CONFORMATIONAL HETEROGENEITY IN LINEAR PEPTIDES IN SOLUTION: A CARBON-13 NMR STUDY OF [Pro^3, Pro^5]-ANGIOTENSIN-II

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Received 10 November 1975

1. Introduction

Angiotensin-II, (Asp–Arg–Val–Tyr–Val (or Ile)–His–Pro–Phe), is a natural pressor agent which has undergone considerable conformational investigation by theoretical energy calculations [1] and by physical [2] as well as by chemical and biochemical techniques [3]. Minimum energy conformational calculations have shown a number of possible folded low energy conformations for angiotensin-II [1]. Electrometric titrations of [Ile^5]-angiotensin-II and of a number of homologs and analogs [4] have been interpreted as indicating that the N- and C-terminal ends of [Ile^5] angiotensin-II are in closer proximity than expected in a random coil conformation. It was further proposed that intramolecular electrostatic interactions are present between the N- and C-termini. Carbon-13 (^{13}C) nuclear magnetic resonance studies on [Ile^5]-angiotensin-II have shown that the His–Pro peptide bond is in the *trans* conformation about the peptide link; ^{13}C spin–lattice relaxation time (T_1) measurements indicated that all the α-carbons in the peptide backbone have almost equal mobility [5]. The T_1 values are compatible with an overall folded conformation.

^{13}C n.m.r. spectroscopy is proving useful in the study of conformationally heterogeneous cyclic and linear peptides in solution [5-11]. ^{13}C Spin–lattice relaxation time studies are gradually increasing our knowledge of the relative mobilities [6,8–10] and rates of rotation about individual bonds in peptides [11] and peptide hormones [5,12]. The purpose of the present study is to investigate the conformational properties of an angiotensin analogue which is not believed to fold to the same extent as does [Ile^5] angiotensin-II [4]. Such studies are necessary in order to relate structure to biological activity. If hormones and analogues can display a number of conformations in solution, determination of the nature and relative importance of the various conformers may lead to greater insight into the conformers which can interact with receptor molecules. Furthermore conformational heterogeneity may be responsible for different activities of peptide hormones in different tissues. The greater dispersion afforded by high field n.m.r. spectrometers has allowed the observation of conformational heterogeneity in [Pro^3,Pro^5]-angiotensin-II. Spin–lattice relaxation times give insight into the relative mobilities of individual amino acyl residues.

2. Materials and methods

[Pro^3,Pro^5]-antiotensin-II was synthesized as previously described [4]. The methods used to obtain ^{13}C chemical shifts and T_1 values are described in reference [10]. The ^{13}C spectra at 90.5 MHz were obtained on a Bruker HX-360 spectrometer at Spectrospin AG, Zürich, with the kind help of Mr W. Schittenhelm. All spectra were obtained in D_2O; 'pH' values were measured directly in D_2O and were corrected for the deuterium isotope effect at the glass electrode [13]. The 'pH' of samples was adjusted using CD_3COOH or NH_4OH dissolved in D_2O.

