ELSEVIER

Biophysical Chemistry 52 (1994) 173–181

Biophysical Chemistry

The conformational equilibria of a renin inhibitor peptide in solution

Graeme J. Anderson [a,*], Parvez I. Haris [b], Dennis Chapman [b], Alex F. Drake [c]

[a] *Department of Chemistry, Manchester Metropolitan University, Chester Street, Manchester M1 5GD, UK*

[b] *Department of Protein and Molecular Biology, Royal Free Hospital School of Medicine, University of London, Rowland Hill Street, London NW3 2PF, UK*

[c] *SERC Chiro-optical Laboratory, Department of Chemistry, Birkbeck College, University of London, 20 Gordon Street, London WC1H 0AJ, UK*

Received 8 March 1994; accepted in revised form 13 June 1994

Abstract

The conformational equilibrium of a decapeptide renin inhibitor (Renin Inhibitory Peptide (RIP), NH–P–H–P–F–H–F–F–V–Y–K–CO_2H) in water, methanol and trifluoroethanol has been investigated. The value of a combined spectroscopic approach was apparent, with the need to define conformational states that were mixtures of conformational forms. Similarities between this study and that of the Melanin Concentrating Hormone (MCH) core peptide (5–14) are notable [1]. In water, two β-turn conformations and an extended form were found to be in equilibrium, with cis/trans isomerism at Pro-3. Extended conformations associated with the P_{II} helix and irregular forms were more favoured in aqueous environments. In MeOH and TFE, two β-turn conformations associated with overlapping sequences and cis/trans isomerism at Pro-3 amide bond were seen to be in equilibrium. 2D ROESY and chemical-exchange cross-peaks were detected by ^{1}H NMR and used to build up detailed models of the interconverting β-turn conformations of RIP.

Keywords: Renin inhibitor peptide; Conformation; Fourier Transform infrared; Nuclear magnetic resonance; Circular dichroism

1. Introduction

In contrast to the situation found in proteins, peptides exhibit great flexibility and normally exist with multiple conformations in solution. The use of low temperatures and/or viscous media are often needed to investigate these complex conformational equilibria [2,3]. Understanding the conformational behaviour of small peptides which act as local hormones, neurotransmitters, toxins etc., is important when considering binding and inhibition studies of peptides to enzymes and receptors, and in investigating the mechanisms of protein folding.

We report here the conformational investigation of a linear decapeptide inhibitor of the enzyme renin, of modest potency (IC_{50} = 8 mM), by the use of perturbation circular dichroism (CD), Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy. Renin inhibitory peptide (RIP) [4,5] (see Table 1 for sequence) has been previously

Abbreviations: CD: circular dichroism; FTIR: Fourier transform infrared; NMR: nuclear magnetic resonance; RIP: renin inhibitory peptide; 2D ROESY: two-dimensional rotating frame nuclear Overhauser enhancement spectroscopy; ROE rotating frame nuclear Overhauser enhancement

* Corresponding author.

SSDI 0301-4622(94)00095-2

Table 1
Chemical shift values obtained for RIP in H_2O/D_2O (8:1, pH 3.15), CD_3OH and CF_3CD_2OH.

