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PROBING THE ROLE OF PROLINE IN PEPTIDE HORMONES

NMR STUDIES OF BRADYKININ AND RELATED PEPTIDES

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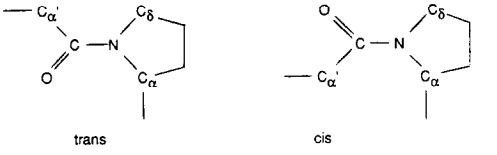
Abstract—The use of NMR methods to study conformational and dynamic aspects of the proline residues in the nonapeptide bradykinin is reviewed. NMR analyses involve considerations of bistable equilibria which include the *cis/trans* conformational heterogeneity of the imide bond, the *cis'/trans'* regions of conformational stability which characterize rotation about the C_α—CO bond (dihedral angle ψ), and the interconversion of the pyrrolidine ring of proline between puckered C_γ-endo and C_γ-exo conformations. These conformational features are all characterized by different kinetic behavior, are interdependent with peptide bond conformation, and exhibit sensitivity to amino acid substitutions. Thus, the substitution of Gly^6 for Ser^6 increases the fractional *cis* probability of the sixth peptide bond from 0.1 to 0.35. Substitutions of α-aminoisobutyric acid (AIB) residues for proline introduce conformational constraints analogous to those in *cis'* proline. Correlations of pyrrolidine ring conformation and dynamics with the *cis/trans* ratio of the imide bond have also been observed in model systems. Conformational and activity analyses of [AIB^7]-bradykinin provided a stimulus for the development of the first bradykinin antagonist by Stewart and Vavrek (Vavrek RJ and Stewart JM, Competitive antagonists of bradykinin. *Peptides* **6**: 161–164, 1985).

Crystallographic studies of proteins and enzymes have demonstrated that proline residues play a unique role as determinants of secondary structure; for example, proline residues terminate α helicies and frequently occur in β turns, thereby defining the limits of β sheets [1]. Peptide hormones frequently contain proline residues; three of the nine residues in the peptide under study (bradykinin: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) are proline. This raises the general question of what structural or functional roles proline may play in these systems. The action of small peptide hormones such as bradykinin is generally thought to arise from interactions with specific receptors localized on the exterior surface of the cell; however, the lack of any major stabilizing interactions due to hydrogen bonding or ionic bonding, and the relatively modest magnitude of potential hydrophobic interactions compared with, for example, aromatic amino acids, suggest that, in general, the major function of proline residues may involve the imposition of structural constraints, as in the case of proteins. It is clear, however, that in order for many of the sidechains of small peptides to remain available to interact with receptors, structural constraints may act primarily to limit or preclude inter-residue interactions among these sidechains as would occur in globular proteins so that they remain solvated and exposed for potential receptor contact. The importance of understanding the role of proline in peptide hormones is underscored by the fact that after evaluation of hundreds of bradykinin analogs, the first competitive antagonist of bradykinin was obtained by substituting D-phenylalanine for proline-7 [2]. NMR data can provide unique insight into the conformation and dynamics of proline in peptide hormones; applications related to bradykinin are summarized here.

Cis-trans *Equilibrium of the imide bond*

Although the difference in stability between *cis* and *trans* peptide bonds (defined in terms of the orientation of successive α carbons) is sufficient to make *cis* bonds a rare occurrence, the additional substitution of the amino group in the corresponding imide bonds formed with proline, hydroxyproline or sarcosine destabilizes the *trans* conformation; the *cis* and *trans* conformations exhibit sufficiently similar stabilities so that in short peptides both forms are typically observed [3–8]. Since the interconversion barrier is sufficiently large (>20 kcal/mol) to lead to slow exchange on the NMR time scale [9–11], peptides containing proline residues typically exhibit two sets of resonances corresponding to both *cis* and *trans* imide bond conformations:



In general, large fractional probabilities of peptides with the *cis* bond conformation are observed only if proline or another N-substituted amino acid, such as sarcosine, occupies the C-terminal position [3–8]. Thus, in bradykinin which does not contain a C-terminal proline residue, the fractional population of *cis* bonds is ~10% for each of the imide bonds [12]. Additionally, the fractional *cis* bond conformation in peptides containing titratable groups such as His-

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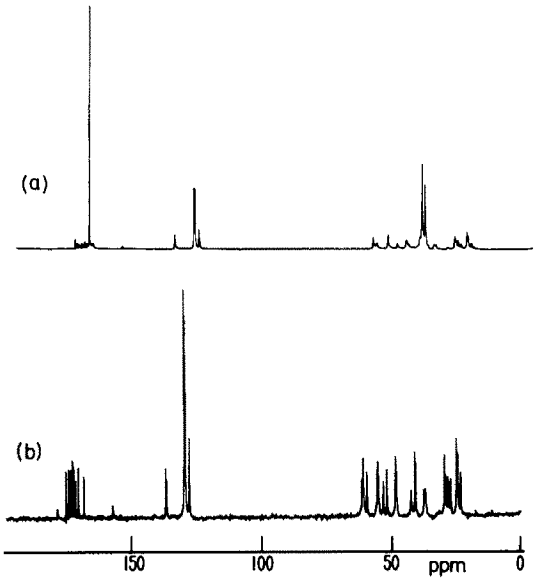


Fig. 1. Proton decoupled ^{13}C NMR spectra of [20%-1,2-$^{13}C_2$-Gly^6]-bradykinin (a) and bradykinin (b). The selective enrichment of three resonances at 41.57, 42.71 and 169.83 ppm (relative to external Me_4Si) corresponding to Gly^6 C-2 *cis*, Gly^6 C-2 *trans*, and Gly^6 carbonyl, respectively, is apparent. Reprinted with permission from *J Am Chem Soc* **101**: 2455–2462, 1979. Copyright (1979) American Chemical Society. [Ref. 15].

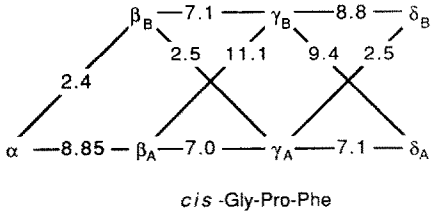
Pro, thyrotropin releasing hormone [13] or angiotensin [14] is found to be pH dependent.

Carbon-13 NMR studies have demonstrated that the replacement of Ser^6 of bradykinin with a glycine residue leads to a significant increase in the fractional *cis* probability of the sixth peptide bond, from 10 to 35% [15]. From a spectroscopic standpoint, the chemical shifts of the glycine are surprising, with the methylene and carbonyl groups exhibiting *cis/trans* shift differences of 1.14 and 0.0 ppm respectively (Fig. 1). Recent ^{19}F NMR studies indicate that the fluorine resonance in [Gly^6,*p*-fluoroPhe^8]-bradykinin is similarly sensitive to the *cis/trans* equilibrium [16]. Conformational analyses of several model peptides such as Gly-Pro-Phe indicate extensive conformational rearrangements involving the phenyl sidechain and the proline ring conformation [17], and support the conclusion that the relatively high *cis/trans* ratio observed in [Gly^6]-bradykinin reflects primarily the unfavorable steric interaction between the glycine carbonyl oxygen and the phenylalanine side chain (Fig. 2a). This is analogous to the unfavorable electrostatic interaction between the glycine carbonyl and the carboxyl group of C-terminal proline residues which similarly destabilizes the *trans* conformation, leading to a high *cis/trans* ratio (Fig. 2c) [8]. Additional stabilization of the *cis* bond conformation may also reflect an attractive electrostatic interaction between the π electrons of the phenyl ring and the Gly carbonyl carbon (Fig. 2b).

