 1670 cm^{-1} turn assignments and is sufficiently intense that one cannot exclude the possibility of contributions from both extended chain and turn structures in this band. Likewise a turn component might contribute to the spectrum near 1663 cm^{-1}. There are, however, no bands that can be uniquely attributed to turn structures in the PKI(5–22)-amide Fourier-transform i.r. spectrum. The areas under the Amide I′ bands calculated by curve-fitting the Fourier-deconvolved spectrum of PKI(5–22)amide peptide indicate that the peptide is approx. 26% α-helix. This agrees well with the previous c.d. estimate (Reed *et al.*, 1987).

The mono-substituted [Ala^{10}]PKI(5–22)amide gave a Fourier-transform i.r. spectrum very closely similar to that of the parent unsubstituted peptide (Fig. 1*b*); parallel results were also obtained with several other mono-

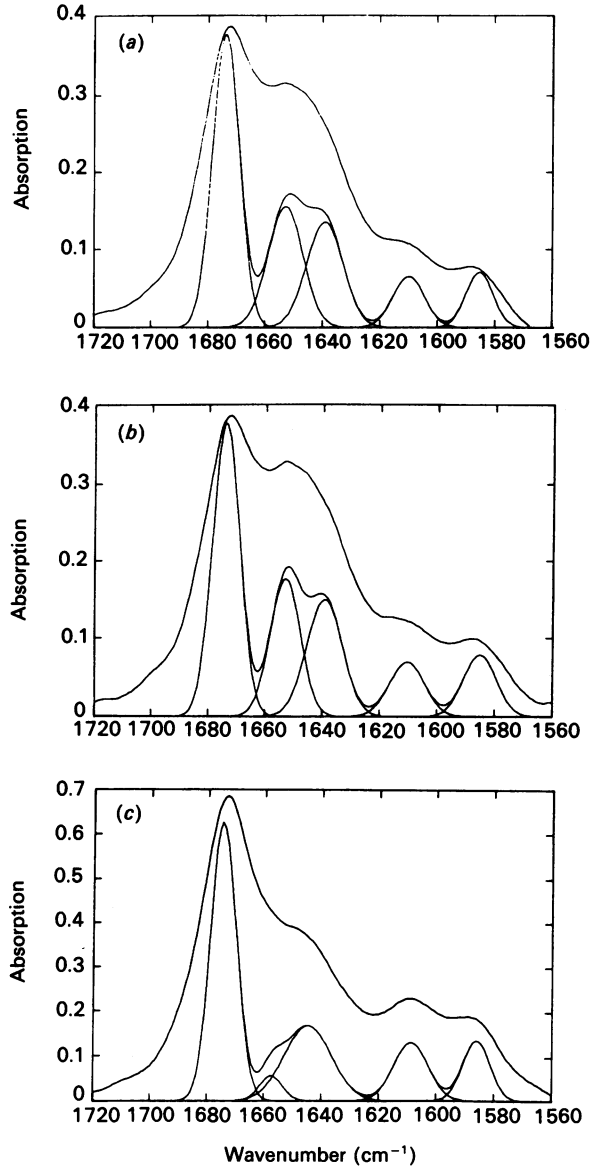

Fig. 1. Fourier-transform i.r. spectra for (*a*) PKI(5–22)amide, (*b*) [Ala^{10}]PKI(5–22)amide and (*c*) PKI(14–22)amide peptides

In each case the outer envelope is the buffer-subtracted spectrum, inside which is shown the least-squares fit of the Fourier-deconvolved spectrum showing each of the component peaks. The frequency assignments (in cm^{-1}) and relative intensities (as % of total Amide I′ region) for the three resolved Amide I′ peaks are for (*a*) and (*b*) 1639 and 26%, 1653 and 26%, and 1674 and 49%, and for (*c*) 1645 and 31%, 1658 and 5%, and 1674 and 63%.

substituted 18-amino acid-residue peptide analogues (spectra not shown). For [Ala^{10}]PKI(5–22)amide the maximum differences in amplitude compared with the parent peptide were less than 3% and probably not significant. It, like the native peptide, was calculated to contain 26% α-helix.

The Fourier-transform i.r. spectrum of PKI(14–22)-amide exhibits results in marked contrast with those obtained with the longer peptides (Fig. 1*c*). The buffer-subtracted spectrum shows a marked decrease in the Amide I′ region between 1640 and 1660 cm^{-1} compared with the parent peptide, and the two peaks in this region also had somewhat different frequency assignments. The deconvolved spectra give frequency assignments at 1645, 1658 and 1674 cm^{-1} (together with bands at 1585 and 1610 cm^{-1}). Of these, the two prominent peaks at 1645 and 1674 cm^{-1}, constituting 31% and 63% respectively of the total relative intensity in the Amide I′ region, can be assigned to extended-chain unordered structure. The 1658 cm^{-1} peak of the PKI(14–22)amide spectrum lies between the Byler & Susi (1986) assignments for α-helix and turn structures, but at most such contributed 6% of the total relative intensity. Similar results were obtained with three substituted PKI(14–22)amide analogues (Leu^{17}, Ala^{20} and Leu^{17},Ala^{20}-substituted peptides; spectra not shown). These Fourier-transform i.r. spectra indicate that, distinct from what is observed for the parent octadecapeptide PKI(5–22)amide, these shorter peptides no longer contained any significant degree of α-helix. This would be consistent with, although not fully diagnostic of, the α-helix in PKI(5–22)amide being located in the *N*-terminal residue-5–13 portion that had been eliminated in the truncated peptides.

^{1}H-n.m.r. spectra of PKI(5–22)amide and peptide analogues

Fig. 2 displays the 500 MHz ^{1}H-n.m.r. spectrum of the synthetic inhibitor peptide PKI(5–22)amide in ^2H_2O solution at neutral pH. It can be clearly seen that non-degenerate resonances are present for the three alanine and three threonine methyl protons and that the α-CH region is much more complex than would be expected for a random-coil peptide. These n.m.r. features show that the peptide PKI(5–22)amide is structured in aqueous solution, an observation compatible with the Fourier-transform i.r. data reported above.

