^{13}C and ^{1}H Nuclear Magnetic Resonance Studies of Bradykinin and Selected Peptide Fragments†

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ABSTRACT: Complete ^{13}C chemical shift assignments of the nonapeptide bradykinin have been made on the basis of pH titration studies and the examination of peptide fragments. Hysteresis effects previously reported for the ^{13}C shifts of the Arg^9 resonances as a function of pH and interpreted to reflect an intramolecular salt bridge involving the Arg^1 guanido and the Arg^9 carboxyl (Ivanov, V. T., et al. (1975) *Bioorg. Khim. 1*, 1241; *Proc. 4th Am. Pept. Symp.*, 151) were not observed in the present study. Chemical shifts of the proline carbons indicate that the trans configuration about the three X-Pro peptide bonds is strongly favored in all cases, although the minor cis resonances accounting for ~10% of the proline intensity can also be observed. In addition, there appears to be no significant pH sensitivity of the cis ⇄ trans equilibria. Studies of the solvent dependence (water → methanol) of the carbonyl shifts are consistent with the possibility of an intramolecular hydrogen bond involving the Ser^6 carbonyl oxygen. Preliminary ^{1}H nuclear magnetic resonance studies of the amide proton region of bradykinin and the C-terminal tetrapeptide Ser-Pro-Phe-Arg are also reported. In the latter peptide, the Phe^8 and Arg^9 amide proton resonances appear as two sets of resonances corresponding to the cis and trans configurations of the Ser-Pro bond. The temperature dependence of the proton shifts of the amide resonances is independent of whether the Ser-Pro bond is cis or trans, indicating that any intramolecular hydrogen bonding existing only for the trans peptide is insufficient to significantly reduce this parameter. Measurements of the ^{13}C spin–lattice relaxation times indicate rapid internal motion for all of the peptide side chains, supporting the interpretation that bradykinin exists in solution primarily in a disordered state. However, the possibility of a time-averaged interconversion between a disordered and a partially ordered conformation as proposed by J. R. Cann et al. ((1973) *Biochemistry 12*, 3780) cannot be excluded.

The ^{13}C NMR studies of small peptide hormones have been shown to be particularly rewarding since nearly every carbon resonance can frequently be resolved (Smith et al., 1973; Deslauriers & Smith, 1976, and references therein). Chemical shift and relaxation studies can, therefore, provide a very detailed understanding of the solution conformation and dynamics of such peptides. In most of the cases which have been investigated, the shifts can be predicted very well, based on the constituent amino acids (Deslauriers et al., 1975), and the spin–lattice relaxation behavior indicates that most residues are free to rotate internally relative to the peptide backbone (Allerhand & Komoroski, 1973; Deslauriers et al., 1974a–d, 1976; Deslauriers & Somorjai, 1976; Keim et al., 1973a,b, 1974). Previous CD studies (Cann et al., 1973, 1976) suggest that the nonapeptide bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, interconverts between a disordered and a partially ordered structure containing an intramolecular hydrogen bond, most probably a 3→1 type bond bridging Pro^7 (γ turn). However, on the basis of ^{13}C NMR studies and studies with spin-labeled bradykinin, Ivanov et al. (1975a,b) have recently concluded that one particular conformation of the peptide, in which the Arg^1 guanidinium group is ionically bonded to the Arg^9 carboxyl group, is predominant.

In the present study, we report the complete assignments of the ^{13}C NMR resonances of bradykinin based on pH titration studies and on a comparison with the shifts observed in five peptide fragments. Conformational information based on the titration shifts, ^{13}C spin–lattice relaxation times, solvent dependence of the ^{13}C shifts in going from H_2O to methanol, and temperature-dependent ^{1}H NMR studies are presented.

Materials and Methods

Bradykinin and its peptide fragments were synthesized by the solid phase method (Stewart & Young, 1969) as described previously (Cann et al., 1973, 1976). Characterization of the synthesized peptides is summarized in Table I of Cann et al. (1973, 1976).

Single sweep proton NMR spectra of solutions of bradykinin (100 mg/mL) and Ser-Pro-Phe-Arg (50 mg/mL) were recorded using a Varian HR-220 spectrometer operated in the CW[1] mode. Internal *tert*-butyl alcohol was used as a chemical shift reference. Proton decoupled ^{13}C NMR spectra at 27 °C of the same solutions and of solutions of Arg-Pro-Pro, Arg-Pro-Pro-Gly-Phe, Ser-Pro-Phe-Arg, Phe-Ser-Pro-Phe-Arg, and Gly-Phe-Ser-Pro-Phe-Arg (all at ~50 mg/mL) were obtained at 25.2 MHz on a Varian XL-100-15 spectrometer interfaced to a Data General Nova computer, using a D_2O capillary as an external lock.

pH titration studies were accomplished with minimum sample dilution by addition of stock solutions of 6 M NaOH and 6 M HCl. pH values were not changed monotonically but were varied somewhat randomly between the extreme values of 1.1 and 11.0. Since no hysteresis effects were observed, the additional NaCl concentration appears to have a negligible effect on the ^{13}C chemical shifts. In the methanol–water study of bradykinin, the aqueous sample (pH 7.0) was initially evaporated and then dissolved in [^{12}C]methanol (2 mL). Due

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[1] Abbreviation used: CW, continuous wave.

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to its low solubility in methanol, much of the salt accumulated in the titrations of the aqueous solution was eliminated. Aliquots of H_2O were then added until a value of f_{MeOH} of 0.55 was reached. The sample was then evaporated to dryness and dissolved in H_2O, and aliquots of [^{12}C]MeOH were added to obtain ^{13}C spectra corresponding to low values of f_{MeOH}. The final pH measured in 35% methanol was 6.6, in fair agreement with the initial value.

