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Conformational Flexibility of Angiotensin II. A Carbon-13 Spin–Lattice Relaxation Study†

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ABSTRACT: Carbon-13 spin–lattice relaxation times (T_1) have been determined for the carbons in the octapeptide hormone [5-isoleucine]-angiotensin II in aqueous solution. Two possible models for molecular motion are considered: isotropic overall motion of the hormone with internal motion of some residues and anisotropic overall molecular motion. The data are interpreted in detail using the former model. The α carbons of the peptide backbone are all equally restricted in their motion. The correlation time for overall molecular reorientation, calculated from an average T_1 value of 95 msec for the α carbons in the peptide backbone, is *ca.* 5×10^{-10} sec. The carbons in the side chains are more mobile than those in the peptide backbone, with the exception of the side chain of the Tyr residue which does not undergo rapid segmental motion. We propose that [5-isoleucine]-angiotensin II has a restricted backbone conformation and that the α carbons of the N- and C-terminal residues are constrained to nearly the same extent as the remaining α carbons in the peptide backbone. Chemical shift data indicate that the Pro residue adopts the trans conformation about the His-Pro bond and that the imidazole ring of His has a strong preference for the N^{τ}-H tautomer.

The conformations of linear peptide hormones have been more difficult to elucidate than those of cyclic peptides of similar size due to the larger number of degrees of freedom of the peptide backbone. The conformations of angiotensin II, Asp-Arg-Val-Tyr-Val(or Ile)-His-Pro-Phe, a natural pressor agent, and its synthetic analog angiotensinamide II, Asn-Arg-Val-Tyr-Val(or Ile)-His-Pro-Phe,[1] have been extensively investigated by thin film dialysis (Craig *et al.*, 1964; Franze de Fernandez *et al.*, 1968), gel filtration (Ferreira *et al.*, 1969), ultracentrifugation (Paiva *et al.*, 1963), hydrogen–deuterium exchange (Paiva *et al.*, 1963), hydrogen–tritium exchange (Printz *et al.*, 1972b), infrared and Raman spectroscopy (Fermandjian *et al.*, 1972a,b), circular dichroism (Fermandjian *et al.*, 1972a,b), and ^{1}H nuclear magnetic resonance spectroscopy (^{1}H nmr) (Vine *et al.*, 1973; Glickson *et al.*, 1973; Marshall *et al.*, 1973; Weinkam and Jorgensen, 1971; Bleich *et al.*, 1973b). A number of conformations have been proposed for angiotensinamide II such as the random coil (Paiva *et al.*, 1963), the antiparallel β-pleated sheet (Fermandjian *et al.*, 1972a,b), the α-helix (Smeby *et al.*, 1962), the γ-turn (Printz *et al.*, 1972a; Bleich *et al.*, 1973a), as well as other conformations peculiar to this peptide (Weinkam and Jorgensen, 1971). Fermandjian and coworkers (1972a,b) have stressed the point that the conformation of angiotensamide II varies greatly depending on experimental conditions. A recent ^{1}H nmr study by Glickson *et al.* (1973) revealed that angiotensin either exists in a unique conformation, different from the α-helix, the β-turn, and γ-turn models, or is in rapid equilibrium ($>10^3$ sec^{-1}) between various conformations. ^{1}H nmr spectra also reveal conformational transitions associated with titration of the α-amino and/or imidazole group as well as with titration of the phenol group in tyrosine. The exact nature of the conformational transitions has not been elucidated (Glickson *et al.*, 1973).

Zimmer *et al.* (1972) have investigated the structure of [5-valine]-angiotensinamide II in aqueous medium using ^{13}C nmr spectroscopy. ^{13}C resonances were identified on the basis of the titration behavior of specific peaks, on the chemical shifts of resonances in the constituent amino acids, as well as by comparison with the spectrum of the C-terminal tetrapeptide. All the pK values obtained for the titratable groups in [5-valine]-angiotensinamide II were determined by following the pH dependence of the ^{13}C chemical shifts. The ^{13}C spectra did not give any evidence of conformational rearrangements as a function of changes in pH similar to those observed in the ^{1}H nmr spectra.

The present ^{13}C nmr studies were undertaken in order to measure the spin–lattice relaxation times (T_1) of the backbone and side-chain carbons in [5-isoleucine]-angiotensin II and the antagonist [5-isoleucine,8-leucine]-angiotensin II.

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[1] The nomenclature used in this paper follows the Recommendations of the IUPAC-IUB Commission on symbols for amino acid derivatives and peptides ((1972), *J. Biol. Chem. 247,* 977).

These values are compared with each other and with those obtained for linear (Keim *et al.*, 1973a,b; 1974; Gurd *et al.*, 1972) and cyclic peptides (Allerhand and Komoroski, 1973; Deslauriers *et al.*, 1974a–c) in order to deduce the conformational characteristics of the peptide backbone and the degree of segmental motion[2] of side chains in [5-isoleucine]-angiotensin II.

Experimental Section

Materials. [5-Isoleucine]-angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and [5-isoleucine,8-leucine]-angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Leu) as well as the lower homologs of angiotensin II, Tyr-Ile-His-Pro-Phe, Val-Tyr-Ile-His-Pro-Phe • HCl, and Arg-Val-Tyr-Ile-His-Pro-Phe, were prepared by solid phase synthesis (Merrifield, 1963; Stewart and Young, 1969; Paiva *et al.*, 1973, 1974). The peptides were purified by countercurrent distribution and ion exchange chromatography. The criteria for purity were: (a) the amino acid analysis of acid hydrolysates yielded a molar ratio within 3% of the theoretical value for each amino acid; (b) the peptide content determined by amino acid analysis, spectrophotometry (ϵ_{275} 1375), and titration agreed within 1%; (c) only one spot was detected with Pauly, ninhydrin, and Sakaguchi reagents after the tlc of a 0.1-mol sample with three solvents and high voltage paper electrophoresis with three different buffers (pH 2.8, 4.9, and 9.9).