Resonance assignments for PKI(5–22)amide were made on the basis of the chemical-shift and spin–spin-coupling patterns of the constituent residues (Wüthrich, 1976, 1986), together with the use of peptides with specific residue substitutions as a further criterion. [The method of sequential assignments (Wüthrich, 1986) could not be employed since one- and two-dimensional nuclear-Overhauser-effect experiments (results not shown) failed to detect inter-residue nuclear Overhauser effects. It is probable that the peptides, because of their size, have correlation times on the order of the reciprocal of the Larmor frequency. In such cases the nuclear Overhauser effect approaches zero (Morris, 1983; Motta *et al.*, 1987).] Spectral assignments of PKI(5–22)amide and six mono-substituted peptides are listed in Table 2. Overall there was a very strong similarity between the spectra of all of the mono-substituted peptides and that of the parent PKI(5–22)amide, indicating that all have a very similar solution structure. This was also indicated by the Fourier-transform i.r. spectra, illustrated above in Fig. 1 in the comparison of PKI(5–22)amide and the alanine-10 substitution analogue. The single amino acid replacements of PKI(5–22) peptides used for these studies, i.e. either alanine or leucine, would not have tended to break any α-helical structure present (Chou & Fasman, 1978,1979). Further, with the exception of the replacement of phenylalanine-10, the replacement of the other residues had minimal to no effect on the K_i for inhibition of the protein kinase (Table 1).

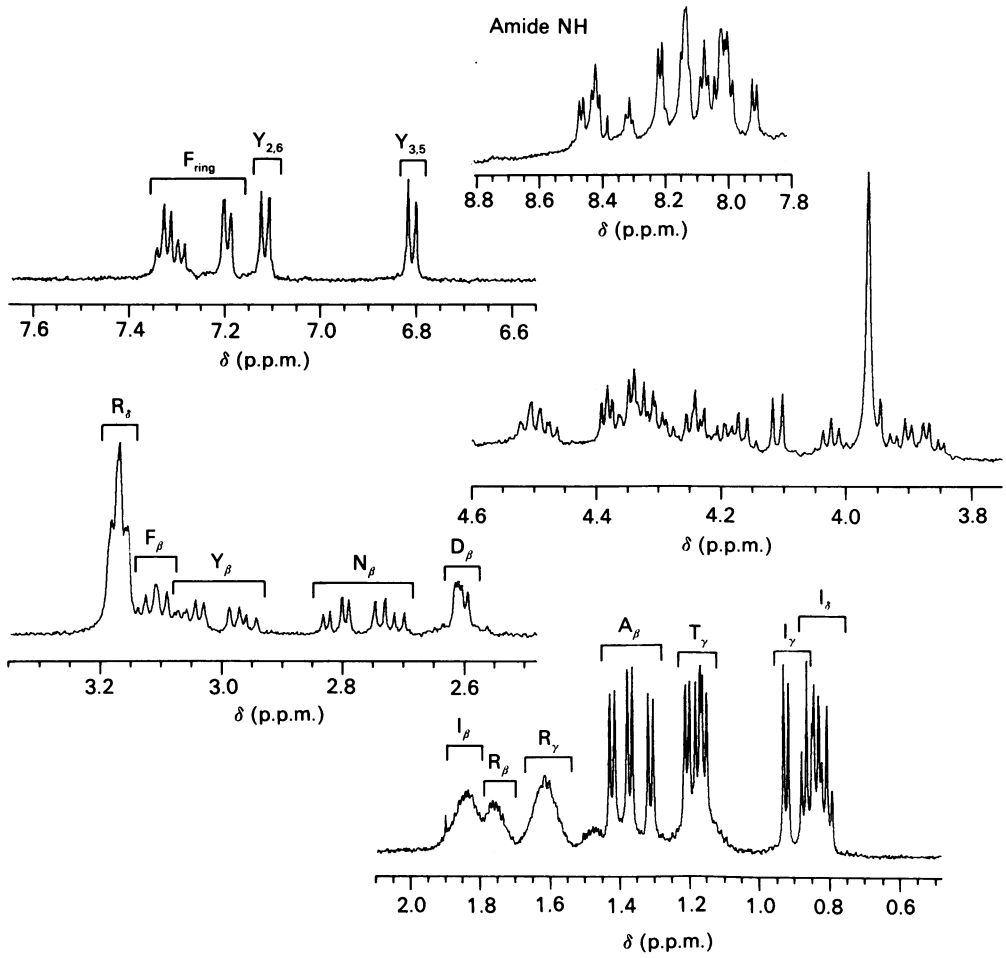

Fig. 2. ^{1}H-n.m.r. spectra of PKI(5–22)amide in ^2H_2O in 50 mM-potassium phosphate buffer, pH 7.0 (non-exchangeable protons), and $^1H_2O/^2H_2O$ (9:1, v/v) (amide protons)

The spectrum of non-exchangeable protons did not differ significantly from that of unbuffered peptide in ^2H_2O at pH 6.85. Spectra were accumulated as described in the Experimental section.

The substituted peptides were utilized to assist in making many of the resonance assignments. For example, the more downfield of the pairs of isoleucine λ-CH_3 doublets and δ-CH_3 triplets could be assigned to isoleucine-22 on the basis of its *C*-terminal position. This left the upfield resonances to be assigned to isoleucine-11; this is confirmed by their disappearance in the Ala^{11}-substituted peptide. Similarly, the identification of the most upfield of the alanine CH_3 resonances (1.31 p.p.m.) as that of alanine-8 could be made owing to the absence of a resonance at this position in the Leu^8-substituted peptide, [Leu^8]PKI(5–22)amide, the other two alanine CH_3 signals being unaffected by this substitution. In the β-CH_2 region, the relatively invariant position of the asparagine CH_2 resonances, coupled with the elimination of phenylalanine, tyrosine and aspartic acid in turn in the Ala^{10}-, Ala^7- and Ala^9-substituted peptides respectively, allowed a clear assignment of the occasionally overlapping signals.