3. Results and discussion

3.1. *Chemical Shifts*

The ^{13}C chemical shifts of [Pro^3,Pro^5]-angiotensin-II are given in table 1. Assignments were made in part by comparison with those observed in [Ile^5]-angiotensin-II [5]. However, the presence of proline residues in peptides is known to perturb the ^{13}C chemical shifts of residues attached to the nitrogen atom of proline [14]. The 'γ-steric' effect of proline results in an upfield shift of approx. 1 part per million (ppm) in the α-carbon of the residue preceeding proline. To confirm the assignments of the various carbon resonances we varied the 'pH' of the sample sufficiently to titrate the aspartic acid, phenylalanyl and histidyl residues. The influence of pH on the ^{13}C chemical shifts in a peptide is confined almost exclusively to the residue undergoing titration [1,15]. In the case of [Pro^3,Pro^5]-angiotensin-II this method is not unambiguous because the pK_a values of the α-carboxyl group of phenylalanine (pK_a = 3.34) and the β-carboxyl group of aspartic acid (pK_a = 3.02) are similar [4]. We therefore studied the dipeptide Asp–Phe-NH_2 and titrated only the aspartic acid residue which allowed us to determine the chemical shifts of the aspartyl residue in a peptide at all 'pH' values. The ^{13}C chemical shifts of the aspartyl residue in Asp–Phe-NH_2 at 'pH' 0.7 are as follows (in ppm): Asp α-CH, 50.39. β-CH_2, 35.72; γ-C=O, 173.54; carbonyl C=O, 169.33. Titration of the carboxyl group followed by that of the amino group yields changes in aspartyl resonances only: α-CH, + 2.8; β-CH_2, + 7.6; γ C=O, + 4.8; carbonyl, + 10.4 ppm (a positive sign indicates a downfield shift). The phenylalanyl residue is

Table 1
^{13}C Chemical shifts[a] in [Pro^3,Pro^5]-angiotensin II in D_2O

Residue		'pH' 1.8	'pH' 5.2	'pH' 9.6	Residue		'pH' 1.8	'pH' 5.2	'pH' 9.6
Asp	αCH	50.67	51.59	53.40	Pro	αCH	61.58	61.89	61.48
	βCH_2	36.03	38.19	43.32		βCH_2	30.36	30.21	30.05
	γC=O	173.77	176.97	177.63		γCH_2	25.64	25.59	25.50
	C=O	169.41	170.33	≃172.8		δCH_2	49.03	48.90	48.94
						C=O	174.51	174.40	174.4
Arg	αCH	52.58	52.43	52.71	His	αCH	51.45	51.59	51.97
	βCH_2	28.59	28.57	28.89		βCH_2	26.98	27.13	29.30
	γCH_2	25.06	25.02	25.02		γC	129.13	129.02	126.64
	δCH_2	41.73	41.70	41.74		δCH	134.53	134.68	137.28
	ζC		157.79			εCH	118.77	118.79	119.19
	C=O	172.15	172.21	≃172.8		C=O	170.67	170.91	≃172.8
Pro	αCH	61.58	61.61	61.48	Pro	αCH	61.58	62.04	61.86
	βCH_2	30.36	30.21	30.05		βCH_2	30.36	30.21	30.05
	γCH_2	25.64	25.59	25.50		γCH_2	25.64	25.59	25.50
	δCH_2	49.03	48.90	48.94		δCH_2	49.03	48.90	48.94
	C=O	174.51	174.40	174.4		C=O	174.88	173.95	173.9
Tyr	αCH	54.00	53.91	53.94	Phe	αCH	55.24	57.31	57.23
	βCH_2	36.72	36.71	36.86		βCH_2	37.51	38.48	38.60
	γC	129.13	129.28	127.75		γC	137.58	138.52	138.57
	δCH	131.85	131.82	131.86		δCH	130.38	130.47	130.49
	εCH	116.50	116.47	117.15		εCH	129.82	129.57	129.64
	ζC	155.55	155.60	157.80		ζCH	128.25	127.85	127.93
	C=O	172.50	172.44	≃172.8		C=O	176.70	178.60	178.52

[a]Chemical shifts are reported in parts per million downfield from external $(CH_3)_4Si$. Accuracy of chemical shifts ± 0.05 ppm. Carbonyl carbon assignments are tentative. 'pH' values are pH-meter readings in D_2O uncorrected for the deuterium isotope effect.