Methods. Proton-decoupled ^{13}C nuclear magnetic resonance spectra were obtained on a Varian CFT-20 spectrometer and on a Varian XL-100-15 spectrometer in the Fourier transform mode, using a Varian 620L computer with 16K memory. Spin-lattice relaxation time (T_1) measurements were performed by the inversion–recovery method as described by Freeman and Hill (1970) using a pulse sequence (180°–τ–90°– T_∞ . . .) where τ is a variable delay time and T_∞ is at least five times longer than the longest T_1 to be measured. The accuracy of the T_1 values is ±15%. T_1 values were determined by a least-squares fit of a semilogarithmic plot to the best straight line. Samples were studied in tubes of outside diameter 12 mm (XL-100) or 10 mm (CFT-20). Chemical shifts are reported downfield from external tetramethylsilane ($(CH_3)_4Si$) contained in a concentric inner tube of outside diameter 5 mm. Spectra were obtained at 32°. pH values reported in tables and figures are uncorrected pH meter readings taken in D_2O (pD = pH meter reading + 0.4; Glascoe and Long, 1960). CD_3COOH and HCl diluted in D_2O were used to adjust the pH of samples. Samples used for T_1 measurements were run at a concentration of 100 mg/ml. Nuclear Overhauser enhancements (NOE) were measured by comparing the integrated intensities of the ^{13}C resonances in proton noise-decoupled spectra and coupled spectra. Integrations were performed separately on the carbonyl carbons and on the carbons of aromatic residues. The α- and β-carbon regions were integrated as units. This was necessary due to the complexity of the coupled spectra. The proton-bearing α, β, and aromatic carbons yielded enhancements of 3.0 ∓ 0.1.

Results

Spectral Assignments. The calculated and observed ^{13}C chemical shifts of [5-isoleucine]-angiotensin II and related peptides (Figure 1) are given in Table I. The calculated ^{13}C chemical shifts for [5-isoleucine]-angiotensin II were based on the chemical shifts of free amino acids in D_2O (Horsley *et al.*, 1970; Voelter *et al.*, 1971) which were then corrected for incorporation into a peptide (Christl and Roberts, 1972; Keim *et al.*, 1973a,b; 1974) as well as for the effect of amino acid titration (Quirt *et al.*, 1974, Freedman *et al.*, 1971; Reynolds *et al.*, 1973; Christl and Roberts, 1972; Gurd *et al.*, 1971; Keim *et al.*, 1973a). The assignments of the carbon resonances in the pentapeptide Tyr-Ile-His-Pro-Phe were based on the chemical shifts calculated for the peptide in a random coil conformation as well as on the perturbations caused by titration of various residues. The assignments of resonances for larger homologs were based on those of the next smaller peptide, corrected for the addition of one new residue. Spectral changes were found to occur only in the penultimate residue, as each additional residue was added. Changes in pH produced no effects other than the chemical-shift changes expected from titration of various residues. The observed ^{13}C chemical shifts of [5-isoleucine]-angiotensin II and those calculated for a random coil of similar sequence agree within *ca.* 1.0 ppm. The proline residue is in the trans conformation about the His-Pro bond as judged by a chemical shift of 25.5 ppm for the γ carbon (Dorman and Bovey, 1973). In [5-isoleucine)-angiotensin II and the shorter oligomers examined in this work, no resonance occurred at 23.4 ± 0.3 ppm which would be characteristic of the cis isomer about the His-Pro bond. We therefore conclude that the conformation about the His-Pro bond in [5-isoleucine]-angiotensin II is 100% trans. The chemical-shift changes which occurred upon changing the pH of the solution of [5-isoleucine]-angiotensin II from 4.5 to 10.4 are characteristic of a *ca.* 4:1 predominance of the N^τ–H tautomer of histidine at basic pH (Reynolds *et al.*, 1973) similar to the preference found in thyrotropin-releasing hormone (Deslauriers *et al.*, 1974a). The C-5 and C-2 resonances of the imidazole ring shifted downfield 4.6 and 2.6 ppm, respectively. The calculated carbonyl carbon chemical shifts agree within <1.0 ppm with the experimental values and therefore if intramolecular hydrogen bonding of the carbonyl carbons does occur it does not perturb the ^{13}C chemical shifts to an appreciable extent.

^{13}C Spin-Lattice Relaxation Time Measurements. The NT_1 values of individual carbon atoms in [5-isoleucine]-angiotensin II in D_2O are given in Figure 2, where N is the number of directly attached hydrogens. The T_1 values for the backbone are remarkably similar, *i.e.* all values are between 74 and 110 msec. This contrasts with the observation of Keim *et al.* (1973a,b; 1974) on Gly-Gly-X-Gly-Gly pentapeptides (X being any amino acid) where differences of factors of 2–4 were observed in the NT_1 values of the C_α of the X residue and the terminal residues. The C- and N-terminal residues have T_1 values slightly longer than most of the α carbons. At pH 4.5 the C_α resonance of the N-terminal aspartic acid residue overlapped with that of the histidine residue and only an average T_1 value could be measured. It was not possible to determine T_1 values at neutral pH due to insufficient solubility of the peptide. Decreasing the pH of the sample to 1.0 using dilute HCl allowed resolution of the histidine and aspartic acid C_α resonances. This pH change caused an overall increase in T_1 values of ~50% for the backbone carbons as well as for the side-chain carbons.

The NT_1 values for the angiotensin antagonist [5-isoleu-

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[2] Segmental motion is defined as the motion of two moieties of fixed conformation about a bond connecting the moieties. An implicit assumption is that the motion is independent of the conformations of the two moieties with respect to all other bonds, and that these other conformations do not change during motion about the bond in question.