Residue	NH	α	β	γ	δ	ε	Additional protons
RIP chemical shifts in water/deuterium oxide							
Pro (1)	–	4.12	2.23	1.80	3.22	–	–
His (2)	8.60	4.76	2.99/296	–	–	–	8.36(C2)/7.06(C4)
Pro (3)	–	4.21	2.06/1.58	1.77	3.52/3.34	–	–
Phe (4)	8.17	4.29	2.78	–	–	–	6.94(2,6)/7.02,7.05(3,5)/7.09(4)
His (5)	7.98	4.32	2.90/2.86	–	–	–	8.29(C2)/6.80(C4)
Phe (6)	7.83	4.26	2.76/2.71	–	–	–	6.96,694(2,6)/7.11,7.08(3,5)/7.04(4)
Phe (7)	7.91	4.30	2.70/2.67	–	–	–	6.90(2,6)/7.03(3,5)/7.09(4)
Val (8)	7.60	3.77	1.69	0.62/0.60	–	–	–
Tyr (9)	7.99	4.29	2.82/2.75	–	–	–	6.95(2,6)/6.61(3,5)
Lys (10)	7.64	3.97	1.77/1.58	1.12	L44	2.76	–
RIP chemical shifts in trifluoroethanol-d2							
Pro (1)	–	–	4.38	2.45/2.03	2.03	3.44/3.39	–
His (2)	Exch. Broad.	4.95	3.13/3.10	–	–	–	8.04(C2)/7.18(C4)
Pro (3)	–	4.45	2.25/1.92	1.95	3.68/3.31	–	–
Phe (4)	8.37	4.63	3.09	–	–	–	7.19(2,6)/7.32(3,5)/7.28(4)
His (5)	7.95	4.57	3.17/3.14	–	–	–	8.21(C2)/7.00(C4)
Phe (6)	7.73	4.50	3.05/2.98	–	–	–	7.17(2,6)/7.30(3,5)/7.26(4)
Phe (7)	7.70	4.56	3.11/3 03	–	–	–	7.20,7.30(2,6)/7.35(3,5)/7.47(4)
Val (8)	7.25	4.04	2.00	0.82	–	–	–
Tyr (9)	7.62	4.59	3.11/2.96	–	–	–	7.13(2,6)/6.84(3,5)
Lys (10)	7.40	4.24	1.86/1.72	1.41	1.72	3.00	–
RIP Chemical shifts in methanol-d3							
Pro (1)	–	4.32	2.36/1.96	1.96	3.71/3.46	–	–
His (2)	Exch. Broad	4.75	3.17	–	–	–	8.10(C2)/7.23(C4)
Pro (3)	–	4.38	2.19/1.88	1.85	3.63/3.57	–	–
Phe (4)	9.02	4.56	3.10	–	–	–	7.19(2,6)/7.25(3,5)/7.22(4)
His (5)	8.18	4.48	3.12	–	–	–	8.35(C2)/7.00(C4)
Phe (6)	8.05	4.47	3.01/2.92	–	–	–	7.18(2,6)/7.26(3,5)/7.23(4)
Phe (7)	8.16	4.57	3.07/2.91	–	–	–	7.23(2,6)/7.29(3,5)/7.16(4)
Val (8)	7.69	4.01	1.94	0.84/0.76	–	–	–
Tyr (9)	7.97	4.53	3.07/2.82	–	–	–	7.07(2,6)/6.67(3,5)
Lys (10)	8.02	4.29	1.89/1.78	1.42	1.64	2.88	–

investigated by ^1H NMR in dmso-d^6 solution, but the results were inconclusive and the problems of multiple conformations was not addressed [6]. Our previous studies of RIP in cryogenic solvents [7] identified an interconversion of the amide bond at Pro-3, with a mixture of predominantly these two isomers at room temperature and the cis isomer being 'frozen out' at low temperatures. The results presented here represent a further refinement of the conformational equilibria for RIP in solution.

2. Materials and methods

RIP was synthesised using standard Merrifield Boc solid-phase peptide synthesis techniques [8]. Amino acids were purchased from Nova Biochemicals, and used without further purification. Coupling procedures employed dicyclohexyl carbodiimide (DCC) and hydroxy benzotriazole (HOBt) active esters. Peptide was cleaved from the resin by liquid HF at $-4°C$, and purified by C_{18} reverse-phase HPLC. Characterisation

of product included ion-exchange HPLC, FAB-MS, ^{1}H NMR and amino acid analysis.

CD spectra were recorded on a JASCO J40 spectrometer in a 0.02 cm cell using spectroscopic grade methanol and trifluoroethanol (Aldrich Chemicals); distilled, de-ionised water was used for the aqueous studies. A solvent blank was recorded and subtracted from each spectrum shown. Peptide concentrations were 0.2–0.5 mg/ml.

FTIR spectra were recorded with a Perkin-Elmer 1750 spectrometer in D_2O (pH 3.15), deutero-TFE (CF_3CD_2OD) and deutero-methanol (CD_3OD). All deuterated solvents used for FTIR and NMR were purchased from Fluorochem Ltd. Sample concentrations were typically 0.7 mg per 100 μl solvent. 200 scans were collected per spectrum and the absorption and second derivative spectra generated, after subtraction of solvent spectra.