The spectrum of PKI(5–22)amide in Fig. 2 deviates in several significant respects from that expected for the same residues in a random-coil peptide. The chemical shifts of several of the non-exchangeable protons differ from the given random-coil position, and in cases where a particular amino acid is present more than once this leads to a marked degree of non-degeneracy. This is especially noticeable in the methyl proton region 0.20–2.0 p.p.m. Thus, although the location of an isoleucine at the *C*-terminal end of the molecule might be expected to result in shifts of its γ-CH_3 and δ-CH_3 resonances downfield from the random-coil position (as is observed for one of the two isoleucine residues present), the non-degeneracy of the three alanine methyl resonances and two of the threonine methyl signals must arise from other influences. (The third threonine is *N*-terminal.) Changes in chemical shifts of this degree in the spectrum of PKI-(5–22)amide are greater than those reported for alanine C_β protons owing to nearest-neighbour effects in a peptide with a random-coil structure. With peptides in this size range that contain multiple alanine residues but are known to be unstructured in solution, fully degenerate alanine methyl proton resonances are observed. This, for example, has been shown with both the peptide Leu-Arg-Arg-Ala-Ala-Leu-Gly (Granot *et al.*, 1981) and a synthetic 21-residue troponin C fragment (Gariépy *et al.*, 1982). Under denaturing conditions (Fig. 3), as would be expected, the resonances are found more nearly at the expected random-coil position and non-degeneracy is decreased. Thus with denaturation (Fig. 3*b*) two of the three alanine CH_3 signals become fully degenerate (the

Table 2. Chemical shifts (δ) of the non-exchangeable protons in PKI(5–22)amide and substituted and truncated derivatives

PKI(5–22)amide is:

5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile

Abbreviations for peptides are: peptide (5–22), PKI(5–22)amide; Ala^6, [Ala^6]PKI(5–22)amide; Ala^7, [Ala^7]PKI(5–22)amide; Leu^8, [Leu^8]PKI(5–22)amide; Ala^9, [Ala^9]PKI(5–22)amide; Ala^{10}, [Ala^{10}]PKI(5–22)amide; Ala^{11}, [Ala^{11}]PKI(5–22)amide. Resonance values are given in p.p.m. calibrated with an accuracy of approx. ± 0.005 p.p.m. Superscripts to the resonance numbers denote residue number in the sequence, with T denoting the *C*-terminal, R.c.p. indicates the random-coil position according to Wüthrich (1986).

R.c.p.		Native peptide (5–22)	Mono-substituted peptides					
			Ala^6	Ala^7	Leu^8	Ala^9	Ala^{10}	Ala^{11}
0.89	Ile $CH_3\delta$	0.86^T	0.86	0.87	0.86	0.86	0.87	0.86
		0.81^{11}	0.81	0.85	0.83	0.81	0.83	Absent
0.95	Ile $CH_3\gamma$	0.93^T	0.92	0.93	0.93	0.93	0.93	0.92
		0.84^{11}	0.84	0.88	0.86	0.84	0.88	Absent
0.94	Leu $CH_3\delta$				0.84^8			
1.23	Thr CH_3	1.18	Absent	1.21	1.18	1.16	1.18	1.20
		1.18	1.24	1.24	1.20	1.17	1.18	1.17
		1.21	1.21	1.22	1.21	1.21	1.22	1.21
1.39	Ala CH_3	1.31^8	1.31	1.34	Absent	1.31	1.35	1.27
		1.36	1.35	1.38	1.37	1.32	1.38	1.34
			1.37	1.42		1.37	1.40	1.37
		1.42	1.42	1.42	1.42	1.41	1.42	1.42
1.70	Arg $CH_2\gamma$	1.62	1.62	1.62	1.62	1.62	1.62	1.62
1.79	Arg $CH_2\beta$	1.76	1.76	1.76	1.76	1.76	1.76	1.76
1.90	Ile $CH_2\beta$	1.83	1.83	1.84	1.84	1.84	1.84	1.84
2.75	Asp $CH_2\beta$	2.70	2.64	2.62	2.60	Absent	2.67	2.62
2.75	Asn $CH_2\beta$	2.77	2.77	2.77	2.77	2.77	2.77	2.77
3.02	Tyr $CH_2\beta$	3.00	3.00	Absent	3.02	2.98	3.01	2.98
3.10	Phe $CH_2\beta$	3.10	3.10	3.12	3.11	3.08	Absent	3.10
3.20	His $CH_2\beta$							
3.32	Arg $CH_2\delta$	3.18	3.18	3.19	3.18	3.18	3.19	3.18
6.86	Tyr ring-3,5	6.81	6.82	Absent	6.81	6.80	6.82	6.81
7.15	Tyr ring-2,6	7.12	7.12	Absent	7.12	7.12	7.13	7.12
7.30	Phe ring-2,6	7.20	7.20	7.24	7.22	7.20	Absent	7.24
7.39	Phe ring-3,5	7.32	7.32	7.34	7.32	7.32	Absent	7.32

third resonance probably remains somewhat upfield owing to some shielding from the neighbouring tyrosine-7), and two of the three threonine methyl resonances become fully degenerate (the third threonine remaining shifted downfield owing to its location at the *N*-terminus). These data support the view that the native peptide is structured.