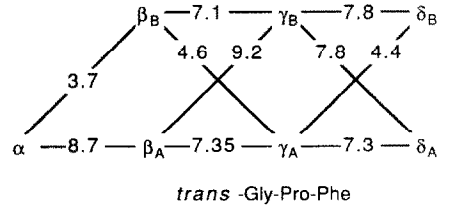
Physiological assays have indicated that [Gly^6]-bradykinin is slightly less active than bradykinin itself [15]. This difference could be related to the conformational perturbation or to the elimination of the interaction of the serine sidechain with the receptor. This analog potentially provides a unique opportunity to study the effects of *cis* peptide bond conformation on physiological activity. Studies of the hydrolysis of X-pro dipeptides by the enzyme prolidase have demonstrated biphasic kinetics reflecting the *cis/trans* conformational distribution of the peptide bond [18–20]. An analogous biphasic response could characterize the physiological response to [Gly^6]-bradykinin, although the time course of most assays precludes such a determination.

Puckering of the pyrrolidine ring of proline

Theoretical calculations indicate two regions of conformational stability for the pyrrolidine ring of proline, which correspond conformationally to C_β-C_γ half chairs [21]. However, in contrast to the case of *cis/trans* conformational equilibria which are separated by an energy barrier of 20 kcal/mol, the energy barrier separating envelope or twist conformations is only ~2.7 kcal/mol [21]. Hence, interconversion is sufficiently rapid on the NMR time scale so that only single resonances with chemical shifts and coupling constants which are weighted averages of the puckered conformations are observed. This interconversion of puckered ring conformations is sufficiently rapid to perturb the relaxation rates of the proline nuclei. A description based on a two-site jump model has been developed, which is a function of the parameters τ_A, τ_B, p_A and p_B corresponding to the lifetimes and probabilities of the two puckered conformations, and the angles β and θ, where the jump is modeled as the limit of a rapid rotation in which β corresponds to the angle between the relaxation vector and the rotation axis, and θ corresponds to the half-range of the motion [22] (Fig. 3). These parameters are not all independent. The lifetimes are related by the usual equilibrium constraint, $p_A/\tau_A = p_B/\tau_B$, the fractional populations are normalized ($p_A + p_B = 1$), and there is only one independent angle since for an instantaneous jump between two states the path is not specified [22]. Simulations for a number of proline-containing peptides indicate lifetimes for the puckered states in the range 10^{-11} to 10^{-12} sec, and ranges of motion 2θ of 50–70°. These conclusions can be refined by including additional data, for example, the vicinal coupling constant data for *cis* and *trans* Gly-Pro-Phe:



cis -Gly-Pro-Phe



trans -Gly-Pro-Phe

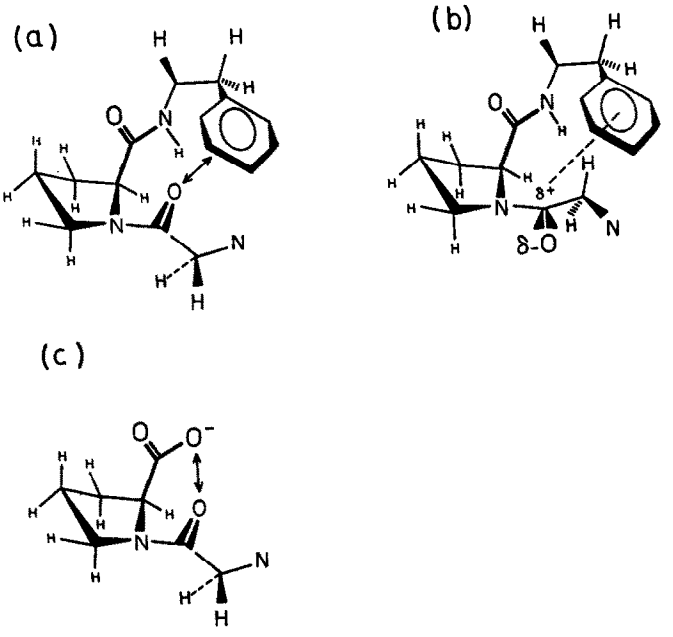


Fig. 2. Predominant *trans* (a) and *cis* (b) conformations of Gly-Pro-Phe based on analysis of ^1H coupling constants. The upfield shift of the Gly C-2 and the pro-R ^1H resonances in the *cis* conformation is seen to reflect the proximity of the phenylalanine ring. The analogous *trans* structure of glycyl-proline indicating the repulsive carboxyl–carbonyl interaction which destabilizes the *trans* conformation is shown in (c).