Spin-lattice relaxation times (T_1) were measured using the (180°-τ-90°-T) pulse sequence with values of T = 2.0 s, well in excess of the longest T_1 values measured. The spin-lattice relaxation measurements were made subsequent to the pH titration and, therefore, at relatively high salt concentrations.

Results and Discussion

Several experimental approaches were used to obtain information on the structure and dynamics of bradykinin: (1) a pH titration of the chemical shifts; (2) measurements of the spin-lattice relaxation times of the ^{13}C resonances; (3) a study of the solvent dependence (water to methanol) of the ^{13}C resonances; (4) a preliminary proton NMR study of the amide proton region of bradykinin and Ser-Pro-Phe-Arg in H_2O. The results of these studies are summarized below.

1. *pH Titration.* The pH dependence of the ^{13}C chemical shifts of bradykinin and several related peptides is summarized in Figure 1. A complete pH titration of the resonances was undertaken in order to facilitate resonance assignments, to study the possible pH dependence of proline cis-trans isomerism which we have found to be significant in the N-terminal tripeptide Arg-Pro-Pro (Cann et al., 1976), and to look for possible evidence of long-range interactions resulting from titrations of the terminal carboxyl and amino groups. Most of the resonance assignments have been based on the pH data, comparison of the shifts with data obtained by Keim et al. (1973a,b, 1974) on Gly-Gly-X-Gly-Gly pentapeptides and comparisons with the data obtained from the N-terminal fragments Arg-Pro-Pro and Arg-Pro-Pro-Gly-Phe and the C-terminal fragments Ser-Pro-Phe-Arg, Phe-Ser-Pro-Phe-Arg, and Gly-Phe-Ser-Pro-Phe-Arg.

In general, the effect of titrating the terminal residues can be observed for both the terminal and the penultimate amino acid peaks (Zimmer et al., 1972). Interestingly, Arg^1 C-2, which was assigned by comparison with the resonance position in Arg-Pro-Pro (Cann et al., 1976), exhibits no pH dependence despite titration of the directly bonded amino group. Presumably this reflects cancellation of the inductive and electrostatic contributions to the shift. Similar effects have been observed in other systems (Christl & Roberts, 1972; Flohé et al., 1972). In a few cases where the resonances of repeated residues overlap, the entire resonance position appears to shift, probably due to a change in the relative positions of the constituent amino acid peaks. Thus, the composite Pro^{2,3,7} C-5 resonance is sensitive to the Arg^1 titration, although it is reasonable to expect that only the Pro^2 C-5 resonance is shifting.

The carbonyl region of the ^{13}C NMR spectrum of bradykinin is surprising in that, despite the frequency of repeated residues in the peptide, nine carbonyl resonances are distinguishable at neutral pH. The carbonyl peaks of the C and N terminal arginines are readily distinguishable by the pH behavior as are the carbonyl peaks of Pro^2 and Phe^8. These assignments have been checked and the remaining carbonyl peaks assigned on the basis of comparisons with the N and C terminal fragments (Figure 1). The data obtained for Arg-Pro-Pro are not included in the figure since a complete pH ti-

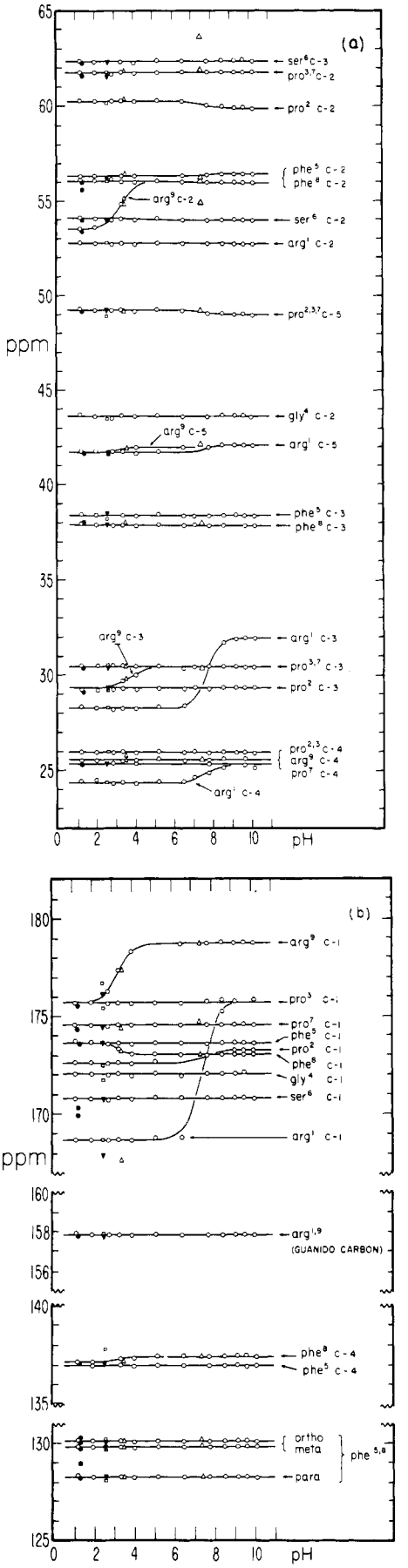

FIGURE 1: ^{13}C chemical shifts of bradykinin and several fragments as a function of pH. Spectra were obtained at 27 °C in H_2O: (○) bradykinin; (△) Ser-Pro-Phe-Arg; (●) Phe-Ser-Pro-Phe-Arg; (□) Arg-Pro-Pro-Gly-Phe; (▼) Gly-Phe-Ser-Pro-Phe-Arg. (a) Upfield region; (b) downfield region.