For the majority of the assigned protons, only a single signal is present. The bulk of the peptide in solution thus appears to exist in one favoured configuration, or multiple conformations that are in fast exchange (on an n.m.r. time scale). Either way, the single resonance produced by each proton argues against multiple large structural changes and for a favoured average conformation. However, The $C_{(\beta)}$ protons of phenylalanine, tyrosine, asparagine and aspartic acid residues appear as complex multiplets, since restricted rotation about the $C_{(\alpha)}$–$C_{(\beta)}$ bond results in non-equivalent β-CH_2 protons that couple differently to the α-CH proton, giving rise to multiplets characteristic of AB parts of ABX spin systems (Wüthrich, 1976).

Fig. 4 shows the effect of pH titration on the chemical shifts of the non-exchangeable protons. Deflexions at pH 3–5 would be typical of the titration of the β-carboxylate group of the aspartic acid residue. The deflexions at pH 10–11 seen in the tyrosine ring and β-CH_2 protons, in two of the three threonine methyl protons, and in one isoleucine methyl resonance (isoleucine-11) are characteristic of the pK_a of the tyrosine hydroxy group. The aspartic acid CH_2 protons deflect in the pH region expected for the carboxylate group and also show indications of a deflexion with a $pK_a > 11$; the latter could not be defined further because the peptide begins to hydrolyse at the higher pH.

α-Helix assessment from amide proton exchange and $^3J_{HN\alpha}$ values

Analysis of the kinetics of hydrogen exchange from the backbone amide groups can give an indication of the presence and relative stability of hydrogen-bonded structures. For PKI(5–22)amide the use of peptides with specific substitution, together with chemical shifts, was a primary tool for the assignment of amide proton resonances. The half-times for amide exchange rates

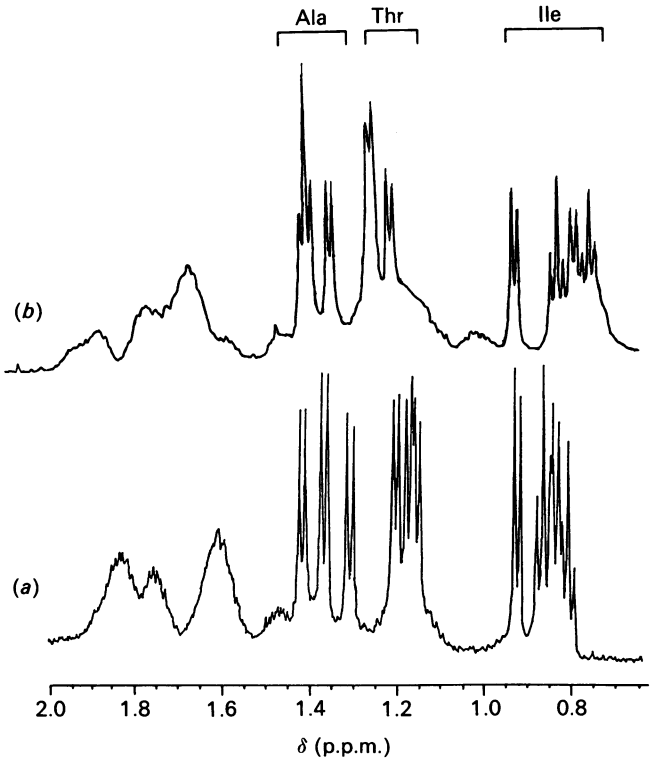

Fig. 3. Downfield (2.00–0.5 p.p.m.) portions of ^{1}H-n.m.r. spectra of PKI(5–22)amide in ^2H_2O

Conditions were as follows: (*a*) peptide in ^2H_2O at pH 7; (*b*) peptide in 100% trifluoroacetic acid; similar results were obtained in dimethyl sulphoxide at 120 °C or 4 M-LiCl at 90 °C. Chemical shifts (δ) have been adjusted for acid-induced downfield shifts.

measured at pH 4.5 (Fig. 5) indicate a number of slowly exchanging protons. The phenylalanine and isoleucine residues (probably isoleucine-11, given the position and the induction data), with a $t_{\frac{1}{2}}$ of approx. 20 min, have the most highly stabilized amide protons, which would tend to locate them at the core of any structure present. These kinetics of hydrogen exchange would be compatible with the presence of an α-helical segment near the *N*-terminus, as suggested above by the Fourier-transform i.r. data. If such were present, phenylalanine-10 and isoleucine-11 would be hydrogen-bonded to the carboxy groups of threonine-6 and tyrosine-7 respectively. Aspartic acid-9 also showed a lower rate of exchange than most of the other residues, with a $t_{\frac{1}{2}}$ of approx. 5 min; such would be compatible with it being within an α-helix and hydrogen-bonded with threonine-5. In addition to these three amide protons, a fourth moiety, probably that of one of the three arginine residues, also exhibited exchange-protected amide protons.

Pardi *et al.* (1984) have calibrated the relationship between $^3J_{HN\alpha}$ and ϕ, the dihedral angle, and determined that segments with three or more spin–spin-coupling constants $^3J_{NH\alpha} < 6$ Hz serve as an independent criterion for the presence of helical structure. ^{1}H-n.m.r. spectra of PKI(5–22)amide and the substituted peptides were measured in $^1H_2O/^2H_2O$ (9:1, v/v) in order to obtain $^3J_{HN\alpha}$ values from the amide proton signals. Table 3 gives those $^3J_{HN\alpha}$ values, and in the lower half of the Table are indicated, above each residue in the PKI(5–22)amide sequence, values consistent with the presence of an α-helical segment. As can be seen from this diagram and the data of the Table, the *N*-terminal portion of the molecule contains a stretch of sequence with a high probability of α-helix with residues having $^3J_{HN\alpha} < 6$ Hz,