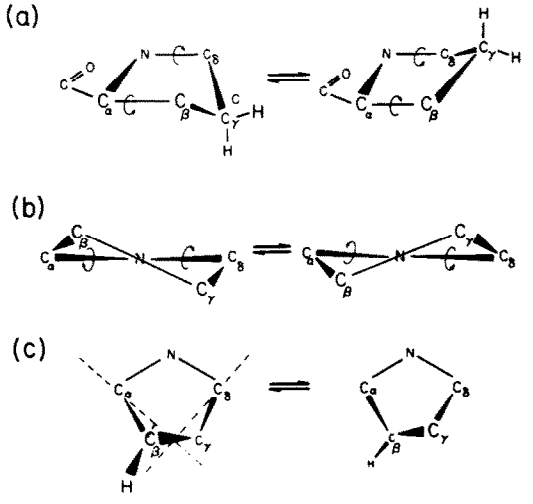


Fig. 3. Puckering motions of the pyrrolidine ring of proline: (a) C_γ-exo–C_γ-endo interconversion with $\beta = 70.5°$ for C_β-H and C_δ-H, and $\beta = 90°$ for C_γ-H; (b) C_β-endo-C_γ-exo–C_β-exo-C_γ-endo interconversion with $\beta = 70.5°$ for C_α-H and C_δ-H, and $\beta = 75.4°$ for C_β-H and C_γ-H. The latter value was calculated assuming a C—C—C bond angle of 104°, C—C bond length of 1.53 Å, and an effective rotation axis shown in (c), which illustrates the jump in (b) viewed perpendicular to the C_δ-N-C_α plane. The two effective jump axes are indicated by the broken line. If the overall motion is isotropic, the first model predicts $T_1^\gamma > T_1^\beta = T_1^\delta = T_1$, while the second predicts that $T_1^\gamma = T_1^\beta > T_1^\delta = T_1$. Each pattern has been observed in different proline-containing peptides, but the most typical pattern, $T_1^\gamma > T_1^\beta > T_1^\delta$ presumably reflects a modified form of the jump illustrated in (b) in which the range of motion for C_γ-H exceeds that for C_β-H. Reprinted with permission from *J Am Chem Soc* **100**: 2678–2685, 1978. Copyright (1978) American Chemical Society. [Ref. 22].

From the above data, the symmetry of the coupling interactions indicates the presence of a mixture of C_γ-endo–C_γ-exo conformers, with ratios calculated as 60:40 for the *trans* isomer and 70:30 for the *cis* [17]. For the bistable model discussed above, a maximum relaxation effect of the puckering motion, and hence the largest increase for the T_1 of the γ carbon, is predicted for $\tau_A = \tau_B$, while the effect of puckering vanishes in the limit of one state becoming much more stable than the other [22]. Hence, if the rates and range of the puckering motion remain relatively constant, the coupling constant data would predict that the parameter NT_1^γ/NT_1^q (where N is the number of directly bonded protons) should be larger for the *trans* isomer. This is indeed the case, with ratios of 1.74 and 2.02 measured for the *cis* and *trans* conformers respectively [17]. Modeling the dynamic behavior of the proline by assuming overall isotropic motion and $\tau_{endo} = 10^{-12}$ sec, leads to a jump range of 56° for both conformations.

We have extended the formalism of the bistable jump model to the case of overall anisotropic motion [23]. In this case, additional data must be obtained for the system under study to characterize the anisotropic nature of the overall motion. Although in the case of bradykinin, overlap of the ^{13}C resonances has precluded an analysis of the dynamics of individual proline residues, it is now possible using two-dimensional NMR techniques to obtain separate resonances for each of the proline residues, and consequently to analyze differences in relaxation behavior [24]. It has also been demonstrated recently that ^2H NMR studies of deuterated proline in the solid state can provide a useful means of studying the dynamics of puckering of the pyrrolidine ring [25].