TABLE I: ^{13}C Spin–Lattice Relaxation Times for Bradykinin[a]

Residue	Carbon	NT_1 (ms)	Residue	Carbon	NT_1 (ms)
Arg[1]	C-2	186	Ser[6]	C-2	161
	C-3	318*		C-3	242
	C-4	434	Pro[7]	C-2	176*
	C-5	558		C-3	286*
Pro[2]	C-2	173		C-4	480[b]
	C-3	318*		C-5	208*
	C-4	410[b]	Phe[8]	C-2	168*
	C-5	208*		C-3	242
Pro[3]	C-2	176*		C ortho, meta	297*
	C-3	286*		C para	178*
	C-4	480[b]	Arg[9]	C-2	168*
	C-5	208*		C-3	286*
Gly[4]	C-2	154		C-4	410[b]
Phe[5]	C-2	168*		C-5	455
	C-3	232			
	C ortho, meta	297*			
	C para	178*			

[a] Data taken at 27 °C in H_2O, pH 7.0 at 25.2 MHz. Pairs of poorly resolved peaks at pH 7.0 are indicated with the symbol * (see Figure 1). [b] NT_1 values for Pro[2,3,7] C-4 and Arg[9] C-4 are uncertain due to assignment uncertainties. Data in the table are based on the assumption that the peak at 26.0 ppm, which represents two carbons, corresponds to Pro[3,7] C-4 since these two residues give similar shifts for the other carbons. The two poorly resolved peaks at 25.3 and 25.6 ppm are assumed to correspond to Pro[2] C-4 and Arg[9] C-4.

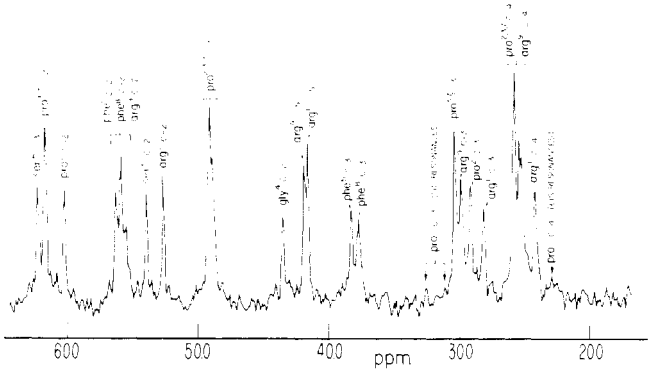

FIGURE 2: Upfield region of bradykinin ^{13}C NMR spectrum, pH 4.0. Presumed *cis*-proline C-3 and C-4 peaks are indicated.

tration curve has been previously reported (Cann et al., 1976). As expected, extraneous peaks corresponding to terminal residues in fragments, e.g., Phe in Phe-Ser-Pro-Phe-Arg, clearly do not correspond to any of the resonances in bradykinin and in most cases these are not included in the figure. Since the effect of creating a terminal resonance extends at least two residues into the chain, such comparisons must be made with caution. Thus, the Ser[6] C-1 resonance corresponds more closely to one of the C-1 peaks in Gly-Phe-Ser-Pro-Phe-Arg than in Phe-Ser-Pro-Phe-Arg. However, the fit at pH 2.55 obtained by summing the resonances from Arg-Pro-Pro-Gly-(Phe) + (Gly)-Phe-Ser-Pro-Phe-Arg, where the resonances corresponding to the residues in parentheses are not included, is remarkably good. None of the peaks in this sum differ by more than 0.4 ppm from the positions in bradykinin and most are considerably closer. The fact that we are able to obtain such an excellent simulation of the bradykinin spectrum in this way suggests that long-range interactions involving the two halves of the bradykinin peptide do not significantly perturb the structure. Thus, no evidence of shifts due to ring current effects or formation of a salt bridge are observed. The upfield shift of the Phe[8] C-1 peak upon deprotonation of the terminal carboxyl is analogous to an effect seen in dipeptides and tripeptides (Christl & Roberts, 1972; Cann et al., 1976) and is consistent with hydrogen bonding of the protonated terminal carboxyl at low pH to the Phe[8] carbonyl.

The shifts of the most intense resonances of the proline residues are consistent with the dominance of the trans conformation about all of the X-Pro peptide bonds (Thomas & Williams, 1972). Nevertheless, some low intensity resonances do occur at approximately the positions expected for *cis*-proline, indicating a small population of *cis*-proline peptide linkages (Figure 2). Due to the very poor signal/noise attainable for these peaks, and the fact that in many cases they tend to be obscured by the arginine C-3 and C-4 resonances, a quantitative estimate of the overall cis/trans ratio is difficult, and only the rough estimate ~10% can be set for the cis population. Similar effects have been observed in [Asn[1], Val[5]]-angiotensin (Galardy et al., 1976), and in [Pro[3], Pro[5]]-angiotensin II (Deslauriers et al., 1976). In contrast to the results which we have observed in the N-terminal fragment Arg-Pro-Pro (Cann et al., 1976), there seems to be no pH dependence of the cis/trans ratio but, rather, the first two peptide bonds in bradykinin at all pH values adopt conformations similar to those in Arg-Pro-Pro at acid pH.