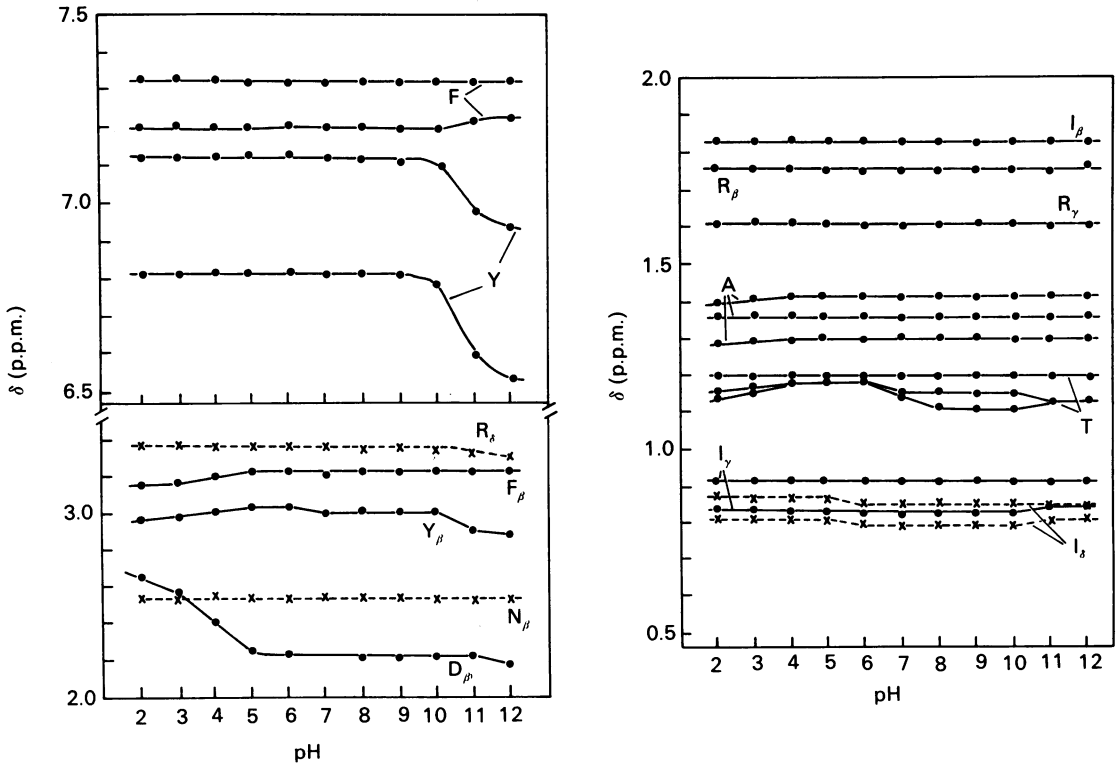

Fig. 4. Chemical shifts (δ) of non-exchangeable protons in PKI(5–22)amide as a function of pH

Conditions were as given in the Experimental section.

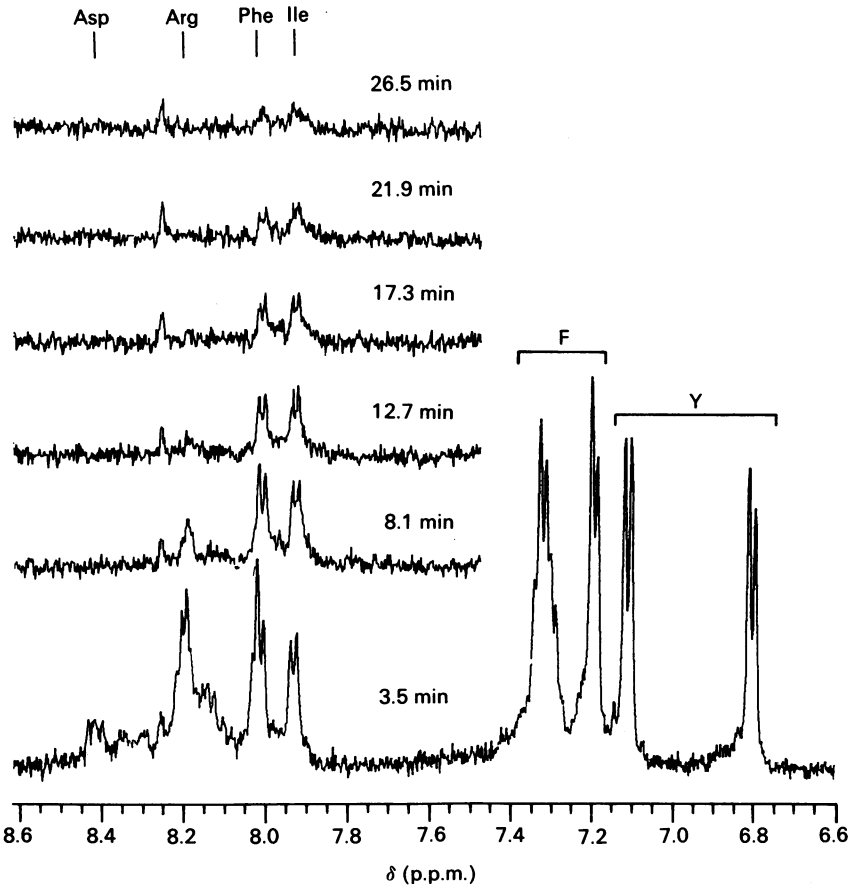

Fig. 5. $^1H/^2H$-exchange ^1H-n.m.r. spectra of PKI(5–22)amide at pH 4.5

Accumulation time was 2.3 min; only every other spectrum is shown. $t_{\frac{1}{2}}$ values, determined by linear regression, were: isoleucine, 22 min; phenylalanine, 21 min; arginine, 10 min; aspartic acid, 5 min. For all determinations of $t_{\frac{1}{2}}$, $r > 0.95$.