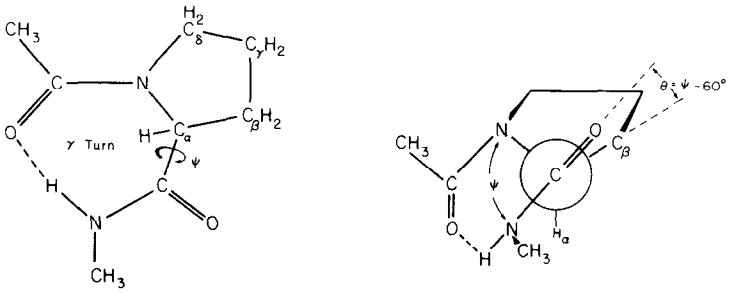


Fig. 4. A model of acetylproline *N*-methyl amide in the γ turn conformation. The nearly eclipsed orientation of the proline C_β and carbonyl oxygen atoms in this conformation, which is associated with a significant upfield shift of the proline C_β resonance, is illustrated. Reprinted with permission from *Int J Pept Protein Res* **14**: 377–387, 1979. Copyright (1979) Munksgaard Int. Publishers Ltd., Copenhagen, Denmark. [Ref. 29].

Gamma turn conformations

Circular dichroism (CD) studies of bradykinin and related analogs have suggested the presence of a γ turn involving the Phe[8] amide proton and the Ser[6] carbonyl oxygen, bridging the Pro[7] residue [26, 27]. ^{1}H NMR [28] and ^{13}C NMR [29] studies of the model peptide acetylproline *N*-methylamide have been carried out which provide insight into the NMR parameters associated with such γ turns. These studies show that in nonpolar solvents the *trans* isomer exists predominantly in the γ turn structure with an intramolecular hydrogen bond involving the amide proton and the acetyl oxygen. Alternatively, the *cis* isomer tends to form aggregates by intermolecular hydrogen bonding. Bases on ^{13}C NMR studies of a series of conformationally constrained peptides, an empirical correlation between the chemical shift difference $\Delta_{\beta\gamma}$ ($=\delta_\beta - \delta_\gamma$) and the value of ψ was deduced [30]. This