NMR spectra were performed using a Bruker AM500 spectrometer. All spectra shown are recorded at 25°C. RIP was dissolved in H_2O/D_2O (8 : 1), pH 3.15, trifluoroethanol-d^3 (CF_3CD_2OH) and methanol (CD_3OH) at concentrations of 2–4 mM. Complete proton spectral assignment in all three solvents was obtained by a combination of HOHAHA [9] (mixing time 65–80 ms) and ROESY [10] (τ_m = 50–200 ms) two-dimensional (2D) experiments. In each of the solvents rotating frame NOE (ROE) cross-peaks were identified and assigned as strong, medium or weak from their intensities and related to short (1–5 Å), medium (2.5–5 Å) or long range (3.5–5 Å) distances. ROEs were then classified into several mutually exclusive categories, from which several different structures could be built; no single structure could be derived from the ROE data obtained for any of the solvents.

RIP was monomeric under the conditions studied, as determined by NMR and CD concentration studies (10 to 0.1 mg/ml and 1 to 0.05 mg/ml respectively). Spectra showed no variation in chemical shift or line-width (NMR) or changes in λ_{max} or $\Delta\varepsilon$ (CD) over the concentration ranges studied.

3. Results

3.1. CD studies

The CD spectra of RIP in MeOH, TFE and water (Fig. 1) were not typical of linear oligopeptides, as they could not be simply assigned in terms of backbone amide electronic transitions which would be required for secondary structure analysis. The RIP sequence is rich in aromatic amino acid residues which provided aromatic contributions in the CD spectra [10]. Nevertheless solvent variations did reveal that several conformational components must co-exist in solution.

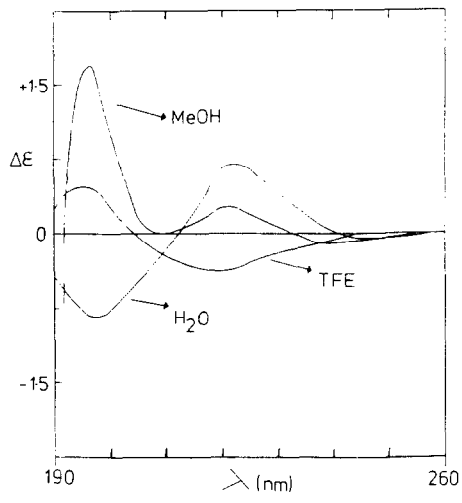


Fig. 1. Circular dichroism (CD) spectra of RIP recorded in methanol (MeOH), trifluoroethanol (TFE) and water (H_2O).

Isodichroic points in a set of CD spectra are characteristic of systems where two states are in equilibrium [2]. In the MeOH/H_2O and TFE/H_2O titrations such points were not apparent (Fig. 2) and the conformational equilibrium is therefore multiple. Three possible states, consistent with the NMR data, were suggested from the TFE/H_2O titration curve (Fig. 2c) corresponding to 100% water, ~60% TFE/water and 100% TFE. This behaviour with linear peptides has been observed previously [11]. For the non-aqueous solvent titration (MeOH/TFE) the previous identification of an isodichroic point suggested a smooth, two-state transition between the MeOH and TFE states [7].

3.2. FTIR studies

Fig. 3 shows the second derivative FTIR spectra [12] in (a) D_2O, (b) CF_3CD_2OD and (c) CD_3OD,