Table I: Calculated and Observed ^{13}C Chemical Shifts of [Ile^5]-Angiotensin II and Related Peptides.[a]

	Calcd Chem Shifts at pH	Angiotensin II at pH		[Leu^8]Angiotensin II at pH			Peptide 4–8 at pH		Peptide 3–8 at pH		Peptide 2–8 at pH				
	4.5	4.5	10.4	1.0	3.9	4.9	3.5	6.3	2.9	5.4	1.1	3.9	4.5	5.1	6.5
Asp α-CH	51.9	51.61	53.74	50.64	51.62	51.72									
β-CH$_2$	37.8	37.96	43.64	36.06	38.00	38.33									
γ-C=O	178.4	177.01	N.O.	173.44	176.42	177.01									
C=O	170.1	170.57	N.O.	169.35	170.26	170.65									
Arg α-CH	54.5	54.54	54.45	54.69	54.59	54.55					53.76	53.64	53.64	53.63	53.91
β-CH$_2$	28.5	29.37	29.59	29.44	29.43	29.46					29.35	29.29	29.30	29.30	29.88
γ-CH$_2$	24.8	25.53	25.56	25.63	25.51	25.48					24.57	24.52	24.52	24.51	24.62
δ-CH$_2$	41.7	41.67	41.86	41.78	41.74	41.77					41.66	41.52	41.52	41.52	41.63
ζ-C	157.5	157.86	N.O.	N.O.	157.90	157.88					157.88	157.83	N.O.	157.83	157.85
C=O	173.7	173.68	N.O.	173.44	173.41	173.60					170.76	170.63	170.90	171.00	171.70
Val α-CH	60.1	60.39	60.62	60.46	60.47	60.44			59.73	59.70	60.59	60.44	60.47	60.47	60.48
β-CH	30.8	31.59	31.53	31.61	31.60	31.60			31.51	31.50	31.52	31.56	31.54	31.47	31.56
γ-CH$_3$	19.1	19.51	19.70	19.59	19.58	19.59			19.01	19.00	19.59	19.47	19.54	19.53	19.55
γ-CH$_3$	17.9	19.01	19.12	19.08	19.02	19.10			18.33	18.30	19.10	18.95	18.97	18.97	19.04
C=O	173.8	173.68	N.O.	173.44	173.41	173.60			171.10	171.06	173.61	173.28	173.53	173.54	173.62
Tyr α-CH	56.8	55.81	56.13	55.90	55.89	55.84	54.99	55.33	56.41	56.40	56.00	55.90	55.99	55.99	56.15
β-CH$_2$	37.5	37.29	37.54	37.31	37.70	37.29	36.89	37.38	37.58	37.60	37.37	37.34	37.33	37.31	37.44
C-1		129.08	126.54	129.27	129.14	129.35	125.99	126.54	129.08	129.10	129.27	129.11	129.20	129.12	129.04
C-2	130.5	131.61	131.52	131.71	131.70	131.70	131.70	131.85	131.89	131.80	131.79	131.66	131.67	131.67	131.75
C-3	117.5	116.46	117.73	116.50	116.50	116.47	116.61	116.81	116.84	116.70	116.58	116.41	116.43	116.42	116.51
C-4	156.4	155.63	N.O.	155.62	155.66	155.63	156.21	156.27	156.02	156.00	155.62	155.59	155.59	155.58	155.68
C=O	173.5	173.68	N.O.	173.44	173.41	173.60	170.51	170.45	173.60	173.40	173.61	173.28	173.53	173.54	173.62
Ile α-CH	60.0	58.97	58.93	59.09	59.03	58.97	59.07	59.08	59.05	59.00	59.07	58.99	59.00	58.99	58.81
β-CH	36.8	37.29	37.54	37.31	37.31	37.29	37.20	37.38	37.58	37.60	37.37	37.34	37.33	37.62	38.29
γ-CH$_2$	25.7	25.53	25.56	25.63	25.51	25.75	25.40	25.53	25.84	25.90	25.73	25.60	25.60	25.60	25.58
γ-CH$_3$	15.9	15.79	15.88	15.99	15.82	15.79	15.39	15.56	15.99	15.90	15.86	15.74	15.76	15.75	15.76
δ-CH$_3$	12.5	11.10	11.22	11.10	11.08	11.09	10.96	11.17	11.29	11.20	11.10	11.06	11.06	11.05	11.14
C=O	173.5	173.68	N.O.	173.44	173.41	173.60	172.95	173.09	173.40	173.20	173.61	173.28	173.53	173.54	173.62
His α-CH	52.7	51.61	52.98	51.54	51.62	51.72	51.27	51.95	51.90	51.90	51.54	51.41	51.50	51.57	52.27
β-CH$_2$	26.9	27.13	29.59	27.07	27.10	27.91	26.75	27.73	27.10	27.40	27.03	27.08	27.14	27.17	28.71
γ-C-5		129.08	133.70	129.27	129.14	129.35	128.78	N.O.	129.30	129.56	N.O.	N.O.	N.O.	129.41	N.O.
C-2	134.7	134.64	137.26	134.73	134.75	134.83	134.49	135.49	134.90	135.04	134.73	134.69	134.69	134.78	136.15
C-4	118.6	118.79	118.74	118.89	118.90	118.90	118.62	118.80	119.10	119.10	118.90	118.86	118.78	118.78	118.78
C=O	171.5	170.96	N.O.	170.42	170.41	170.65	169.81	171.50	170.20	169.98	170.19	170.03	170.21	170.23	172.60
Pro α-CH	61.4	61.87	61.99	61.75	61.76	61.79	61.47	61.87	61.90	62.20	61.68	61.83	61.93	61.94	61.94
β-CH$_2$	30.6	30.33	30.26	30.66	30.62	30.61	30.16	30.20	30.70	30.62	30.54	30.38	30.31	30.37	29.88
γ-CH$_2$	25.6	25.53	25.56	25.63	25.51	25.48	25.40	25.53	25.90	25.84	25.73	25.60	25.60	25.60	25.58
δ-CH$_2$	48.9	49.10	49.10	49.34	49.34	49.36	48.93	49.11	49.50	49.40	49.28	49.15	49.16	49.15	49.15
C=O	173.0	173.98	N.O.	175.04	174.46	174.38	174.14	173.89	175.00	174.08	174.96	174.03	174.04	174.02	174.12