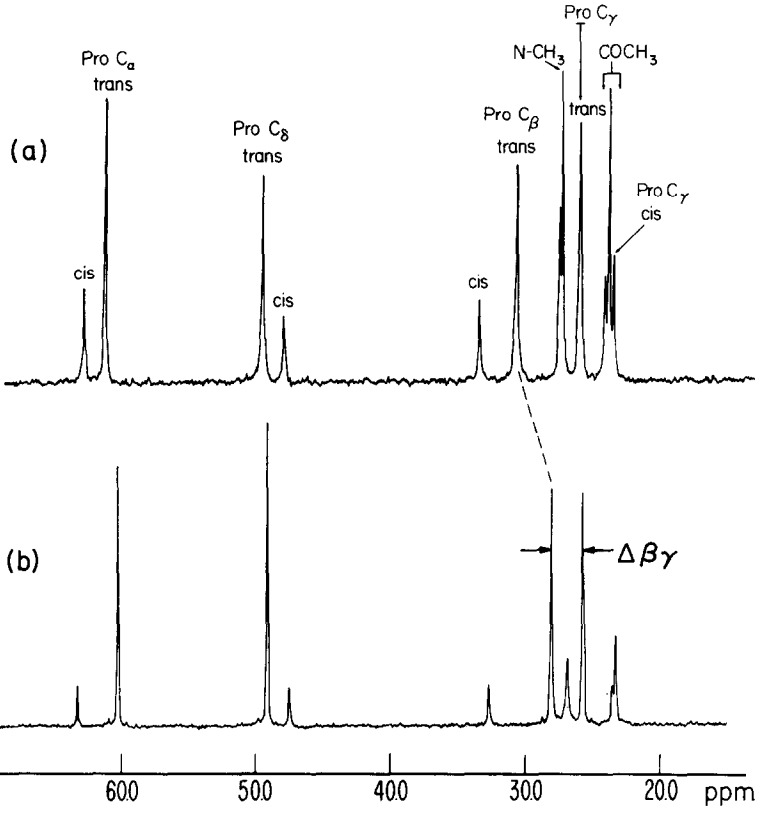


Fig. 5. Proton decoupled ^{13}C NMR spectra of acetylproline *N*-methyl amide obtained in deutero chloroform, illustrating the effect of dilution: (a) 3 M peptide; (b) 0.147 M peptide. The decrease in the *cis/trans* ratio as well as the upfield shift of the *trans* C_β and C_α resonances accompanying peptide dilution are readily observed. It is noted that an additional effect of high concentration is a reduction in the T_1 values so that the methyl resonances are considerably reduced in (b). Reprinted with permission from *Int J Pept Protein Res* **14**: 377–387, 1979. Copyright (1979) Munksgaard Int. Publishers Ltd., Copenhagen, Denmark. [Ref. 29].

correlation reflects a "γ effect" type of interaction between the proline carbonyl oxygen and the proline C_β. Since the formation of a $3 \rightarrow 1$ hydrogen bond bridging proline will require a value of ψ close to 60° resulting in a nearly eclipsed orientation of C_β and CO (Fig. 4), an upfield shift is expected for the C_β resonance of proline in peptides adopting this conformation. Dilution of the model peptide in chloroform increases the fraction of intramolecularly bonded *trans* conformers. This is reflected in the decreased intensity ratio of *cis*/*trans* resonances and in the upfield shift of the proline C_β resonance (Fig. 5). Extrapolation to infinite dilution suggested that the chemical shift difference $\Delta_{\beta\gamma}$ decreases to a value near zero. Hence, the magnitude of this parameter may be used to provide a rough estimate for the fractional γ turn probability. If the peptide is assumed to exist as a mixture of conformations which include the γ turn ($\Delta_{\beta\gamma} = 0$) and the more stable *cis'* and *trans'* conformations ($\Delta_{\beta\gamma}$ = 5–6 ppm), an upper limit on the fractional γ turn probability can be estimated. Based on analysis of the Pro^7 resonances of bradykinin, it is concluded that, in aqueous solution, the γ turn probability is <25% [29].

Carbon-13 relaxation behavior in carbon-13-labeled bradykinin

Measurements of the spin-lattice relaxation times for the protonated carbon-13 nuclei of bradykinin yield the typical pattern observed for peptides observed to be largely disordered in solution: NT_1 values for backbone carbons increase towards the C and N terminals of the peptide, and out along the sidechains [31]. An approach to the problem of studying the dynamics of the peptide involves the introduction of multiply labeled amino acids so that carbon–carbon dipolar interactions can be probed [32]. The ^{13}C NMR spectrum of [90%-1,2-$^{13}C_2$-Gly^6]-bradykinin is shown in Fig. 6. If the enrichment is <100%, comparison of the relaxation behavior among the various isotopomers allows an accurate quantitation of the small carbon–carbon dipolar term. In the extreme narrowing limit, such interactions are only significant for non-protonated carbons (Fig. 7). However, carbon–carbon interactions can become considerably more significant in slowly tumbling molecules [32]. For the case of [90%-1,2-$^{13}C_2$-Gly^6]-bradykinin, the correlation time τ_{CC} derived from the carbon–carbon dipolar interaction is significantly longer than the value of τ_{CH} derived from the relaxation of the glycine methylene group. This result presumably reflects the fact that motion of the carbon—carbon bond is slower than torsional motion of the C—H bonds about the carbon—carbon bond. It would be useful, however, to obtain additional data on such effects for isotopically labeled rigid molecules in order to obtain further insight into the interpretation of this result.