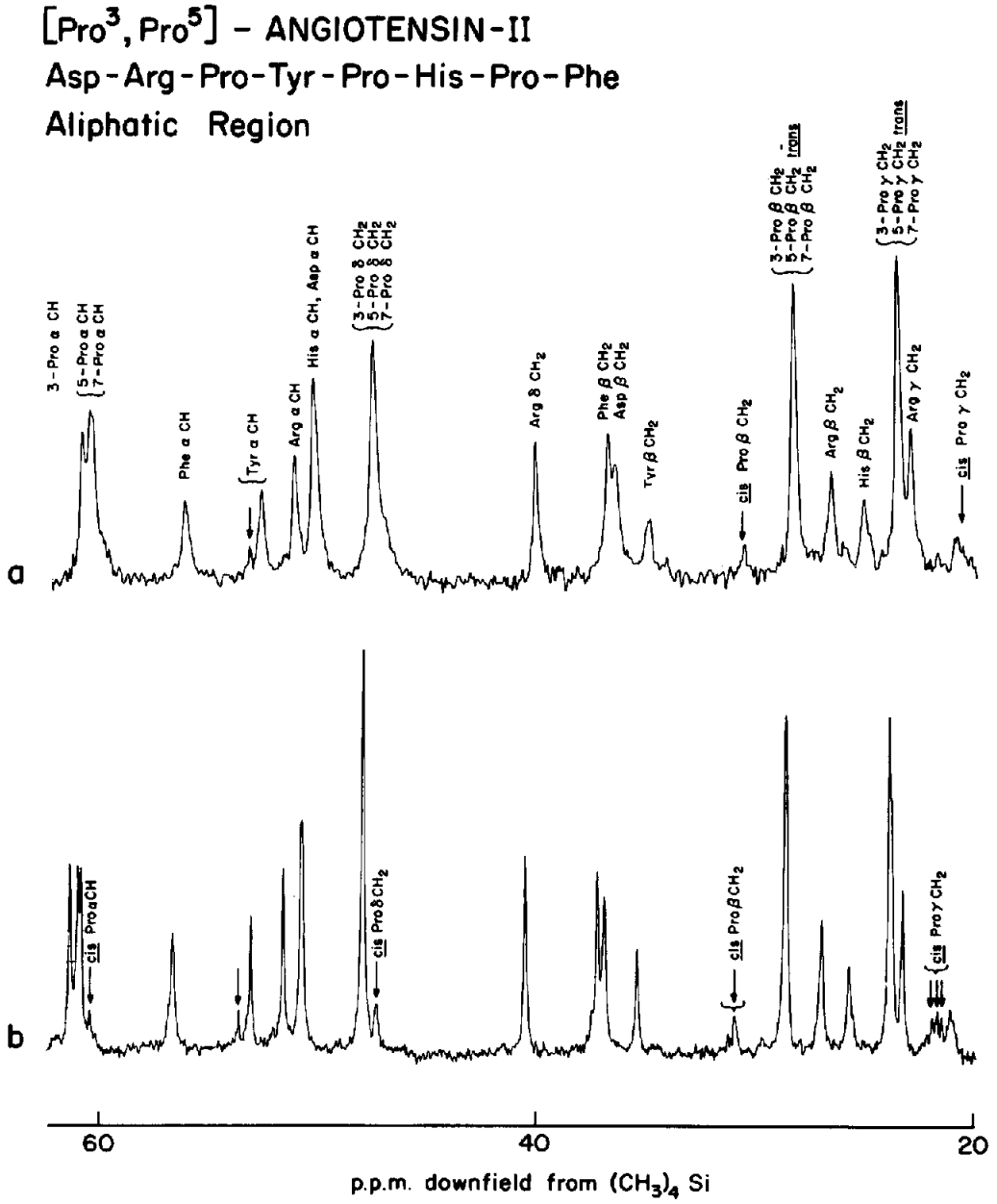