The chemical shift data obtained for proline are of particular interest in terms of the solution conformation of bradykinin. The dependence of the shift of proline C_β on the dihedral angle ψ about the proline C_α–CO bond was first noted by Blout and coworkers (Madison et al., 1974). A relationship between the ^{13}C chemical shift difference, $\Delta_{\beta\gamma}$, between the resonances of the proline β and γ carbons and the dehedral angle ψ about the proline C_α–CO bond has recently been proposed (Siemion et al., 1975). The value of ψ corresponding to a γ turn, $\psi = 70°$, corresponds to a very low value of $\Delta_{\beta\gamma} = 1.3$ ppm. The observed values for the three proline residues of bradykinin are in the range $\Delta_{\beta\gamma} = 4.2$–$5.1$ ppm corresponding to $\psi = 150$–$180°$. In Ser-Pro-Phe-Arg at pH 7.4, $\Delta_{\beta\gamma} = 4.8$ ppm. Thus, the data appear to be inconsistent with the possibility that residues 6–8 of bradykinin exist *exclusively* in a γ-turn conformation. On the other hand, this result is consistent either with the conclusion that bradykinin is disordered in solution or that it is partially ordered at room temperature, as has been proposed by Cann et al. (1973).

The pH titration data summarized above differ in several

respects from that obtained by Ivanov et al. (1975a,b). In particular, we have not observed the very unusual hysteresis effects for the Arg^9 resonances previously reported. This discrepancy has been traced to the mistaken use of the bradykinin methyl ester in the previous studies (V. T. Ivanov, private communication).

2. *Spin–Lattice Relaxation Measurements.* The ^{13}C spin–lattice relaxation times of the proton-bearing carbons of bradykinin at pH 7.0, 27 °C, are summarized in Table I. Although the measurements were made in H_2O rather than in the more commonly used D_2O, the spin–lattice relaxation is dominated by the dipolar interaction with directly bonded protons; this interaction will be particularly favorable due to the long rotational correlation time of the molecule. The data obtained are qualitatively similar to measurements made in other small peptides (Allerhand & Komoroski, 1973; Smith et al., 1973; Deslauriers et al., 1974a, 1975, 1976), the most notable feature being the increasing T_1 values for carbons further from the peptide backbone. Comparison of the absolute values obtained for T_1 with values given in the literature is not particularly informative due to the different experimental conditions used in the studies reported, e.g., temperature, concentration, solution viscosity (D_2O vs. H_2O), molecular weight, and magnetic field strength.

In general, the NT_1 values for the α carbons of flexible peptide hormones exhibit a NT_1 gradient such that the longest NT_1 values correspond to residues in the terminal positions (Deslauriers & Somorjai, 1976; Deslauriers et al., 1976). Although the NT_1 value for the Arg^1 C-2 resonance is the longest measured, the effect is considerably less pronounced than in most, but not all other cases which have been studied (Deslauriers et al., 1975). It is unclear at present whether this result reflects the particular experimental conditions of the present study or an unusual mobility gradient. Further studies are in progress to resolve this question.

A second point worth noting is the NT_1 gradient of the Arg^9 residue. If there were a salt bridge between the Arg^9 carboxyl and the Arg^1 guanidinium group as proposed by Ivanov et al. (1975a,b), some restriction in the motion of the Arg^9 side chain could be expected and, for a tight ionic bond, NT_1 of Arg^1 C-5 should be similar to NT_1 of Arg^9 C-2. Clearly, this is not the case: NT_1 of Arg^9 C-2 = 168 ms; NT_1 of Arg^1 C-5 = 558 ms (Table I). Instead, these data can be interpreted using the theory of multiple *free* internal rotations developed by Woessner (1962), Wallach (1967), Levine et al. (1973), and recently applied by Deslauriers & Somorjai (1976) to peptides. Using an isotropic diffusion coefficient $D_0 = 5.9 \times 10^8\ s^{-1}$ and internal diffusion coefficients $D_{23} = 1.5 \times 10^9\ s^{-1}$, $D_{34} = 1.5 \times 10^9\ s^{-1}$, $D_{45} = 1.6 \times 10^9\ s^{-1}$ where D_{ij} represents the internal diffusion about the bond connecting C_i and C_j, we obtain the following set of T_1 values: T_1 (C-2) = 170 ms; T_1 (C-3) = 154 ms; T_1 (C-5) = 216 ms; T_1 (C-5) = 290 ms, in reasonable agreement with the observed Arg T_1 values (Table I). Due to the fact that only some of the two sets of Arg peaks are well resolved, we have not performed a more complete analysis. Thus, the Arg spin–lattice relaxation data are more consistent with an unconstrained side chain rotating internally about each bond with rates approximately three times the rate of overall diffusion of the bradykinin molecule than with the conclusion of a tight intramolecular ionic bond involving the Arg^1 guanido group.