In addition to the comparisons of T_1 relaxation behavior which can be made in multiply labeled

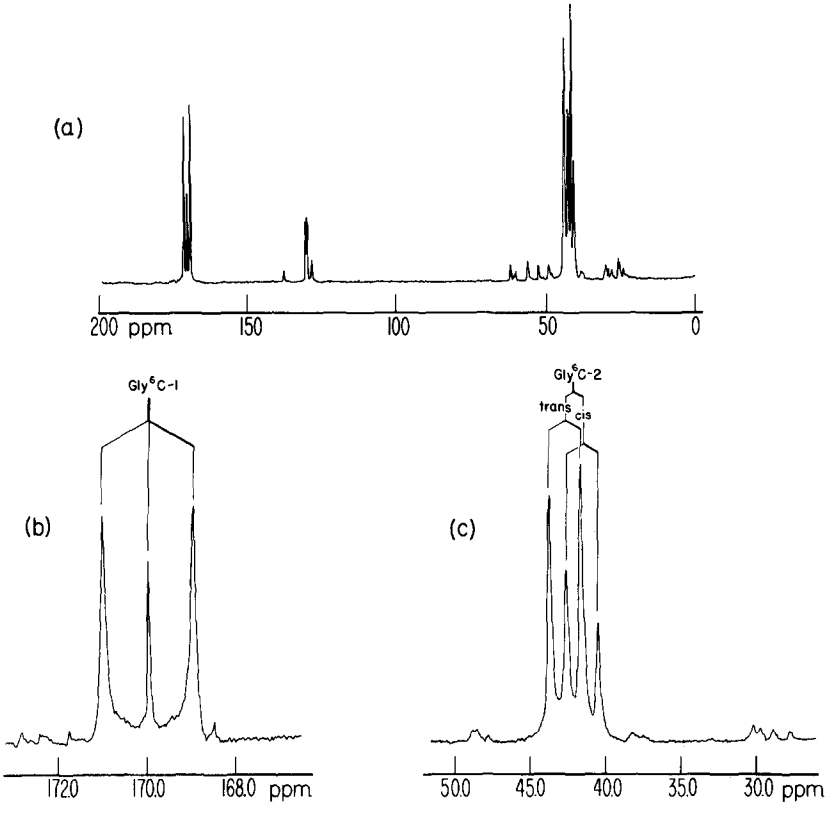


Fig. 6. Proton decoupled ^{13}C NMR spectra of [90%-1,2-$^{13}C_2$-Gly^6]-bradykinin: (a) full spectrum; (b) carbonyl resonances; and (c) methylene resonances. Reprinted with permission from *Biochemistry* **21**: 470–477, 1982. Copyright (1982) American Chemical Society. [Ref. 32].

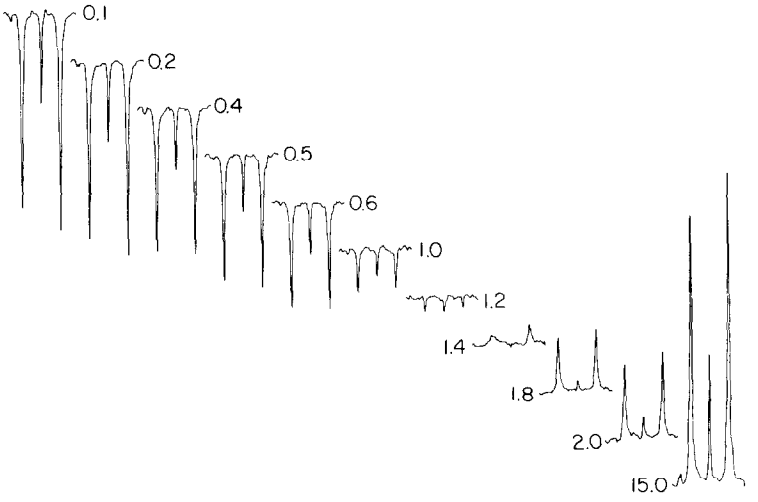


Fig. 7. Inversion recovery study of the carbonyl resonances of [90%-1,2-$^{13}C_2$-Gly^6]-bradykinin. The additional contribution of the carbon–carbon dipolar interaction is most readily observed for the 1.4 sec delay. Reprinted with permission from *Biochemistry* **21**: 470–477, 1982. Copyright (1982) American Chemical Society. [Ref. 32].