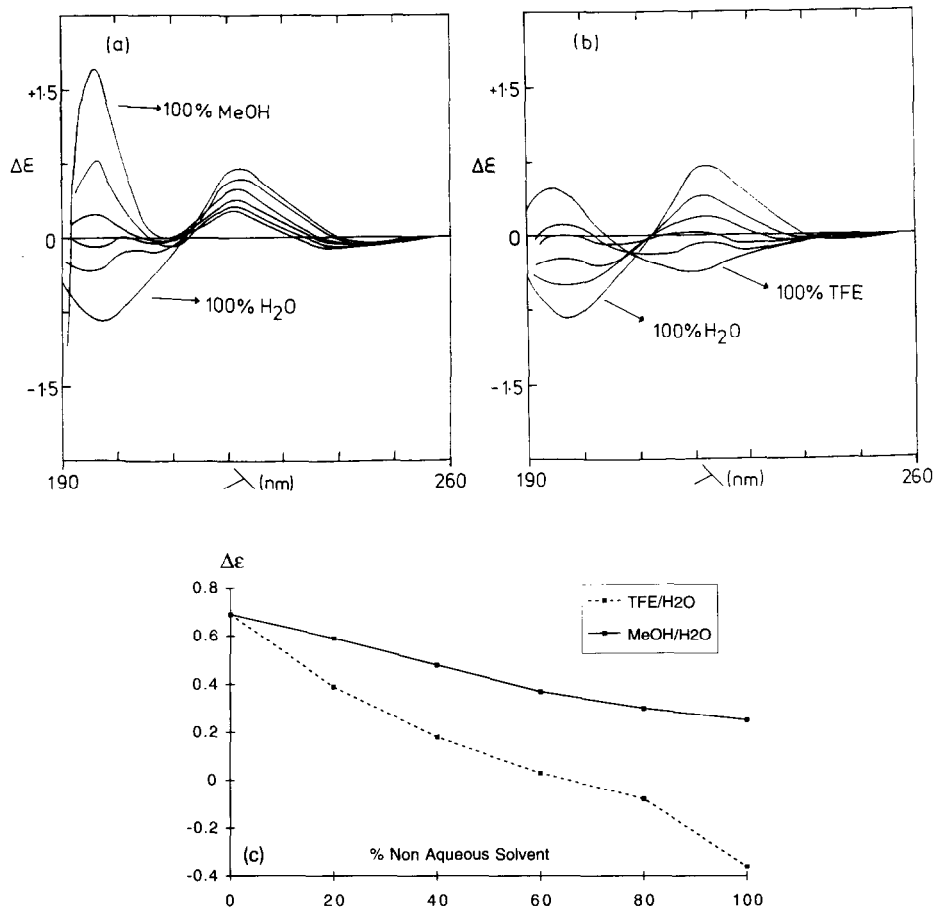


Fig. 2. CD solvent titration between (a) MeOH ↔ H_2O, (b) H_2O ↔ TFE. Each intermediate spectrum corresponds to a 20% change in solvent composition. (c) Plot of $\Delta\varepsilon$ (at 222 nm) versus % non-aqueous solvent for MeOH/H_2O (●) and TFE/H_2O (▲) solvent titrations.

with the frequencies as indicated. Each spectrum represented two major vibrational bands, at 1671–83 cm^{-1} and 1643–57 cm^{-1}, suggesting two predominant conformations in each solvent, from a β-turn component and an additional extended form (see Section 4).

3.3. NMR investigations

Complete proton chemical shift assignments were obtained for RIP in deutero-trifluoroethanol (d^2), deutero-methanol (d^3) and water (H_2O/ D_2O). Table 1 shows the chemical shift values obtained for RIP in each of the solvents. 1D spectra for RIP in the region of the Pro-3 δ(delta) protons, in all three solvents studied, are shown in Fig. 4a. The cis–trans-extended (where applicable) conformations have been identified from 2D ROESY. The extended conformation (δ_e) was absent in non-aqueous solvents. Fig. 4b shows a representative 2D ROESY contour plot in the region of the Pro-3 δ (delta) protons, with 1D plot above, in aqueous solution. Rotating Frame NOE (ROE) and chemical exchange (CE) peaks are as indicated, and were distinguished by opposite phases. Trans isomers