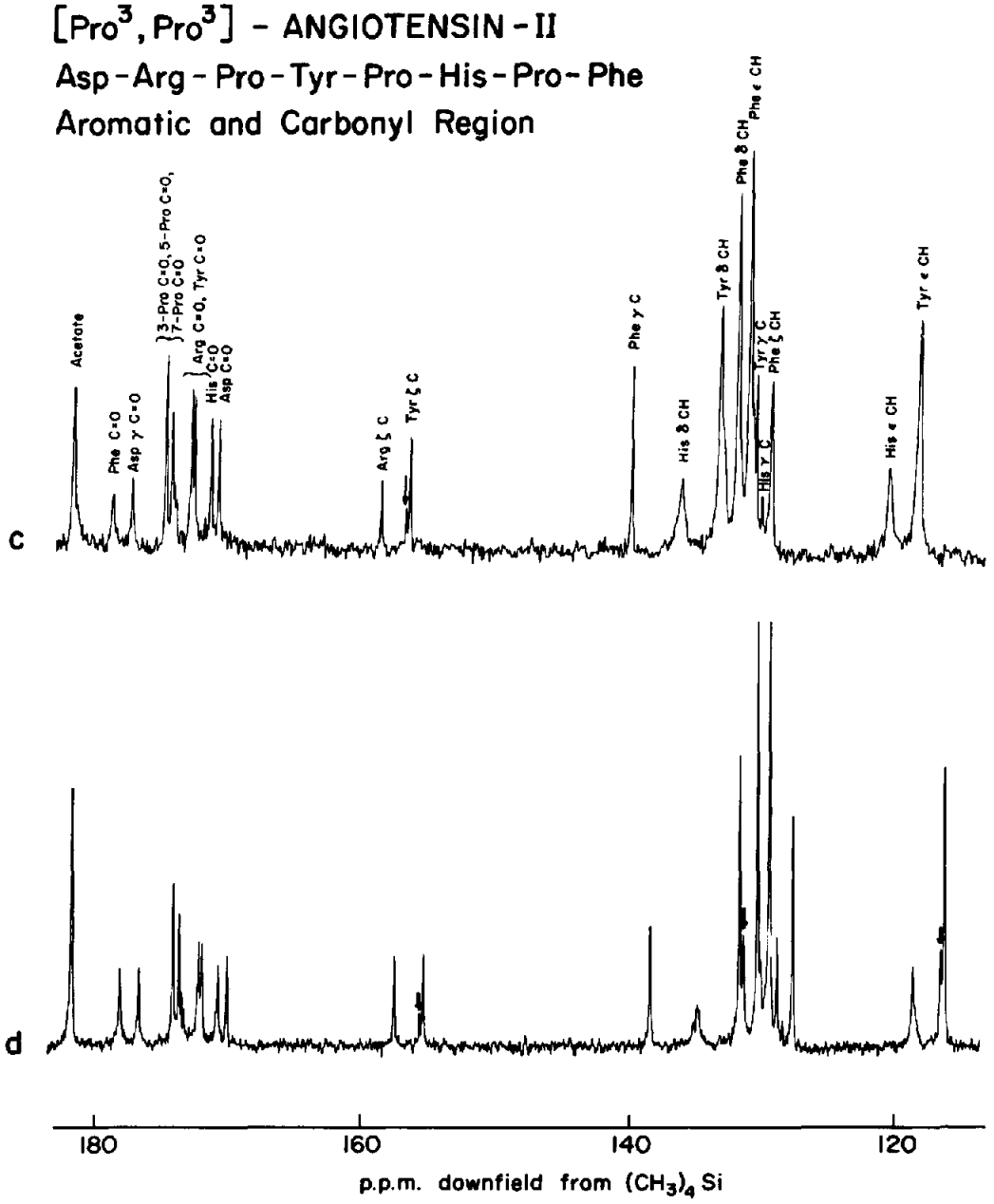