Table I: *(Continued)*

	Calcd Chem Shifts at pH	Angiotensin II at pH		[Leu^8]Angiotensin II at pH			Peptide 4–8 at pH		Peptide 3–8 at pH		Peptide 2–8 at pH				
	4.5	4.5	10.4	1.0	3.9	4.9	3.5	6.3	2.9	5.4	1.1	3.9	4.5	5.1	6.5
Phe α-CH	58.0	56.89	57.19				55.98	57.30	56.00	56.41	55.33	55.66	57.15	57.35	57.34
β-CH$_2$	38.5	38.34	38.82				37.78	38.55	38.00	38.84	37.65	38.29	38.50	38.58	38.68
C-1		138.34	138.53				137.73	138.57	138.10	138.93	137.66	138.20	138.50	138.58	138.59
C-2	131.1	130.54	130.53				130.26	130.53	130.80	130.90	130.53	130.57	130.59	130.59	130.59
C-3	130.7	129.65	129.65				129.47	129.62	130.10	129.95	129.94	129.69	129.69	129.64	129.69
C-4	129.5	127.98	127.98				127.87	127.92	128.50	128.21	128.39	128.03	127.96	127.95	127.96
C═O	178.5	178.46	N.O.				176.72	178.70	176.60	178.48	175.75	177.56	178.25	178.44	178.52
Leu α-CH	55.7			52.68	54.57	55.25									
β-CH$_2$	41.9			40.45	41.46	41.77									
γ-CH	25.9			25.82	25.88	25.75									
δ-CH$_3$	23.2			23.40	23.56	23.61									
δ-CH$_3$	22.1			21.86	22.07	22.15									
C═O	180.1			177.26	180.80	180.82									

[a] The accuracy of the chemical shifts is 0.05 ppm. [b] N.O., not observed.

cine,8-leucine]-angiotensin II in D$_2$O at pH 4.5 are given in Figure 3. The T_1 values of residues in the backbone are *ca.* 10% greater than those of the analogous residues in [5-isoleucine]-angiotensin II.

Dipole-dipole interaction provides the predominant relaxation mechanism occurring for protonated carbon nuclei (Kuhlmann *et al.*, 1970; Doddrell *et al.*, 1972). Nuclear Overhauser enhancements of 3.0 ∓ 0.1 for [5-isoleucine,8-leucine]-angiotensin II further support this statement. In the limit of rapid motion on the ^{13}C nmr time scale, the greater the NT_1 value, the more mobile is the ^{13}C moiety. The NT_1 values can accordingly provide information relating to the mobility of the various residues in a peptide. The mobility can be described as combination of rotational diffusion of the molecule as a whole and intramolecular motion (Allerhand *et al.*, 1971; Allerhand and Komoroski, 1973; Deslauriers *et al.*, 1974b). An effective correlation time (τ_{eff}) for overall molecular reorientation can be obtained from the observed T_1 values in the case where carbons relax *via* dipole-dipole coupling to protons (Allerhand *et al.*, 1971):

$$\frac{1}{T_1} = \frac{1}{T_1^{\text{DD}}} = \left\langle \frac{1}{r^6} \right\rangle \frac{N\hbar^2\gamma_C{}^2\gamma_H{}^2}{10}[f(\omega_H - \omega_C) + 3f(\omega_C) + 6f(\omega_H + \omega_C)] \quad (1)$$

$f(\omega) = \tau_{\text{eff}}/(1 + \omega^2\tau_{\text{eff}}{}^2)$, ω_H and ω_C are the angular resonance frequencies of ^{1}H and ^{13}C, respectively, and $\langle r^{-6} \rangle$ is the vibrationally averaged inverse sixth power of the ^{1}H–^{13}C internuclear distance. In the case of isotropic rotational diffusion (Noggle and Schirmer, 1971), τ_{eff} describes the isotropic rotational diffusion of the C–H internuclear vector and τ_{eff} is defined as equal to $D/6$, where D is the rotational diffusion constant. The rotational diffusion constant can be related to the molecular friction constant β (Noggle and Schirmer, 1971), according to:

$$D = kT/\beta \quad (2)$$

where k is Boltzmann's constant, T is the absolute temperature, and

$$\beta = 8\pi\eta r_0{}^3 f_r \quad (3)$$

(Noggle and Schirmer, 1971), where η is the viscosity of the medium, r_0 is the radius of a spherical solute molecule, and f_r is the microviscosity factor, which for pure liquids $\simeq$ 1/6. For larger molecules undergoing Brownian diffusion f_r is assumed to be 1. For the purpose of the present work f_r is assumed equal to 1:

$$\tau_{\text{eff}} = \frac{1}{6D} = \frac{8\pi\eta r_0{}^3}{6kT} \quad (4)$$

or

$$\tau_{\text{eff}} = V_m\eta/kT \quad (5)$$

where the molecular volume $V_m = (4/3)\pi r_0{}^3$.

When molecular motion is anisotropic two or more correlation times must be introduced (Freed, 1972); τ_{eff} is then a weighted average of various correlation times. A rigid ellipsoidal molecule is characterized by two rotational diffusion constants (Noggle and Schirmer, 1971): the diffusion constant for rotation about the symmetry axis, D_a; and the diffusion constant for reorientation about any axis perpendicular to the symmetry axis, D_b. τ_{eff} in the case of a rigid ellipsoidal molecule in which the C–H internuclear vector