A second conclusion concerning the peptide structure can be drawn from an analysis of T_1 for the ortho, meta, and para carbon atoms of phenylalanine, the relative values for which indicate rapid internal rotation of the aromatic ring about the C-3–C-4 bond. Since this axis is parallel to the C_{para}–H vector, this rotation does not affect the T_1 for C_{para} but will increase the T_1 values of the ortho and meta carbons. Similarly, it is expected that since the rotation about C-3–C-4 does not affect $T_1(C_{para})$, then NT_1(C-3)/NT_1 (C_{para}) should equal 1. Although the measured ratio, ~1.3, is only in fair agreement (perhaps reflecting the fact that most of the ring carbon resonances of the two phenylalanine residues are not resolved), it is within the range of other reported values: 0.83 and 0.98 for Me_2SO and D_2O solutions of Met-enkephalin (Bleich et al., 1976); 1.1 for gramicidin S (Allerhand & Komoroski, 1973); 0.96 (pH 4.1) and 1.1 (pH 1.1) for [Pro^3, Pro^5]-angiotensin (Deslauriers et al., 1976); and 1.4 for [Ile^5]-angiotensin (Deslauriers et al., 1975). Using the theory of free internal rotations discussed above for the case of arginine, values of $D_0 = 5.9 \times 10^8\ s^{-1}$, $D_{23} = 6.8 \times 10^8\ s^{-1}$, and $D_{34} = 8.0 \times 10^8\ s^{-1}$ give the relaxation times: T_1 (C-2) = 170 ms, $2T_1$ (C-3) = $T_1(C_{para})$ = 242 ms; $T_1(C_{ortho,meta})$ = 299 ms. These values are in reasonable agreement with the measured ones and indicate that there is a greater restriction in the mobility about the C-2–C-3 bond of phenylalanine than of arginine, perhaps due to the bulkiness of the side chain. The significant internal motion of the phenylalanine side chains appears to preclude a hydrophobic stacking interaction, a result also suggested by examination of molecular models and ultraviolet absorption and CD studies (Brady et al., 1971).

Finally, we note that the T_1 values obtained for proline also indicate the presence of considerable internal mobility, perhaps due to ring puckering effects. Similar observations have been made in a variety of proline-containing peptides (Deslauriers et al., 1974a; Torchia & Lyerla, 1974; Fossel et al., 1975).

3. *Methanol–Water Studies.* The possibility of using the solvent dependence of both ^1H and ^{13}C chemical shifts to obtain information about intramolecular hydrogen bonding has been demonstrated by Urry and co-workers (Pitner & Urry, 1972; Urry et al., 1974a). This procedure is based on the expectation that intramolecularly bonded carbonyl or amide proton resonances will be more shielded from solvent interactions than the remaining carbonyl or amide proton resonances. Numerous studies have indicated that acceptance of a hydrogen bond by a carbonyl oxygen leads to a downfield shift of the carbonyl ^{13}C resonance (Lauterbur, 1958; Maciel & Traficante, 1965; Maciel & Ruben, 1963). More specifically, Maciel & Ruben find that, relative to neat acetone, a methanol solvent leads to a 3.7-ppm downfield shift and a water solvent to a 9.1-ppm downfield shift of the acetone carbonyl resonance. Thus, for carbonyl groups interacting primarily with the solvent, the change from methanol to water, which is a better hydrogen bond donor, should lead to a downfield shift if no significant conformational changes occur when the solvent is changed.

The observed ^{13}C shifts of bradykinin are plotted as a function of f_{MeOH} = [MeOH]/([MeOH] + [H_2O]) in Figure 3. With few exceptions, all of the peaks are observed to shift downfield as f_{MeOH} is decreased. It should be noted that several resonances were not observed; in particular, the Arg^1 C-1 and C-3 resonances are not well resolved at the near neutral pH of the experiment (Figure 1). It was not possible to obtain reliable shifts for the Arg^9 C-1 resonance, and this may reflect a greater tendency of the peptide to associate with traces of contaminating paramagnetic ions in methanol. In contrast, the $Arg^{1,9}$ guanido carbon resonances became resolved at high f_{MeOH} (Figure 4). In order to estimate the possible effects of susceptibility changes on the observed ^{13}C shifts, the standard 2,2-dimethyl-2-silapentanesulfonic acid was also studied as a function of the methanol/water ratio. For the standard, all of the methylene resonances show very little solvent depen-

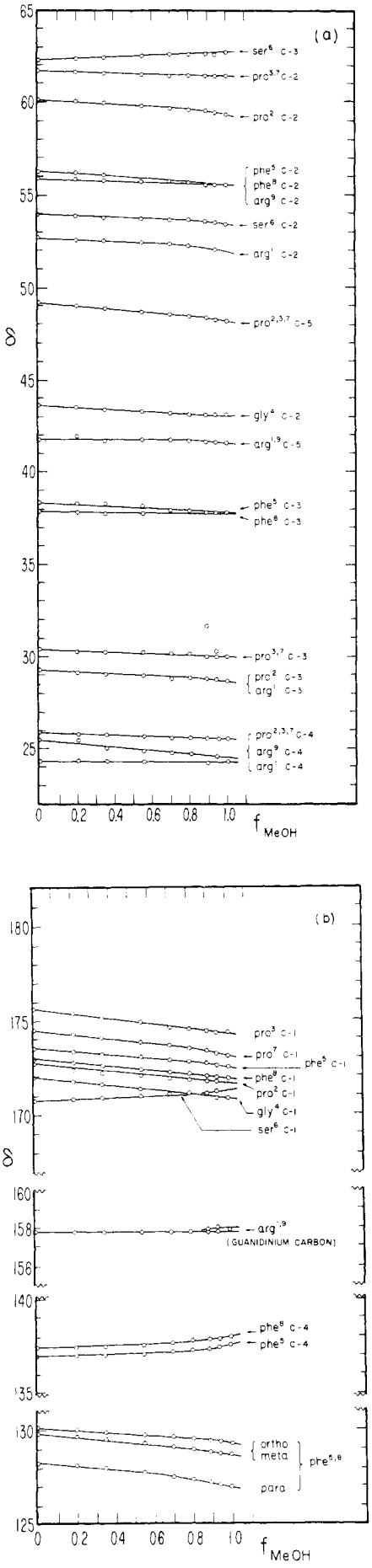

FIGURE 3: ^{13}C chemical shifts of bradykinin as a function of f_{MeOH}: (a) upfield region; (b) downfield region.