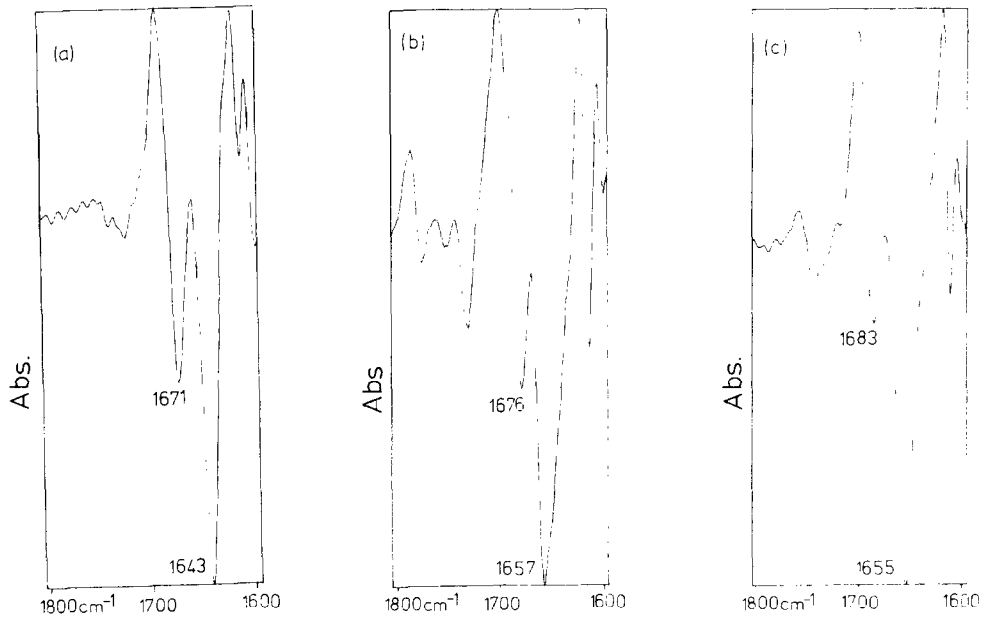


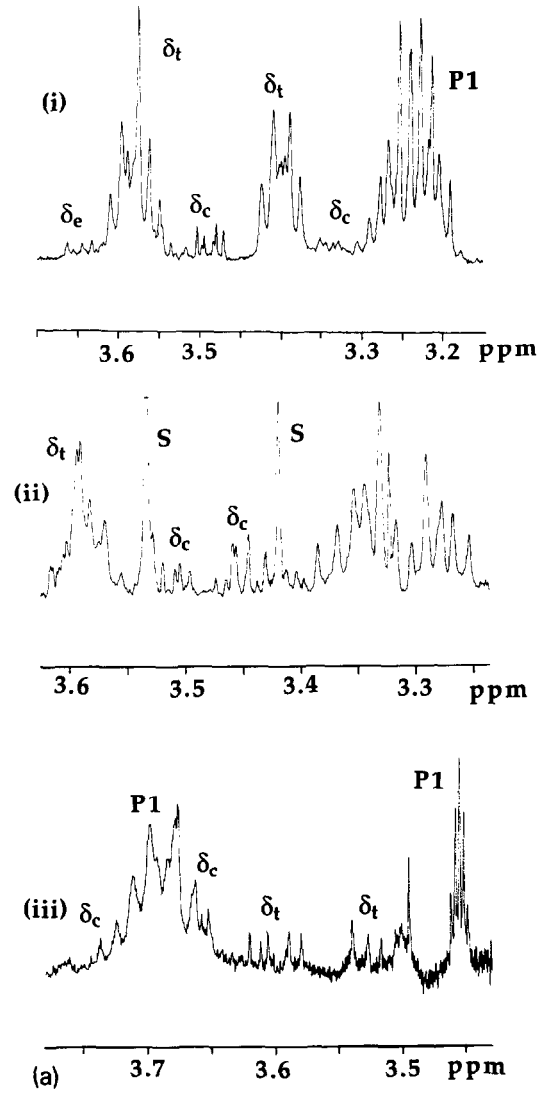
Fig. 3. Second derivative Fourier transform infrared (FTIR) spectra of RIP in (a) D_2O, (b) CF_3CD_2OD and (c) CD_3OD. The positions of the amide I' peptide backbone absorbances are indicated.

(t) were assigned by ROEs to the adjacent α proton (His-2 α), cis isomers (c) were assigned by an absence of these cross-peaks. Two sets of resonance peaks from the Pro-3 δ protons were thus identified (trans and cis) and found to be in chemical exchange from the corresponding cross-peaks. An additional chemical exchange peak was found exclusively in water and was assigned to the resonance from a Pro-3 δ proton in an extended (e) type of conformation (see Sections 3.1 and 4). Similar 2D ROESY plots were observed for RIP in TFE-d^2 and CD_3OH; the extended conformation was not found in these two cases by NMR but may be too small to be observed, or overlapped with other resonances. The large deviations of the Pro-3 δ protons from 'random coil' values, particularly under aqueous conditions, is indicative of folding of the peptide chain and/or hydrogen bonding involving this region of the peptide, confirmed by a number of ROE cross-peaks consistent with the two distinct β-turn conformations. Interestingly, the 'extended' form in water showed chemical shift values for the Pro-3 δ protons close to that of the classical 'random coil' values (3.65 ppm and 3.68 ppm).