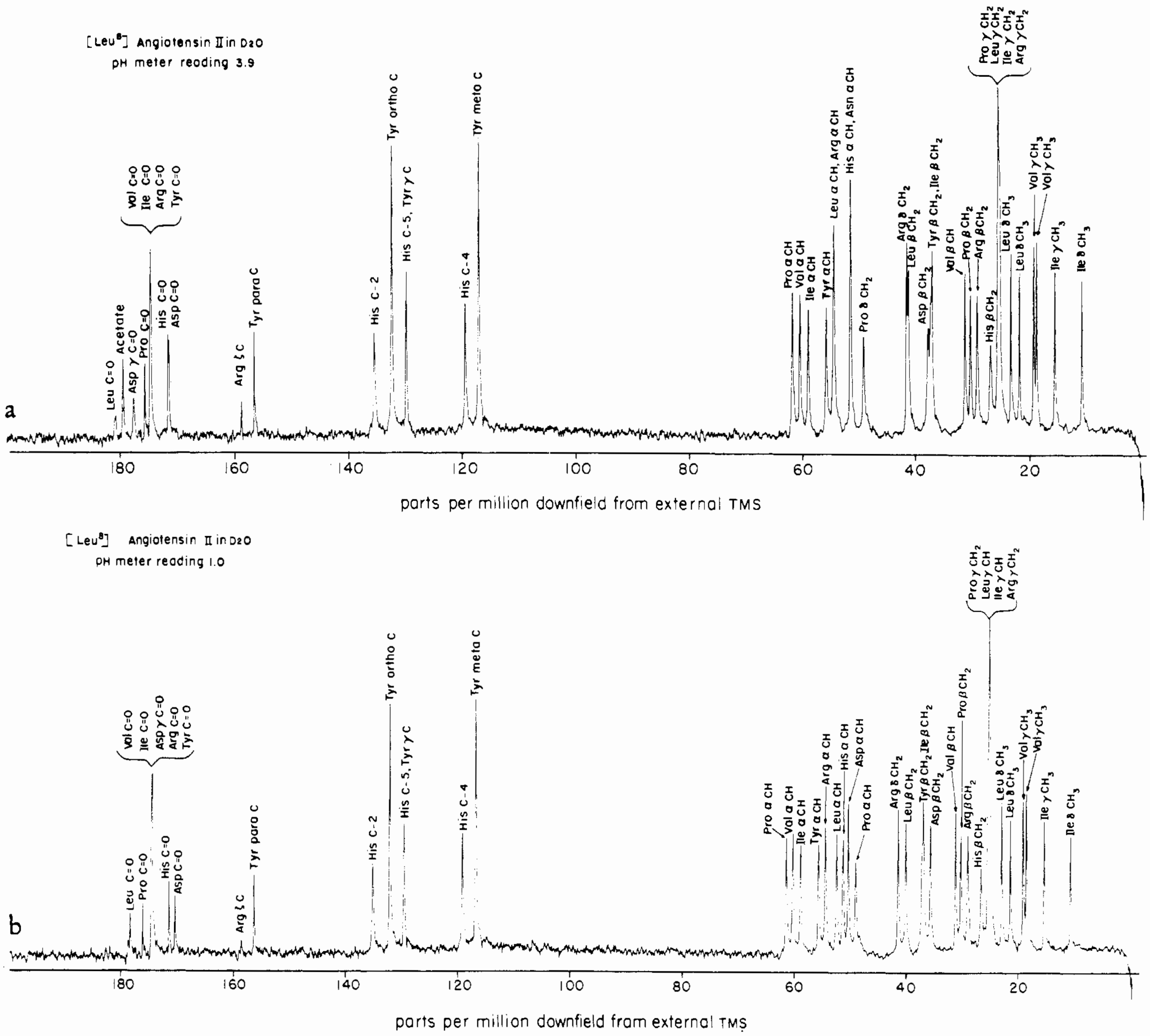

FIGURE 1: (a) ^{13}C nmr spectrum of [5-isoleucine,8-leucine]-angiotensin II in D$_2$O at pH 4.5, 100 mg/ml, 32°, 80,000 scans, cycle time 0.4 sec, pulse width of 45°; (b) pH 1.0, other conditions same as above.

makes an angle θ with the axis of symmetry of the ellipsoid is defined as

$$\tau_{\text{eff}} = \frac{(3\cos^2\theta - 1)^2}{24D_b} + \frac{3\sin^2\theta\cos^2\theta}{(5D_b + D_a)} + \frac{3}{4}\frac{\sin^4\theta}{(2D_b + 4D_a)} \quad (6)$$

where $D_a = kT/\beta_a$, $D_b = kT/\beta_b$, and

$$\beta_a = 32\pi\eta a_2^2(a_1^2 - a_2^2)/3[2a_1 - a_2^2S] \quad (7)$$

$$\beta_b = 32\pi\eta(a_1^4 - a_2^4)/3[(2a_1^2 - a_2^2)S - 2a_1] \quad (8)$$

a_1 and a_2 are the lengths of the semiaxes of the ellipse. When $a_1 > a_2$

$$S = 2(a_1^2 - a_2^2)^{-1/2}\ln\{[a_1 + (a_1^2 - a_2^2)^{1/2}]/a_2\} \quad (9)$$

When $a_1 < a_2$

$$S = 2(a_2^2 - a_1^2)^{-1/2}\tan^{-1}[(a_2^2 - a_1^2)^{1/2}/a_1] \quad (10)$$

(Freed, 1972).

If intramolecular motion occurs at a rate comparable or greater than that of overall molecular motion, the effective correlation time measured according to eq 1 will be a function of the correlation times for overall molecular reorientation (τ_{mol}) and internal motion (τ_{int}). In the extreme narrowing limit $(\omega_H + \omega_C)^2\,\tau_{\text{mol}}^2 \ll 1$, the spin-lattice relaxation time of a protonated carbon which undergoes internal motion as well as overall molecular reorientation will be (Doddrell *et al.*, 1972)

$$\frac{1}{NT_1} = \left\langle\frac{1}{r^6}\right\rangle \hbar^2\gamma_H^2\gamma_C^2\tau_{\text{mol}}\left[A + B\frac{6\tau_{\text{int}}}{6\tau_{\text{int}} + \tau_{\text{mol}}} + C\frac{3\tau_{\text{int}}}{3\tau_{\text{int}} + 2\tau_{\text{mol}}}\right] \quad (11)$$

where

$$A = (3\cos^2\theta - 1)^2/4 \quad (12)$$

$$B = 3\sin^2\theta\cos^2\theta \quad (13)$$

$$C = (3\sin^4\theta)/4 \quad (14)$$

and θ is the angle between the C–H internuclear vector and

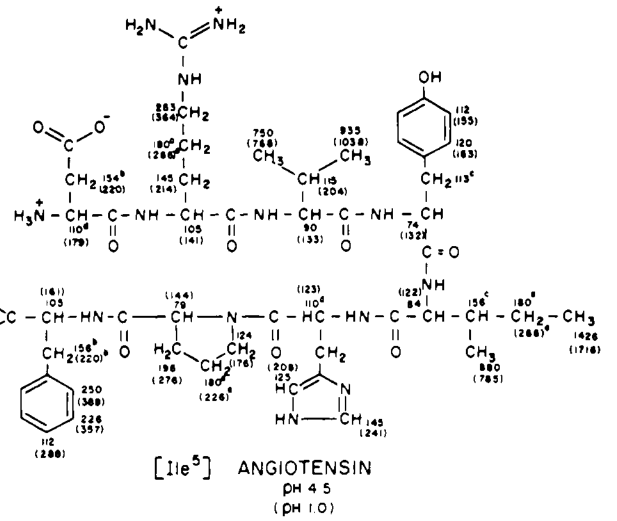

FIGURE 2: Primary sequence of [5-isoleucine]-angiotensin II showing NT_1 values, in milliseconds, of individual carbon atoms in D_2O at pH 4.5; temperature, 32°; sample concentration, 100 mg/ml; T_1 values from a least-squares fit to a straight line determined using 36 spectra, 10,000 scans/spectrum, $T = 2.0$ sec, $\tau = 10$-200 msec (pH 1.0 values in parentheses, solution acidified using dilute HCl in D_2O, T_1 values determined using nine spectra). Superscript letters indicate partially or totally overlapping resonances.