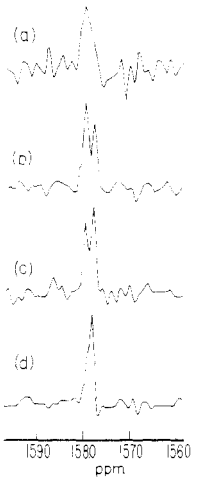

FIGURE 4: The $Arg^{1,9}$ guanidinium carbons of bradykinin measured at f_{MeOH} = 1.0 (a); f_{MeOH} = 0.94 (b); f_{MeOH} = 0.89 (c); and f_{MeOH} = 0.8 (d). The inequivalence of the arginine peaks in a is most probably masked by the broader line widths which were observed for all resonances in pure methanol.

dency with total shifts $\lesssim$0.25 ppm. However, the methyl peaks exhibit slightly greater than a 1-ppm shift downfield in water compared with methanol. Although several groups of resonances exhibit significant solvent shifts, we consider only carbonyl resonances in the following discussion.

With the exception of the Ser^6 carbonyl resonance, all the carbonyl resonances shift upfield as the solvent composition varies from water to methanol rich (0.6–0.7 ppm in the range f_{MeOH} = 0–0.5). Similar upfield water–methanol solvent shifts have been measured by other workers for the carbonyl resonances in a variety of peptides (Grathwohl & Wurthrich, 1976; Urry et al., 1976b). This result is the one expected if the latter carbonyl groups hydrogen bond primarily with the solvent and, in the case of bradykinin, is consistent with the CD studies of Cann et al. (1973) which indicate that at 27 °C the predominant hydrogen-bonding interactions of that peptide are with the water solvent. The unique downfield shift of the Ser^6 carbonyl resonance of bradykinin within this range of solvent compositions is consistent with the interpretation that it is participating in an intramolecular hydrogen bond that is strengthened as the solvent becomes methanol rich. Nevertheless, it is also possible that a solvent-dependent conformational change could produce unusual shift behavior of the Ser^6 carbonyl, particularly if the Phe^5 ring were involved.

4. *High Resolution Proton Magnetic Resonance Studies.* A 220-MHz ^1H NMR spectrum of the downfield region of bradykinin obtained in H_2O at 28 °C, pH 4.9, is given in Figure 5. The resolution of the amide protons is remarkably better than that obtained in the Me_2SO spectrum of bradykinin (Filatova et al., 1973). One reason for this difference may be the greater viscosity and consequently broader resonances observed in Me_2SO. Moreover, previous studies of peptides in Me_2SO indicate enhanced intramolecular hydrogen bonding which could reduce the freedom of internal motion and consequently broaden the lines. In the present spectrum, the five amide proton resonances are clearly distinguishable. The one triplet observed corresponds to glycine. The remaining four amide peaks have not been assigned. However, by comparison with the Ser-Pro-Phe-Arg spectrum the peak furthest upfield may correspond to the Arg^9 amide proton. The coupling constants to the α protons, $J_{NC\alpha}$ are, in order of increasing field, 5.6 Hz, 7.6 Hz, 6.6 Hz, 7.7 Hz, and 7.7 Hz. The similarity in shift and the fact that the coupling constants are similar

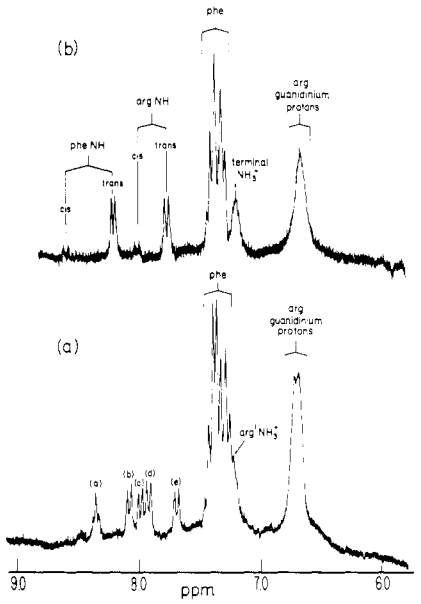

FIGURE 5: The 220-MHz proton magnetic resonance spectrum of the downfield region of (a) bradykinin, pH 4.9, 40 °C. The triplet labeled "a" corresponds to glycine amide proton. (b) Ser-Pro-Phe-Arg, pH 4.7, 28 °C. Two sets of resonances corresponding to each of the amide protons of Phe and Arg are presumed to reflect the cis–trans isomerism of the Ser-Pro bond.

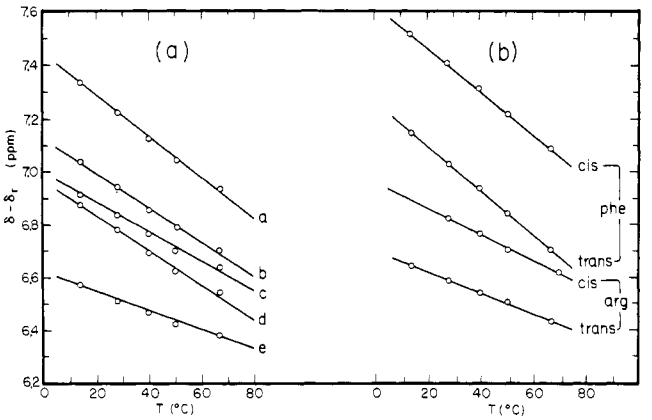

FIGURE 6: ^{1}H chemical shifts of the amide protons illustrated in Figure 5 as a function of temperature. (a) Bradykinin; (b) cis and trans resonances of Arg and Phe in Ser-Pro-Phe-Arg. The internal reference used was *tert*-butyl alcohol with a shift $\delta_r = 1.23$ ppm relative to external Me_4Si.