Fig.1. ^{13}C Spectra of [Pro³,Pro⁵]-angiotensin II (a) Spectral region between 20 and 65 ppm downfield from $(CH_3)_4Si$, 25.2 MHz, 'pH' 5.2, 32 000 scans, 32°C. (b) Same spectral region as in (a), 90.5 MHz, 'pH' 6.0, 39 000 scans, 20°C. (c) Spectral region between 100 and 200 ppm from $(CH_3)_4Si$, other conditions as in (a). (d) Same spectral regions as in (c), other conditions as in (b).


[Pro3, Pro3] - ANGIOTENSIN - II
Asp - Arg - Pro - Tyr - Pro - His - Pro - Phe
Aromatic and Carbonyl Region
Acetate
Phe C=O
Asp γ C=O
3-Pro C=O, 5-Pro C=O,
7-Pro C=O
Arg C=O, Tyr C=O
His C=O
Asp C=O
Arg ζ C
Tyr ζ C
Phe γ C
His δ CH
Tyr δ CH
Phe δ CH
Phe ε CH
Tyr γ C
Phe ζ CH
His γ C
His ε CH
Tyr ε CH
c
d
180
160
140
120
p.p.m. downfield from (CH3)4 Si

unperturbed and its chemical shifts are similar to those in [Pro^3,Pro^5]-angiotensin-II at 'pH' 1.8: α-CH, 56.39; β-CH_2, 37.85; aromatic carbons, 128.34, 129.88, 130.31, 137.37; carbonyl, 176.13 ppm.

Figure 1a) shows the ^{13}C spectrum of [Pro^3,Pro^5]-angiotensin-II at 25.2 MHz. At this frequency most of the resonances of the three prolyl residues are degenerate. A number of weak 'extra' resonances are present in the spectrum. The resonances which occur at 23.2 and 32.4 ppm are typical of *cis* isomers of proline [16]. The approximate amounts of these isomers, as measured from peak intensities vary between 10 and 15%, the larger amount being observed at higher 'pH'. At 25.2 MHz the *cis* proline resonances from individual proline residues are not resolved and one cannot distinguish whether or not all three proline residues show 10–15% *cis* isomer or whether only one residue occurs with 30–45% *cis* isomer. Based on studies of other peptides and hormones [7] the former possibility appears more likely. This is confirmed by the 90.5 MHz spectrum, fig.1b. The γ carbon of proline shows three resonances for the three *cis* isomers. The fourth weak resonance at highest field is that of residual acetate. Caution must be exerted when assigning resonances of minor intensity near 23 ppm to *cis* isomers of proline in peptides as often residual acetate will occur in the same region. However the ambiguity is readily resolved by measurement of spin-lattice relaxation times. The acetate methyl groups show much longer T_1 values than do *cis* isomers of proline. The T_1 values of *cis* and *trans* isomers of proline are of the same order of magnitude [17,18]. Doubling of lines is also apparent on the tyrosyl residue which in [Pro^3,Pro^5]-angiotensin-II is situated between two prolyl residues. These extra resonances may result from a *cis* isomer of tyrosine or simply be a long-range steric effect of the *cis* isomers of proline on the chemical shift of tyrosine.

Comparing spectra of [Pro^3,Pro^5]-angiotensin-II with those of [Ile^5]-angiotensin-II [5] at the same 'pH' we can evaluate the effect of the proline-substitutions on the ^{13}C chemical shifts. The α-, β-, γ and carbonyl carbons of arginine were shifted upfield by 2.1, 1.0, 0.5 and 1.5 ppm respectively. The tyrosyl residue also showed changes: α-CH, −2.1; β-CH_2, −0.6; γ-C, + 0.2; δ-CH, + 0.2; carbonyl −1.5 ppm. All other resonances were affected by less than 0.2 ppm.