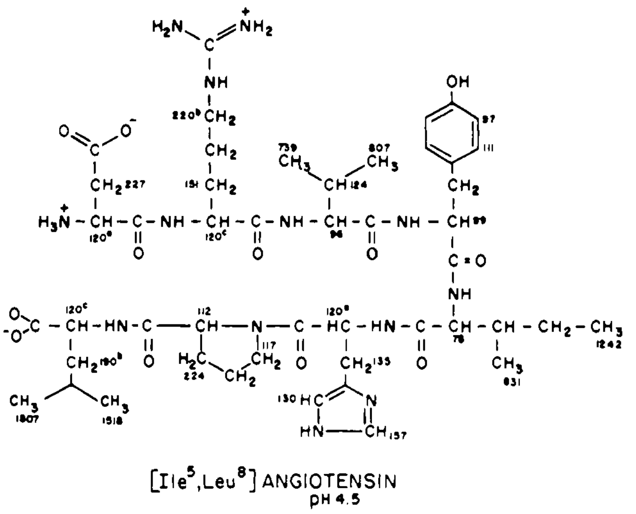

FIGURE 3: Primary sequence of [5-isoleucine,8-leucine]-angiotensin II showing NT_1, in milliseconds, of individual carbon atoms in D_2O at pH 4.5; temperature, 32°; sample concentration, 100 mg/ml; T_1 values determined from a least-squares fit to a straight line using 20 spectra, 10,000 scans/spectrum, $T = 2.0$ sec, $\tau = 10$-200 msec. Superscript letters indicate partially or totally overlapping resonances.

the axis of internal rotation. In the case of rotation about the C-C bond and a tetrahedral carbon, $\cos^2 109° = 1/9$, this then yields $A = 1/9$, $B = 8/27$, and $C = 16/27$. When internal motion is absent or very slow $\tau_{mol} = \tau_{eff}$; when internal motion is very fast ($\tau_{int}/\tau_{mol} \ll 1$), $\tau_{eff} = (1/9)\tau_{mol}$ (Allerhand *et al.*, 1971).

We shall now investigate possible models of motion for angiotensin II in order to describe the observed T_1 values.

Isotropic Diffusion with Internal Motion. From the NT_1 values observed in the backbone of [5-isoleucine]-angiotensin II at pH 4.5 we calculate an average NT_1 value of ~95 msec. Using eq 1 this value yields a $\tau_{eff} = 4.7 \times 10^{-10}$ sec. Assuming a spherical molecule of 8-Å radius as a model for angiotensin (measured on a space-filling molecular model) and using a viscosity of 10.4 mP for D_2O at 32° (Prutton and Maron, 1951; Weast, 1968) we can calculate (eq 5) a value of 5.3×10^{-10} sec for τ_{mol}. Because τ_{mol} is larger than τ_{eff} there is a possible contribution from internal motion to τ_{eff}. The value calculated for τ_{int} assuming an angle of 109° between the C-H vector and the axis of internal rotation is of the order of 1.9×10^{-9} sec. However, because this is slow relative to τ_{mol}, and due to uncertainties in our estimates of (1) the molecular radius, (2) the viscosity, and (3) the measured T_1 values we will assume $\tau_{eff} \simeq \tau_{int}$ only for side chains where NT_1 values differ by more than 50% from the NT_1 values in the backbone. The assumption that $\tau_{eff} = \tau_{mol}$ is not unreasonable for the backbone carbons. This, however, implies that true segmental motion of the peptide backbone is not present, *i.e.* that no portion of the peptide backbone is any more restricted in its motion than any other of the remaining moieties. Support for these assumptions is provided from studies of the T_1 behavior of the octapeptide hormones oxytocin

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH$_2$

and lysine vasopressin

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH$_2$

(Deslauriers *et al.*, 1974a-c). These cyclic peptides have molecular weights of *ca.* 1000, quite similar to that of angiotensin II. The peptides have been studied under conditions of pH and concentration similar to those used here for angiotensin II. The cyclic portions of oxytocin and lysine-vasopressin show T_1 values of ~95 msec for the C_α's of the peptide backbones. Because of the constraints imposed on the peptide backbone by closure of the disulfide bond, segmental motion of the peptide backbone is unlikely (although torsional oscillations about the

$$-\mathrm{N}\underset{\phi}{\curvearrowright}\mathrm{C}_\alpha-$$

bond and

$$-\mathrm{C}_\alpha\underset{\psi}{\curvearrowright}\overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}}-$$

bonds of each residue remain possible). A more significant observation is that the acyclic terminal tripeptides of both oxytocin and lysine vasopressin undergo motion to a larger degree than do the cyclic portions of the peptides, as manifested by NT_1 values 3-4 times larger than those measured in the cyclic moieties.