4. Discussion

Previous studies [7] showed cis–trans isomerism for RIP at residue Pro-3 amide bond (this is not possible at Pro-1) and it is likely that this effect and the several associated conformations were responsible for the room temperature CD spectrum (Fig. 1). The spectra could not be simulated by the use of 100% β-turn and 'extended' components; since six of the ten amino acids are aromatic there was likely to be a significant contribution from these (confirmed from the near UV CD spectra) and hence no simple spectral simulation was possible. Similar observations were noted in previous studies of the MCH core peptide [1].

Coupled with the results of the FTIR measurements, the CD data were consistent with the view that there was a predominant aqueous form, which was present



in smaller amounts in non-aqueous solvents. The lack of isodichroic points in H_2O/TFE and H_2O/MeOH solvent titrations was consistent with greater than two conformational components in these systems. The nature of these states is the basis of this paper and it will be argued that the spectroscopic data can be best rationalised in terms of three states, characterised by different compositions of three major conformational forms, an extended structure (Polyproline II (P_{II}) helix plus irregular forms) and two β-turn forms, identified from FTIR, associated with cis/trans isomerism of the proline-3 amide bond. The loss of water destabilises the P_{II} extended form leaving the non-aqueous solvents dominated by the two β-turn structures [2] (and thus isodichroic points, from a two-state system, were observed here). The P_{II} conformation has been identified previously [2] as being present, along with irregular forms, in the 'random coil' CD spectrum of peptides, and was identified here from its characteristic FTIR band.

The conformational equilibrium has therefore been rationalised in terms of a predominantly three compo-

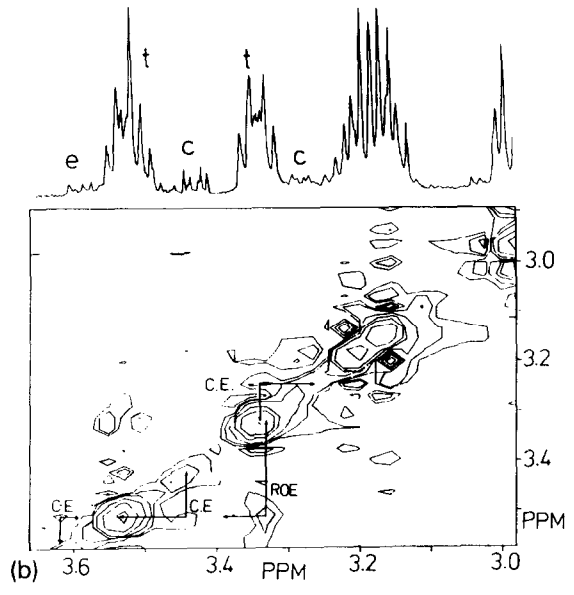
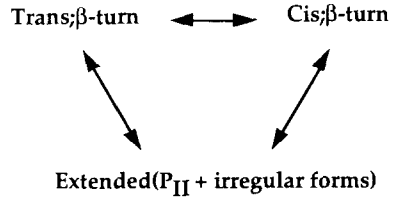


Fig. 4. (a) 1D ^{1}H NMR spectra for RIP in (i) H_2O/D_2O, (ii) TFE and (iii) CD_3OH respectively, in the region of the Pro-3 δ protons. The trans (δ_t), cis (δ_c) and extended forms (δ_e) are as indicated. The 's' in (ii) refers to an impurity from solvent. (b) 2D ROESY spectrum of RIP in H_2O/D_2O (8 : 1) in the region of the Pro (3) and Pro (1) δ (delta) protons, with corresponding one-dimensional spectrum above. t, c and e refer to the trans, cis and extended forms of Pro (3) (see text). Chemical exchange (CE) and rotating frame NOE (ROE) peaks are as indicated and are distinguishable by their different phases.