Table 2
^{13}C Spin–lattice relaxation times in [Pro^3,Pro^5]-angiotensin in D_2O*

Position	Residue	α	β	γ	δ	ε	ζ
1	Asp	275[a]	300				
		(500)	(500)				
2	Arg	185	(200	270	370		
		(270)	(420)	(620)	(730)		
3	Pro	165[b]	210[c]	390[d]	240[e]		
		(280[b])	(420[c])	(540[d])	(340[e])		
4	Tyr	160	190	–	235	225	
		(240)	(300)	–	(430)	(350)	
5	Pro	165[b]	210[c]	390[d]	240[e]		
		(280[b])	(420[c])	(540[d])	(340[e])		
6	His	200[a]	180	–	285	215	
		(235)	(360)	–	(450)	N.O.	
7	Pro	190	210[c]	390[d]	240[e]		
		(260)	(420[c])	(540[d])	(340[e])		
8	Phe	236	270	–	335	335	280
		(365)	(470)	–	(720)	(660)	(430)

*NT_1 values measured at 67.8 MHz are given in msec; N is the number of directly-bonded hydrogens. The hormone concentration was 76 mg/ml in D_2O, 'pH' 4.1, 25°C. The NT_1 values in parentheses were obtained at 'pH' 1.1, 40°C; Superscripts indicate partially or completely overlapping resonances.

3.2. ^{13}C *Spin–lattice relaxation times*

The ^{13}C spin–lattice relaxation times of [Pro^3, Pro^5]-angiotensin-II obtained at 67.9 MHz are given in table 2. The data show that the T_1 values for all parts of the backbone structure of [Pro^3,Pro^5]-angiotensin are quite similar with the exception of the terminal residue which show longer T_1 values. The T_1 values of the aspartyl residue are longer than those of the phenylalanyl residue, indicating greater mobility at the N-terminus than at the C-terminus under both sets of conditions. Segmental motion in arginine is not inhibited by the adjacent prolyl residue nor is rotation about the axis traversing the C_γ–C_ζ bond of the tyrosyl residue. However motion about this axis in tyrosine is slower than in the C-terminal phenylalanyl residue, which was also found for [Ile^5]-angiotensin-II [5]. The prolyl residues show intracyclic motion which is most pronounced at the γ-carbon. The β- and δ-carbons of proline are equally mobile and more mobile than the α-carbon. Although direct comparison of the above data with those obtained for [Ile^5]-angiotensin-II should not be made due to the differences in experimental conditions, we can conclude that the terminal α-carbons in [Pro^3,Pro^5]-angiotensin-II show greater freedom relative to the non-terminal carbons than do the terminal α-carbons in [Ile^5]-angiotensin-II. This may be a consequence of loosening of the peptide backbone in [Pro^3,Pro^5]-angiotensin-II when compared to [Ile^5]-angiotensin-II. The difficulty of accurate temperature regulation on the high field instruments (270 and 360 MHz) during the T_1 experiments makes direct comparison of data obtained at 'pH' 4.1 and pH 1.1 difficult. However, within this uncertainty, it is apparent that the mobilities of all carbon atoms are considerably greater at 'pH' 1.1. An increase in the rate of segmental motion occurs for the arginyl, histidyl, and phenylalanyl residues, but little change occurs for the aspartyl, prolyl, and tyrosyl residues.

4. Conclusion

Our studies have shown that linear peptides can show considerable conformational heterogeneity in solution. Such facts should be considered when testing the biological and pharmacological properties of hormone analogs. It may thus be possible to relate activity to the relative proportions and to the accessibility of specific conformers in linear peptide hormones. The accessiblity of a number of conformations indicates that these conformations differ only slightly in energy. Thus, the energy involved in binding to the receptor site may well be sufficient to force the peptide into a single conformation which is not necssarily one of those observed for the unbound hormone.

The T_1 measurements have shown that the side chains of the residues adjacent to the prolyl residues have not undergone any significant motional restriction due to the presence of the cyclic residues. However the α-carbons of the terminal residues appear to be more flexible than those located in non-terminal positions. This may be a consequence of a looser overall backbone conformations in [Pro^3, Pro^5]-angiotensin-II than in [Ile^5]-angiotensin-II.

Acknowledgements

We are grateful to Bruker Spectrospin (Canada) Ltd. and Bruker AG for their hospitality and the opportunity to use the HX-360 spectrometer in Zürich.

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