Table 3. Spin–spin-coupling values $^3J_{HN\alpha}$ for PKI(5–22)amide residues

* * o * ? * o
T T Y A D F I A S G R T G R R N A I

* indicates $^3J_{HN\alpha}$ is less than 6 Hz. o indicates that there is a 2/3 probability that the $^3J_{HN\alpha}$ values for these alanine residues are less than 6 Hz because two of the three alanine residues are less than this value, but specific residue assignment could not be made. ? indicates that a clear measurement for the determination of the $^3J_{HN\alpha}$ value for phenylalanine was not possible because of overlapping spectra.

Residue	$^3J_{HN\alpha}$ (Hz)	Residue	$^3J_{HN\alpha}$ (Hz)
Thr	5.1	Ile	5.7
	6.4		7.5
	7.0	Ser	6.9
Tyr	4.5	Gly	6.3
Ala	5.0		6.1
	5.5	Arg	7.3
	6.8		6.8
Asp	5.0		5.6
Asn	6.8		

whereas those at the *C*-terminal end are generally greater. Note that the constants measured lie in the medium range, none being very large or small, so that segmental motions about the backbone dihedral angle ϕ are not highly constrained (Nagayama & Wüthrich, 1981).

Subsite interactions within the *N*-terminal domain

Further evidence for a stable folded structure within the PKI(5–22)amide peptide is apparent from the mutual shielding effects seen between the subsite -Tyr^7-Ala^8- on the one hand and -Phe^{10}-Ile^{11}- on the other. Table 2 shows that substitution of an alanine residue for tyrosine-7 results in a downfield shift of the δ-CH_3 (0.81 to 0.85 p.p.m.) and γ-CH_3 (0.84 to 0.88 p.p.m.) protons of the non-terminal isoleucine-11, whereas in contrast replacement of either threonine-6 or aspartic acid-9 left the resonances in the same position as in the parent peptide. Replacement of tyrosine-7 also causes the phenylalanine ring protons to shift from 7.20 to 7.24 p.p.m. Replacement of phenylalanine-10 with an alanine residue causes an isoleucine γ-CH_3 resonance to shift downfield from 0.84 to 0.88 p.p.m. This effect is as expected, since a strongly shielding neighbour (phenylalanine) has been replaced by a non-shielding one; however, this through-bond effect is no stronger than the apparent positional

effects described above with the replacement of tyrosine-7. This would strongly suggest that the through-space tyrosine-7 to -Phe[10]-Ile[11]-shielding is over the short distances typical of a compact structure.

The [Ala[10]]PKI(5-22)amide peptide gave further indications of the through-space interactions between these subsites. The replacement of phenylalanine-10 by alanine affects the other peptide alanine CH_3 protons. Although it is not possible to say exactly which of the four alanine CH_3 resonances is due to alanine-8, the smallest shift downfield, in the absence of phenylalanine-10, must be from 1.31 to 1.35 p.p.m. Again, it appears that the phenylalanine side chain in the PKI(5–22)amide peptide is capable of shielding side-chain protons three and four residues distant. The replacement of isoleucine-11 with alanine has no significant effect on the neighbouring aromatic residue phenylalanine-10, so it is not too surprising that no effect on tyrosine-7 is apparent. An effect is seen in this peptide, however, on the alanine-8 CH_3 signals. In the Ala[11]-substituted peptide, [Ala[11]]PKI(5–22)amide, there are four identifiable alanine CH_3 signals, of which the furthest upfield is at 1.27 p.p.m. As there seems to be no satisfactory way to explain how a substitution of an alanine for an isoleucine could move the signal of even a neighbouring alanine upfield, still less that of one three residues distant, the signal at 1.27 p.p.m. would appear most likely to stem from the newly inserted alanine-11, shielded by proximity to phenylalanine-10. Thus the least that alanine-8 can have been shifted downfield by this substitution is from 1.31 to 1.34 p.p.m.

The shielding effects of the -Phe[10]-Ile[11]- area on -Tyr[7]-Ala[8]- on the one hand, and those of tyrosine-7 on -Phe[10]-Ile[11]- on the other, suggests that in the native PKI(5–22)-peptide these two regions are positioned quite close to one another in a compact structure. Through-bond effects of the nature observed are normally significant only for neighbouring residues because of their severe attenuation with distance. The approximately three-residue interval between the areas is characteristic of α-helical packaging, with a 3.6 residue repeat.

A curious feature of the data is that replacement of aspartic acid-9 by an alanine has little effect on the proton chemical shifts of neighbouring amino acid residues. There is an upfield shift of the phenylalanine CH_2 proton signal in the Ala[9]-substituted peptide, but neither the phenylalanine ring protons nor the alanine-8 CH_3 protons are affected. As the electronegativity of the carboxylate group makes it one of the strongest deshielding influences present in proteins, this is difficult to explain if the aspartic acid side-chain carboxylate group is free in PKI(5–22)amide; this suggests that it is involved in some type of charge-neutralizing interaction such as a salt bridge.

X-ray-scattering solution studies of PKI(5–22)amide

An evaluation of the overall shape of PKI(5–22)amide has been made by using X-ray scattering in solution. Table 4 shows the concentration-dependence of R_g determined from the pair-distance distribution [$P(r)$] analysis, and Fig. 6 shows the $P(r)$ function calculated for infinite dilution by extrapolation of the scattering-intensity data to zero peptide concentration. Plots of I_0/c versus c, where c is the peptide concentration and I_0 the scattering intensity at zero angle, were linear and showed no evidence for aggregation. The calculated M_r from the extrapolation of I_0/c to zero peptide concentration (Levinson *et al.*, 1983), with monodisperse troponin C as the standard (Heidorn & Trewhella, 1988), was in good agreement with theoretical. The $P(r)$ function in Fig. 6 is essentially symmetrical about its maximum at 1.15 nm (11.5Å), indicating that the distribution of scattering density in the peptide is close to spherical. [An elongated molecule, such as in a random coil, would be expected to give a skewed distribution in the $P(r)$ function, with a long tail at the longest vectors (Glatter, 1982).] The R_g and $d_{max.}$ (maximum linear dimension), determined for infinite dilution of the peptide, were 0.90 ± 0.01 nm (9.0 ± 0.1Å) and 2.5 ± 0.2 nm (25 ± 2Å) respectively. For theoretical comparison, also shown in Fig. 6 is the calculated $P(r)$ function curve for PKI(5–22)amide as a prolate ellipsoid of dimensions 2.5 nm × 1.7 nm (25Å × 17Å). These data indicate that PKI(5–22)amide is present in solution in a compact form with, on average, a quite symmetrical distribution of the constituent atoms.