nent system for RIP in TFE/MeOH/H_2O as shown below

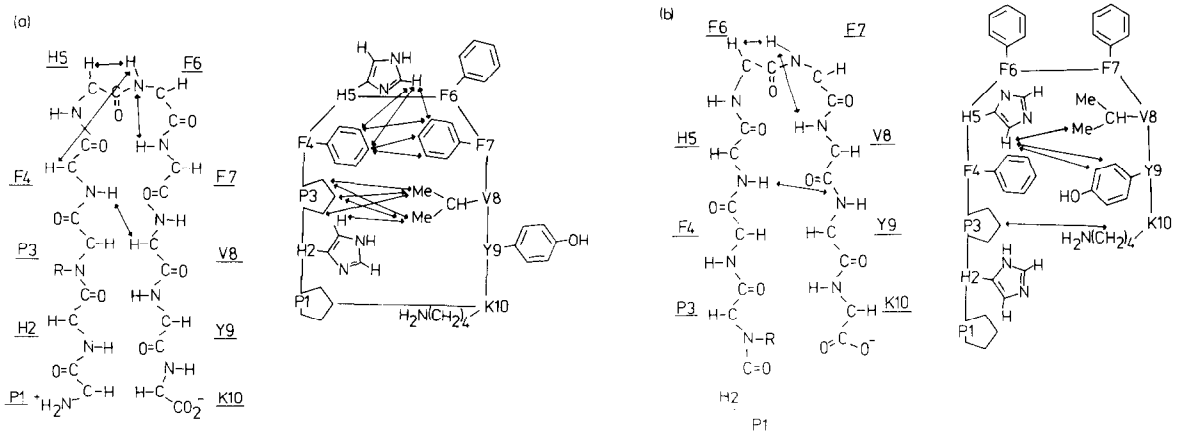


NMR has been able to confirm these conclusions and further refine the β-turn structures. The trans conformation contained a β-turn with a trans amide bond at Pro-3, and was found to predominate by NMR in all three solvents. The cis form (cis Pro-3 amide bond plus β-turn conformation) was present in significant amounts in each solvent (5–15%). The extended structure (poly proline II conformation) was invoked from both FTIR and CD solvent titration studies and detected from chemical exchange ROESY cross-peaks in aqueous solvent (see below). Each of the β-turns detected were associated with a particlar RIP conformation, which gave rise to two distinct classes of ROEs.

The 2D ROESY plots of RIP (Fig. 4) in all three solvents showed chemical exchange cross-peaks between Pro-3 δ protons in cis and trans environments – in aqueous studies an additional peak was observed, in chemical exchange with the other δ protons. This small resonance (~5% by integration) is suggested to be the 'extended' conformation detected by FTIR studies. It was not possible to assign this conformation to a cis or trans amide bond at Pro-3, nor to detect any conformation-specific ROEs at this chemical shift value, presumably because of the very small signal intensity. This extended structure was not found in TFE or methanol NMR studies, but FTIR studies indicated it was present.

By taking cross-sections through the Pro-3 δ trans and δ cis resonances, it was possible to confirm the presence of the two distinct turn conformations and to define approximately the position of the two β-turns within these structures. All other ROEs identified could be classified as belonging to either of these two conformations. The two sets of ROEs are shown in Scheme 1a and 1b. For example, the Pro-3 δ protons (trans) (Scheme 1a) showed a number of close contacts with Val-8 side-chain γ protons in each of the solvents, which defined the approximate position of the β-turn as around the residues 4–5–6–7. Additionally, Pro-1 and Lys-10 ROEs suggested the formation of a salt-bridge ($NH_2^+ \leftrightarrow CO_2$), Phe-4 and Phe-7 benzene rings showed ROEs consistent with a stacking arrangement (responsible for the aromatic dominated CD spectra) and both Phe-4 and -7 residues gave ROEs to the imidazole ring of His-5. A number of other backbone–backbone ROEs (NH–NH and NH–α contacts) also corresponded well with this proposed trans Pro-3 structure.