Using the isotropic diffusion model (eq 11) with internal rotation we calculate τ_{int} values for the side chains of arginine: C_β, $(2.6 \mp 1.9) \times 10^{-10}$; C_γ, $(1.5 \mp 0.8) \times 10^{-10}$; C_δ, $(6.2 \mp 2.7) \times 10^{-11}$. The errors on the values of τ_{int} were estimated by adding +15% and −15% to the observed T_1 values and recalculating τ_{int}. For instance, +15% and −15% errors in the T_1 values of C_δ of the arginyl residue result in −20 and +44% errors in the values of τ_{int}. The error values quoted in the text are the larger ones. It must be noted that for C_γ and C_δ the value calculated for τ_{int} results from motion about more than one C-C bond and therefore is not entirely justified. Another approach is to apply eq 11 successively such that the τ_{eff} of the preceding carbon becomes the τ_{mol} for the next carbon. Using this latter approach we estimate τ_{int} for $C_\gamma = 5.3 \times 10^{-10}$ sec and $C_\delta = 2.0 \times 10^{-10}$ sec. For the valyl residue, τ_{int} for C_β is calculated to be $(5.9 \mp 8.8) \times 10^{-10}$ sec. The CH_3 groups show NT_1 values which are nearly 9 times as long as those of the backbone. Equation 11 is insensitive to variation of τ_{int} in this range and therefore only a qualitative description in terms of rapid internal motion can be assumed. For such rapid rates spin-rotation effects on T_1 become significant (Levy, 1973), eq 11 is no longer applicable, and correlation times cannot be easily estimated from T_1. In the tyrosyl residue τ_{int} is of the order of $(5.7 \mp 8.3) \times 10^{-10}$ sec for the aromatic ring; however, due to the absence of a para hydrogen one cannot determine with confidence if rotation about an axis traversing C_γ and C_ζ can occur. The C_β and C_γ resonances of isoleucine are not resolved from one another and it is not possible to determine τ_{int} for these carbons. As with valine the γ and δ CH_3 groups of isoleucine undergo rapid internal rotation and one cannot easily estimate a correlation time. The prolyl residue cannot undergo true internal motion but from the NT_1 values observed in the ring, ring mobility is more restricted at the C_α and C_δ than at C_β (and possibly C_γ). This behavior has been found in several proline-containing hormones (Deslauriers *et al.*, 1974c). The side chain of phenylalanine undergoes motion about the C_γ-C_ζ axis as judged by NT_1 values greater for the ortho and meta carbons than for the para carbon. τ_{int} for the former carbons is calculated as $(7.8 \mp 3.2) \times 10^{-11}$ sec.

Anisotropic Molecular Diffusion. In order to investigate the effect of molecular shape on the observed T_1 values of angiotensin II we have calculated T_1 and τ values for a molecule of constant volume with varying lengths of semiaxes, and no internal motion. The volume considered was that for a sphere of 8-Å radius. The data are shown in Table II. Maximum T_1 values are observed for a spherical molecule of 8-Å radius. By deforming the molecule and keeping the total volume constant the T_1 values decrease and become dependent on the angle θ between the C–H internuclear vector and the principal axis of the molecule. Varying the ratio of semiaxes from 1:1 to ~3:1 the T_1 values for a C–H bond aligned with the long axis of the molecule decrease by a factor of 2; however, for groups perpendicular to the same axis the decrease is of the order of 20%. This implies that T_1 values are not particularly sensitive to molecular shape when the C–H internuclear vector is nearly perpendicular to the long axis of the molecule, as would be the case with backbone C_α-H groups in a rigid helix. Varying the orientation of C–H internuclear vector makes a difference of a factor of less than two in T_1 values for $\theta = 0°$ and $\theta = 90°$ when the ratio of semiaxes is kept constant at ~3:1. The calculations described above show that T_1 values are not particularly sensitive to molecular shape under the conditions investigated in Table II.

Molecular models demonstrate that any folded conformation (β-turn, γ-turn) leads to a compact, quasispherical shape. To achieve an elongated form it is necessary to extend the backbone. In an extended conformation, dimerization *via* hydrogen bonding could occur in angiotensin thereby forming a β-pleated sheet structure. To make a model of such a structure we have assumed molecular dimensions of 16 × 8 Å for the semiaxes. This yields T_1 values between 28 and 39 msec and τ_{eff} values between 1.6 and 1.1×10^{-9} sec for θ varying between 0 and 90°. Such T_1 values are too short when compared with the observed values. It would then be necessary to invoke internal or segmental motion which would be an order of magnitude faster than the overall motion. This seems unlikely in view of the hydrogen bonding necessary to maintain a β-pleated sheet structure.

Table II: Calculation of τ_{eff} and NT_1 for [Ile^5]-Angiotensin II Assuming Anisotropic Molecular Motion.[a]

Mol Dimensions (Å)	NT_1 (msec)	τ_{eff}(10^{-10} sec)
8.05 × 7.95	85	5.3
9.00 × 7.54	80–85	5.6–5.2
10.00 × 7.16	74–84	6.0–5.3
11.00 × 6.82	68–83	6.6–5.4
12.00 × 6.53	61–80	7.4–5.6
13.00 × 6.27	55–77	8.2–5.8
14.00 × 6.05	49–74	9.2–6.1
15.00 × 5.84	44–70	10.3–6.4

[a] $\eta = 10.4$ mP; $T = 305°K$. First eight entries in table are for a molecule of constant volume, in which only the semiaxes are varied. The NT_1 and τ_{eff} values are the ranges expected for variations in θ between 0 and 90°. θ is the angle between the C–H internuclear vector and the long axis of the molecule.

One possible conformation for angiotensin II is that of a monomer having a backbone in an extended conformation which is strongly hydrogen bonded to the solvent. If the N- and C-terminal residues are strongly immobilzed by interaction with the solvent, no large gradation in T_1 values would be observed between the α carbons of the terminal residues and those of the central residues. However, studies on linear pentapeptides (Keim *et al.*, 1973a,b; 1974) do not provide evidence that this type of interaction severely restricts the terminal residues of peptides.

Discussion

The ^{13}C spectrum of [5-isoleucine]-angiotensin II in D_2O does not reveal any perturbations in chemical shifts due to conformational constraints. Calculated and observed chemical shifts agree within 1 ppm. The proline residue is in the trans conformation about the His-Pro bond. At basic pH, the N^τ-H tautomer of the histidyl residue is predominant. The carbonyl carbon chemical shifts do not show any deviations from the values calculated from the constituent amino acid values corrected for peptide bond formation; therefore no evidence for intramolecular hydrogen bonding is available from ^{13}C chemical shifts.