Table 4. Concentration-dependence of R_g and the maximum linear dimension ($d_{max.}$) of PKI(5–22)amide determined by X-ray scattering

Concn. of peptide (mg/ml)	R_g (nm)	$d_{max.}$ (nm)
10	0.903 ± 0.010	2.5
15	0.885 ± 0.009	2.5
20	0.863 ± 0.008	2.5
40	0.867 ± 0.006	2.4

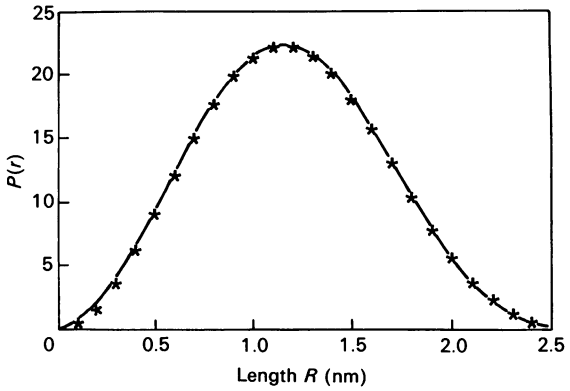

Fig. 6. $P(r)$ functions for PKI(5–22)amide determined from X-ray-scattering data in solution (★) or calculated (——) for a prolate ellipsoid of dimensions 2.5 nm × 1.7 nm (25Å × 17 Å)

Conditions were as given in the Experimental section.

DISCUSSION

The inhibitor protein (PKI) of the cyclic AMP-dependent protein kinase and the active peptide fragment PKI(5–22)amide act by direct interaction at the protein substrate-binding site of the catalytic site of the protein kinase. From all the studies that have been pursued to date it is clear that PKI contains many of the recognition determinants of the substrates of the protein kinase, and, in fact, may have within its structure more recognition determinants than are present in any single protein

substrate (Scott *et al.*, 1985*b*, 1986; Cheng *et al.*, 1986; Glass *et al.*, 1989*a*,*b*). PKI and the peptide PKI(5–22)-amide exhibit a higher affinity for the catalytic site than any native protein substrate. The peptide analogue [Ser^{21}]PKI(14–22)amide is an effective substrate of the protein kinase and, of interest, has both a lower K_m and a higher $V_{max.}$ than any other peptide substrate so far examined whose sequence has been based on recognized protein substrates (Glass *et al.*, 1989*a*). In many ways, therefore, PKI(5–22)amide appears to mirror the interaction of protein substrates. Studies of its recognition determinants serve not only to determine the basis for its high affinity, but also to identify what potential sites may exist on the protein kinase for protein substrate recognition.

A wide array of peptides have been examined to determine which residues are crucial for binding of PKI(5–22)amide by the protein kinase (Cheng *et al.*, 1986; Glass *et al.*, 1989*a*,*b*; Scott *et al.*, 1985*b*, 1986). With deletion, maximum activity is retained in PKI(6–22)amide, but further removal from either the *N*- or the *C*-termini results in significant loss of inhibitory potential. Of the specific residues present in PKI(5–22)-amide it is clear that phenylalanine-10, arginine-15, arginine-18, arginine-19 and isoleucine-22 are major contributors to maximal inhibitory potency. Lesser but significant contributions are also provided by glycine-17 and asparagine-20. Of noted interest, the conservative substitution of any one of the amino acid residues in the stretch of residues from 6 to 11, with the exception of phenylalanine-10, at most only minimally affects activity (Table 1), whereas progressive deletion of these moieties has a more marked effect (Glass *et al.*, 1989*a*). A rationale for this observation is now apparent from the current conformational studies. From the current analysis by Fourier-transform i.r. spectroscopy, n.m.r. spectroscopy and X-ray scattering, and the previous c.d. data (Reed *et al.*, 1987), it is now well documented that PKI(5–22)amide exhibits structure and is a compact molecule. The Fourier-transform i.r. spectra, coupled with the previous c.d. estimates, show that there is a significant content of α-helix. Sequence–conformation correlations, according to Chou & Fasman (1978), suggest that an α-helical segment would probably be located in the *N*-terminal region of the molecule. Multiple lines of evidence now support this conclusion. From n.m.r. studies, substantiation for a hydrogen-bonded structure at the *N*-terminal portion of the peptide is given by (i) the hydrogen exchange rates of the amide protons, (ii) the cluster of $^3J_{HN\alpha}$ values less than 6 Hz, (iii) the mutual shielding of areas spaced roughly three residues apart and (iv) the pH titration data with the occurrence of a pH deflexion in an isoleucine CH_3 resonance at a pK_a characteristic of the tyrosine hydroxy group. Localization of an α-helical segment in the *N*-terminal half of the peptide is also supported by the Fourier-transform i.r. data with the observation that the α-helical contribution evident in the spectra of PKI(5–22)amide and [Ala^{10}]PKI(5–22)amide is absent in the truncated PKI(14–22)amide (Fig. 1). The precise location of this helix has yet to be fully ascertained, but the rates of amide proton exchange suggest that at least threonine-5 to isoleucine-11 is part of this conformation. Such a placement of α-helix provides a rationale for the observations that, whereas conservative single substitutions in this part of the peptide (other than that of phenylalanine-10) did not alter activity, serial deletion markedly diminished inhibitory potency. It would thus appear that the phenylalanine-10 side chain is a protein kinase recognition determinant whose location is optimized by its presence in a stretch of α-helix. When this helix is maintained in the analogues with a conservative substitution that would not disrupt the α-helix, inhibitory potency is maintained. In contrast, even when the phenylalanine is present, deletion of threonine-6, tyrosine-7, alanine-8 and aspartic acid-9 markedly destroys inhibitory potency because the phenylalanine is apparently not optimally located in a stretch of α-helix. Of note, an α-helix in this region would have amphiphilic properties (Fig. 7). The helix may thus serve some role in the orientation of the side chain of phenylalanine, with one surface of the α-helix providing a hydrophobic environment to maximize interaction between the peptide and a hydrophobic cleft or pocket near the catalytic site of the protein kinase. As we have shown elsewhere (Glass *et al.*, 1989*b*), both the hydrophobicity of phenylalanine-10 and the orientation of the phenyl ring make important contributions to maximizing the binding of PKI(5–22)amide to the protein kinase. Of interest, the Fourier-transform i.r. spectra of the parent PKI(5–22)amide and the phenylalanine-10-substituted peptides were within error identical (Fig. 1), with an identical contribution of α-helix. Thus the 100-fold increase in the K_i value upon the substitution of an alanine for phenylalanine-10 (Table 1) clearly seems to be due to the removal of the phenylalanine as a recognition determinant, rather than to a change in the structure of the peptide caused by the substitution. The summation of these and past data (Glass *et al.*, 1989*a*,*b*) thus demonstrates that phenylalanine-10 is a hydrophobic protein kinase recognition determinant and is located within a region of amphiphilic α-helix. What remains to be proven is whether the α-helix is a mandatory requirement for inhibitory potency.