suggest that the "doublet" observed furthest downfield in Me_2SO also corresponds to glycine, although in general the assignments of the amide protons are highly solvent dependent. The $J_{NC\alpha}$ values are typical of those reported for a variety of small peptides (Feeney et al., 1971; Brewster & Hruby, 1973) and represent dihedral angles in the range $\phi_{NC\alpha} = -70°$ to $-100°$ or $+10°$ to $+40°$ if additional criteria are used to rule out other possible values in the solution of the multivalued Karplus equation (Gibbons et al., 1970; Feeney et al., 1971). However, the values are also close to calculations for a random coil assuming complete averaging of gauche and trans orientations (Gibbons et al., 1970).

The temperature dependence of the amide proton chemical shifts observed with bradykinin and with Ser-Pro-Phe-Arg is shown in Figure 6. Values of $d\delta/dT$ for resonances a, b, c, d, and e were found to be 7.8, 6.5, 5.6, 6.6, and 3.6×10^{-3} ppm/°C, respectively. Values obtained for Ser-Pro-Phe-Arg amide proton resonances were 8.4×10^{-3} ppm/°C for the phenylalaninamide proton and 4.0×10^{-3} ppm/°C for the arginine amide proton. Similar values were obtained for the cis amide proton resonances of Ser-Pro-Phe-Arg which apparently arise due to isomerization of the Ser-Pro bond. This result indicates that no hydrogen-bonding interactions dependent on the Ser-Pro amide bond configuration are significantly affecting the association of the amide protons with the water solvent. The values obtained are typical for protons which are not locked tightly in intramolecular hydrogen bonds (Urry, 1976). This result is not inconsistent with the conclusion of Cann et al. (1973) that the secondary structure of bradykinin is a time average of two interconverting structures—one disordered and one partially ordered due to a 3→1 type hydrogen-bond bridging the Pro^7 residue. The NMR method appears best suited to establishing the existence of an all or none hydrogen bond as occurs in certain hydrophobic peptides (Urry, 1976). It should also be noted, however, that the failure to observe a perturbation in the value of $d\delta/dT$ for the Phe^8 amide proton due to intramolecular hydrogen bonding could be caused by the presence of the aromatic ring. Urry (1976) has reported significant perturbations in the $d\delta/dT$ values resulting from aromatic rings which have been eliminated by hydrogenation of the phenylalanine residue to cyclohexylalanine. We also note that, under conditions in which $d\delta/dT$ fails to indicate an intramolecular hydrogen bond, other NMR parameters may still reflect a weak hydrogen-bonding interaction (Higashijima et al., 1977).

As in the ^{13}C NMR spectra, the ^{1}H NMR spectrum of bradykinin is characterized by the appearance of several minor peaks which presumably represent the minor fraction of peptide in which one or more of the proline linkages is in the cis conformation. This effect is more clearly evident in the C-terminal tetrapeptide, Ser-Pro-Phe-Arg (Figure 5). The fractional intensity of the cis peptide is, based on a comparison of peak areas, ~13%. Shifts between cis and trans amide peaks appear to be 0.39 ppm for the Phe NH resonance and 0.24 ppm for the Arg NH resonance. Small downfield shifts (~0.2 ppm) have similarly been observed in poly(Pro-Gly) and poly(Gly-Gly-Pro-Gly) for the *cis*-glycine amide proton resonances relative to the trans resonances (Torchia, 1972). Since downfield shifts are believed to accompany hydrogen bonding interactions, the result suggests stronger hydrogen bonding in the cis peptide. Conversely, model building indicates that the intramolecular hydrogen bond proposed by Cann et al. (1973) can only form in the trans peptide. Presumably, then, the observed cis–trans shift reflects a different degree of intermolecular hydrogen bonding with the solvent. Alternatively, these shifts may also be sensitive to the orientation of the aromatic phenylalanine residue which may in turn be sensitive to whether the Ser-Pro bond is cis or trans. The results differ significantly, however, from those observed by Fermandjian et al. (1972) for the proline amide protons of thyrotropin releasing factor in Me_2SO. In that system, the existence of a tight intramolecular hydrogen bond bridging the proline residue results in a downfield shift of 1.05 ppm for the trans amide proton relative to the cis amide proton. Thus, the Ser-Pro-Phe-Arg cis–trans chemical shift difference does not support a tight hydrogen bonding interaction but may be consistent with a time averaged hydrogen bonded conformation as proposed by Cann et al. (1973).

It is also interesting to note that in the ^{1}H NMR spectrum the two arginine peaks are slightly separated with a shift difference at 40 °C of ~8 Hz. Several possible explanations for this inequivalence can be proposed: (1) electric field effects due to the proximity of the terminal amino and carboxyl groups (the [5-^{13}C]arginine resonances are also not equivalent); (2)

proximity of Arg^9 to Phe^8 which could lead to ring current shifts; (3) salt bridge effects. There is at present insufficient evidence to choose among these alternatives. The two phenylalanine aromatic proton resonances also show a chemical shift inequivalence, although the effect is quite small. There is little evidence of the pronounced upfield shifts which are observed in systems in which hydrophobic stacking interactions occur (Feeney et al., 1971). This is consistent with the ^{13}C relaxation data indicating rapid internal motion of the benzene rings about the C-3–C-4 bond. Such motion would appear to be incompatible with a stacking interaction and is not observed if the motion of the aromatic residue is restricted, e.g., for the tyrosine residue of angiotensin (Deslauriers et al., 1975).