The T_1 values for [5-isoleucine]-angiotensin II in D_2O at pH 4.5 give an estimate of the relative mobilities of side chains and backbone as well as the rate of overall molecular reorientation. A model for angiotensin II which has a radius of 8 Å and rotates isotropically gives a τ_{eff} value of 5.3×10^{-10} sec which is close to the value of 4.8×10^{-10} calculated from the average observed T_1 values of the backbone α carbons. These values are similar to those (~5.0×10^{-10} sec) measured for the cyclic portions of the octapeptide hormones oxytocin and lysine-vasopressin under similar conditions (Deslauriers *et al.*, 1974b). The difference in T_1 values between the middle and end portions of the peptide backbone is less than 50% implying no large gradation in segmental motion. This is in contrast to the terminal tripeptides of oxytocin and lysine-vasopressin (Deslauriers *et al.*, 1974a–c) or the Gly-Gly-X-Gly-Gly pentapeptides studied by Keim *et al.* (1973a,b, 1974) where an increase in NT_1 values of fourfold demonstrated a significant degree of segmental motion. The observations on [5-isoleucine]-angiotensin II are supported by the data from titration (Juliano and Paiva, 1974) and interaction with *p*-nitrophenyl acetate (Juliano *et al.*, 1974) of several angiotensin peptides. These data indicate that the N- and C-terminal ends of angiotensin II are held in closer proximity than that expected in a random conformation, leading to observable electrostatic interactions between the C-terminal carboxyl, the imidazole, the α-amino, and the Asp β-carboxyl groups. Although these electrostatic interactions cannot be responsible for maintaining the folded conformation, it is possible that protonation of the two carboxyl groups would loosen the structure because of repulsion between the positively charged ammonium and imidazolium groups. This might explain the significantly higher T_1 values of [5-isoleucine]-angiotensin II at pH 1.0.

If formation of β-pleated sheets or hydrogen-bonded dimers occurred, the τ_{eff} values calculated would be larger (1.0×10^{-9} sec) than the observed value of $\sim 5 \times 10^{-10}$ sec and a large amount of segmental motion in the backbone would be necessary to account for the observed T_1 values. The hydrogen bonding required to maintain a β structure would make segmental motion unlikely.

From these T_1 studies, a qualitative model emerges for the relative mobilities of backbone and side chains in [5-isoleucine]-angiotensin II in which the molecule is folded on itself and has a radius of ~8 Å. Little segmental motion of the backbone is present; however, the C- and N-terminal residues are slightly more mobile than the other residues.

The side chains demonstrate varying degrees of mobility. The side chain of arginine shows only a factor of 2 in NT_1 values between the δ and α carbons, implying a very slight amount of segmental motion. This behavior contrasts with that of lysine residues in numerous oligomers where a factor $\simeq$ 2 is seen between the NT_1 values of *adjacent* carbons (Saito and Smith, 1974). The small degree of segmental motion observed in arginine is likely to be a consequence of the presence of the bulky guanidino group attached to the δ carbon rather than steric crowding by side chains of adjacent residues, because a similar behavior of arginine has been observed by Keim *et al.* (1974) in the pentapeptide Gly-Gly-Arg-Gly-Gly. In valine, C_β undergoes a slight degree of internal motion whereas the methyl groups undergo fast internal motion. The tyrosine and phenylalanine residues offer a most interesting comparison. In the tyrosine residue, the ortho and meta carbons of the aromatic ring have NT_1 values of 120 and 112 msec, slightly longer than that observed for the α carbon in the backbone, 80 msec. This could be the result of slightly increased motion of the side chain and/or rotation about the axis of the aryl-C_β bond at a rate greater than that of overall molecular reorientation. The phenylalanine residue at the C terminus is much more mobile than the central tyrosine residue. This may be a consequence of the central position of tyrosine as well as the presence of the para hydroxyl group on tyrosine which can hydrogen bond to solvent. The phenylalanine residue clearly shows evidence of rotation of the aromatic ring about an axis coincident with the C_β-C_γ and C_ζ-H bonds. Internal rotation about the C_β-C_γ bond causes a lengthening of the NT_1 values of C_δ and C_ϵ. The C_ζ-H bond is coincident with the C_β-C_γ bond and therefore should not show a NT_1 value greater than that of the β carbon. In the isoleucine residue, the δ CH_3 group shows a greater NT_1 value than does the γ CH_3 group. However, the long NT_1 values for both CH_3 groups would indicate fast internal motion in each group and the difference between each of them may be largely, although not necessarily entirely, a result of a difference in internal motion of the carbons to which each CH_3 group is attached. In [5-isoleucine]-angiotensin II the isoleucine γ CH_3 and δ CH_3 residues show a T_1 ratio of ~1:2 with each other. This is quite similar to that observed in Gly-Gly-Ile-Gly-Gly where the adjacent glycine residues have no side chains which could cause steric interference with the γ CH_3 group. Thus, it would appear that in [5-isoleucine]-angiotensin II the tyrosyl and histidyl side chains do not interfere sterically with the γ CH_3 group of isoleucine and do not cause the γ CH_3 group to be out of the time scale for fast internal motion. Nothing can be concluded from the NT_1 values of the β and γ carbons in isoleucine as these resonances overlapped with other carbon resonances. The side chain of the histidine residue is less rigid than the backbone. In the proline residue, the NT_1 value of the β carbon is greater than that of the α and δ carbons as a consequence of mobility of the β carbon with respect to the plane formed by the α carbon, the δ carbon, and the N atom. This behavior is characteristic of a proline residue occupying a nonterminal position in a peptide (Deslauriers *et al.*, 1974c) and corresponds well with observations on Gly-Gly-Pro-Gly-Gly (Keim *et al.*, 1974).

From these T_1 measurements we can conclude that all segments of the backbone of [5-isoleucine]-angiotensin II are equally restricted in their motion. The rate of molecular reorientation is similar to that observed for the cyclic portions of the neurohypophyseal hormones implying that [5-isoleucine]-angiotensin II exists in a time-averaged folded conformation. The T_1 values exclude large segments of intermolecularly hydrogen-bonded β-pleated sheets but are consistent with families of folded conformations. The folded conformation of this hormone may favor interaction with the hormone receptor by allowing greater access of residues necessary for specific hormone activity.

The antagonist [5-isoleucine,8-leucine]-angiotensin II shows NT_1 values for the peptide backbone which are ~10% greater than those of [5-isoleucine]-angiotensin II (Figure 3). This increase in T_1 values is within the error on experimental values for each individual T_1; however, the overall increase may be significant and reflect a loosening of the conformation of the backbone.

Further studies should involve analogs of angiotensin in which the tyrosyl and phenylalanyl residues have been permuted in order to follow the mobility of these residues as a function of position in the peptide. A study of [3-proline,5-proline]-angiotensin II which is not believed to show spatial proximity of the N-terminal and C-terminal ends (Juliano and Paiva, 1974) should also provide a test for the sensitivity of T_1 measurements to overall molecular shape.

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