The presence of α-helix in the *N*-terminal one-third of PKI(5–22)amide would make an important contribution to the compact structure of the peptide that is evident

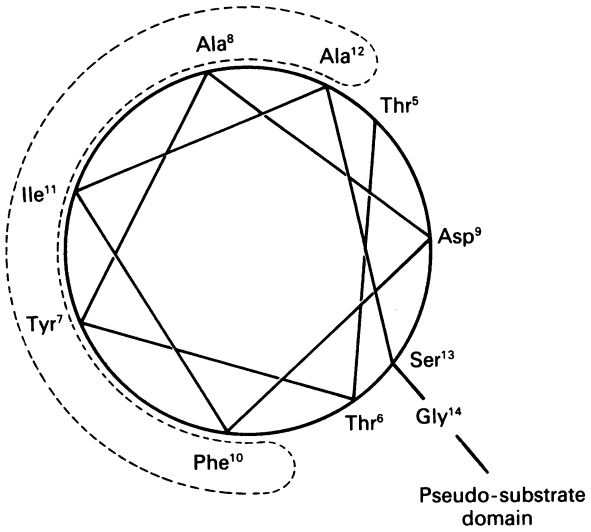

Fig. 7. Helical wheel diagram of the *N*-terminal portion of PKI(5–22)amide

The pseudo-substrate domain includes residues 15–22.

from the X-ray-scattering studies. It alone would not be sufficient to account for the symmetrical distribution of the observed $P(r)$ function demonstrated in Fig. 6, suggesting that some other elements of structure are also present. By using modelling to determine atomic co-ordinates and calculated $P(r)$ functions, several simple possibilities can be readily eliminated; these include structures where the entire peptide is an α-helix or where one-third is helix and the rest extended chain. The calculated $P(r)$ for a prolate ellipsoid, as illustrated in Fig. 6, matches the experimental data well, suggesting that some looping of the *C*-terminal half of the protein into the vicinity of the helical *N*-terminal segment is a strong possibility. Use of atomic co-ordinates from models with potential loops gave calculated $P(r)$ functions close to that observed experimentally. Such a conformation may in some manner account for the observations, briefly referred to in the Results section, that one arginine residue exhibits an amide proton half-life inconsistent with it being in an extended chain, and, from the n.m.r. substitution data, that aspartic acid-9 does not appear to exhibit the effects expected for its potential side-chain electronegativity. Possibly, interactions involving these residues are part of those that stabilize the compact structure.

From the data presented here, in association with previous reports, it appears likely that the secondary-structure elements of the inhibitory peptide PKI(5–22)-amide (and presumably also of the native PKI) contribute directly to its function. From the current data, it is not possible to distinguish whether PKI(5–22)amide in solution adopts a single conformation, or exists in a number of conformations whose average is the proposed type of structure. Given the size of the peptide, it seems likely that it will display some flexibility, and the latter situation may be more likely. The fact that the average structure would be well-defined and compact, as suggested by the n.m.r. and X-ray-scattering data, however, implies a number of conformational sub-states about the average compact form, and is inconsistent with large frequent oscillations between compact and extended conformations. Of interest, the considerable identity of n.m.r. spectra between the various mono-substituted peptides, and also of the Fourier-transform i.r. spectra of PKI(5–22)amide and [Ala^{10}]PKI(5–22)amide, would indicate that each of these substituted analogues adopts a near-identical average structure. Even in the case of the average structure, it is likely that this structure is important in the recognition of the peptide by the active site of the cyclic AMP-dependent protein kinase. Again, because of the small size of the peptide it is possible that binding to the protein kinase will induce a structural rearrangement in the peptide. The solution structure of the free peptide may be of most significance, therefore, to the initial recognition steps for binding rather than reflecting the final conformation of the bound peptide. Of interest, however, is the growing suggestion that binding of the substrates and inhibitor peptides to the protein kinase also induces considerable conformational changes in the kinase (Reed *et al.*, 1987).

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