Conclusions

Cann et al. (1973) have concluded from circular dichroism (CD) data that the secondary structure of bradykinin is a time average of two interconverting structures—one disordered and one partially ordered due to a hydrogen bonded configuration. The hydrogen-bonded configuration was ascribed to a 3→1 type hydrogen bond (i.e., a γ turn) involving the Phe^8 amide proton and the Ser^6 carbonyl oxygen. The NMR data presented here are consistent either with a completely disordered conformation or with an average of an ordered and a disordered conformation. The solvent dependence of the ^{13}C shift of the Ser^6 carbonyl resonance can be interpreted to reflect its involvement in a hydrogen-bonded structure. However, the possibility that the conformation of bradykinin is also solvent dependent precludes a definite conclusion. In particular, if the orientation of the Phe^5 aromatic ring with respect to the Ser^6 carbonyl is dependent on solvent, unusual shift behavior for the carbonyl resonance might arise. Measurements of the temperature dependence for the amide proton shifts provide no evidence for the involvement of either the Phe^8 NH proton or the Arg^9 amide proton (which could form a 4→1 hydrogen bond leading to a β turn) in an intramolecular hydrogen bond. In particular, the temperature dependence of the chemical shifts of the amide protons in Ser-Pro-Phe-Arg is independent of whether the Ser-Pro bond is cis or trans, although an intramolecular hydrogen bond should lead to a significant difference. As in the interpretation of the Ser^6 carbonyl behavior, temperature-dependent studies of amide proton shifts are also subject to error if the bradykinin conformation, and particularly the orientation of the phenylalanine rings, is temperature dependent. The ^{13}C shifts of the Pro C_β and C_γ resonances are inconsistent with the exclusive existence of a γ-turn configuration, although not with a partially ordered structure as defined above. Finally, the spin-lattice relaxation data indicates rapid internal motion of all amino acid side chains. To summarize, the NMR data are consistent with the conclusion that bradykinin exists in solution primarily in a disordered conformation. Any ordering which exists is significant for too low a fraction of time to be reflected unequivocally in the NMR parameters.

Finally, under the conditions of the present study, no evidence for the existence of a tight ionic bond between the Arg^1 guanido and the Arg^9 carboxylate anion has been found. There appears to be no detectable interaction between the hydrophobic phenylalanine rings indicating that the hydrophobic residues are not folded inward but are poised in solution and consequently ready to interact with a hydrophobic binding site as the receptor. Presumably, intramolecular interactions between such residues are prevented from occurring as a consequence of the primary structure of bradykinin. This observation may be related to the presence of proline residues which constitute one-third of bradykinin. The rotational constraints about the N–C_α bond imposed by the side chain and about the C_α–CO bond imposed by steric factors sharply limit the rotational conformational possibilities of the amino acid and, consequently, of the entire bradykinin molecule. This, in turn, suggests that a primary function of the prolines may be to prevent the possible intramolecular hydrophobic interactions due to the lack of flexibility which they impart to the molecule. The above hypothesis is consistent with the observation that substituting the much more flexible amino acid L-alanine for prolines-2 and -7, although not proline-3, leads to drastic reductions in the activity of bradykinin (Schroder, 1964). Thus, the common occurrence of proline in many peptide hormones can perhaps be rationalized as preventing key functional groups from interacting intramolecularly and freeing them for interaction with the appropriate receptors.

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^{13}C Nuclear Magnetic Resonance Study of the Cis–Trans Isomerism in X-Pro-Pro Tripeptides†

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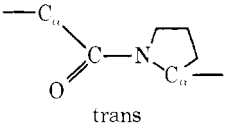
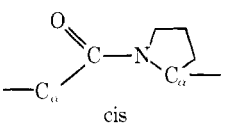
ABSTRACT: ^{13}C nuclear magnetic resonance has been used to characterize quantitatively the cis–trans isomerism about both peptide bonds in the tripeptides Ser-Pro-Pro and Arg-Pro-Pro. Detailed pH titration data indicate that the configuration about both peptide bonds is closely linked to titration of the terminal carboxyl group and, to a lesser extent, to titration of the terminal amino group. The Pro^2 C-3 resonance has been found particularly useful for interpretation due to its sensitivity to the isomerization about both peptide bonds. Analysis of the probabilities of the trans–trans, cis–cis, cis–trans, and trans–cis isomers in aqueous solution indicates a stability decrease in the order given. Similarities in the isomerization behavior of the two peptides indicate that side chain interactions involving the first residue have very little effect on the observed cis/trans ratios. The sensitivity of the cis/trans ratio to titration of the terminal amino group is most readily explained on the basis of an indirect effect on carbonyl–carbonyl repulsion.

The interdependence of the configurations about various bonds in model peptides is a conformational question of great importance to understanding the structure and function of proteins and peptide hormones. The oligopeptides containing proline are particularly useful models because, on the one hand, the rotational constraints introduced by the pyrrolidine ring simplify structural analyses considerably and yet, on the other hand, the presence of an X-Pro linkage can introduce structural heterogeneity corresponding to two possible conformations about the X-Pro bond.

Although peptide bonds preceding α-amino acids show an overwhelming preference for the trans configuration, both cis and trans isomers have been observed for peptide bonds preceding the imino acids sarcosine, proline, and hydroxyproline:


trans


cis

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