A cis configuration at Pro-3 amide bond should, by definition, disrupt any structure in the (two) residues preceding in the sequence, and also remove the stabilising effect on the N↔C terminal salt-bridge [13]. Observation of ROEs through Pro-3 cis δ protons (Scheme 1b) indicated that the salt bridge was no longer present, as Pro-3 and Lys-10 were now found to



Scheme 1. Models of RIP structures in solution. (a) Intra-residue ROEs observed for the trans conformational form between backbone/backbone and sidechain/ sidechain contacts (↔ = NOE contact). (b) Intra-residue ROEs observed for the cis conformational form between backbone/ backbone and sidechain/ sidechain contacts (↔ = NOE contact). The ROEs determined are consistent with two different structures and cannot be combined to form only one structure.

be in close proximity, giving the position of the β-turn between residues 5–6–7–8. Several other ROEs were found which agreed with this conformation, such as His-5 ↔ Tyr-9 backbone/backbone and sidechain/ sidechain and Phe-6 ↔ Phe-7 NH–NH and NH–α contacts. None of these ROEs could be incorporated into the structure derived in Scheme 1a, and have been used to determine an approximate conformation for RIP with a cis amide bond at residue Pro-3 (Scheme 1b).

The sets of data presented in detail here for an aqueous environment were also observed, and found to be qualitatively similar, in TFE and methanol i.e. two distinct conformations could be seen and ROEs showed comparable structures to those obtained for RIP in water. Interestingly, in both TFE and methanol-d^3, the His-2 NH proton was exchange-broadened and not visible by ^1H NMR, because of its proximity to the locus of exchange at Pro-3. In H_2O/D_2O solution, the His-2 NH resonance was significantly broadened, but still detectable by NMR. The small differences observed in conformation between solvents were most likely from changes in sidechain rotamer populations, backbone angles and hydrogen-bonding to solvent molecules.

It is hoped to be able to further these studies by recording NMR spectra at reduced temperatures in order to remove flexibility and 'freeze out' one predominant conformation. This should allow a more rigorous set of ROEs to be obtained and to proceed to molecular modelling studies. Following the CD studies, NMR measurements at various TFE/water concentrations may also better define the relative populations of the two β turn conformations.

The investigation of the complex conformational behaviour of such small linear peptides in solution has applications both in the study of the folding/unfolding of proteins and in the biological activity of these peptides. In particular, the flexibility exhibited by RIP in solution may be important in the recognition and binding to the enzyme renin. Synthesis of conformationally constrained analogues based upon spectroscopic and modelling studies may help to determine the important factors involved in eliciting/modifying the desired biological response.

References

[1] G. Siligardi, M.M. Campbell, W.A. Gibbons and A.F. Drake, Eur. J. Biochem. 206 (1992) 23–29.
[2] A.F. Drake, G. Siligardi and W.A. Gibbons, Biophys. Chem. 31 (1988) 143–146.
[3] A. Motta, D. Picone, T. Tancredi and P.A. Temussi, Tetrahedron 44 (1988) 975–990.
[4] J. Burton, K. Poulsen and E. Haber, Biochemistry 14 (1975) 3892–3898.
[5] J. Burton, J.R. Cody, J.A. Herd and E. Haber, Proc. Natl. Acad. Sci. 77 (1980) 5476–5479.

[6] S. Srivastava, R.S. Phadke and G. Govil, Mag. Res. Chem. 27 (1989) 455–459.
[7] G.J. Anderson, C.H. James and W.A. Gibbons, in: Peptides: chemistry, structure and biology, eds. J.E. Rivier and G.R. Marshall (Escom, Leiden, 1989) pp. 964–966.
[8] J.M. Stewart and J.D. Young, in: Solid phase peptide synthesis, 2nd Ed. (Pierce Chemical Co., Rockford, 1984).
[9] D.G. Davis and A. Bax, J. Am. Chem. Soc. 107 (1985) 2820–2821.
[10] A.A. Bothner-By, R.L. Stephens and J-M. Lee, J. Am. Chem. Soc. 106 (1984) 81–813.
[11] G. Siligardi, A.F. Drake, P. Mascagni, D. Rowlands, F. Brown and W.A. Gibbons, Intern. J. Pept. Prot. Res. 38 (1991) 519–527.
[12] P.I. Haris and D. Chapman, Trends Biochem. Sci. 17 (1992) 328–333.
[13] G.E. Schulz and R.H. Schirmer, in: Principles of protein structure (Springer, Berlin, 1979).