systems, linewidth comparisons have led to an appreciation of an alternative broadening mechanism which can arise in scalar coupled spin 1/2 systems [32]. Thus, as a consequence of the relatively short spin-lattice relaxation time for nuclei in larger molecules, the multiplet structure begins to collapse, analogous to the effect typically observed for spin 1/2 nuclei coupled to quadrupolar nuclei. The carbonyl resonances of [90%-1,2-$^{13}C_2$-Gly^6]-bradykinin illustrate this behavior (Fig. 6b). The relatively rapid relaxation of the glycine methylene group broadens the lines of the carbonyl doublet which correspond to the doubly labeled molecules. Alternatively, the center line corresponding to molecules labeled at the carbonyl but not the methylene carbon is significantly narrower. As discussed previously, this effect will generally lead to a contribution of $1/2\pi T_1$ to the linewidth of the nucleus under observation, where the T_1 in the formula corresponds to the nucleus which is scalar coupled to the observed nucleus. This mechanism is probably responsible for the general broadening of coupled proton resonances of biological macromolecules by ~1 Hz [33].

β Turn structure and α-Aminoisobutyric acid substitution

As noted above, the principle role of proline residues in peptide hormones is more likely to involve the imposition of structural constraints than specific interactions with receptors. The statistical preference of proline residues for positions 2 and/or 3 of β turn conformations in globular proteins has been well documented [1]. The structural constraint introduced by the cyclic nature of the sidechain, requiring that $\phi = -60°$, in turn requires that for the case of *trans* X-Pro, the proline residue occupy position 2 of a type I or II β turn, or positions 2 and/or 3 of a type III β turn, with $\psi = -30°$ in the type I and III turns, or $\psi = 120°$ in the type II turn [34]. These two ranges of values for ψ have been referred to as *cis'* ($\psi = -40°$) and *trans'* ($\psi = 140°$), since the proline nitrogen is in a *syn* or *anti* orientation relative to the nitrogen on the succeeding amino acid (Fig. 8). [35–37]. For the *cis* X-Pro bond, proline can occupy position 3 of a type VI turn. Although relaxation studies [12] and ^1H coupling constant data [38] suggest that the solution conformation of bradykinin involves rapid fluctuations among a broad range of

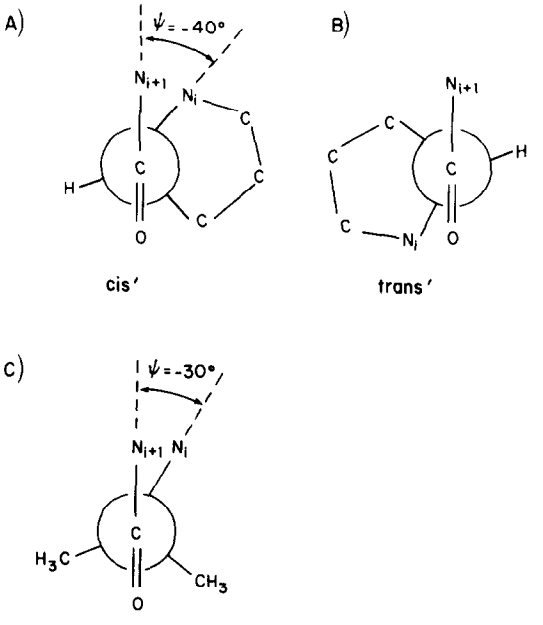


Fig. 8. The *cis'* (A) and *trans'* (B) conformations of proline-containing peptides having the proline and succeeding nitrogen atoms in *syn* or *anti* relationship. The analogous *cis'* conformation of AIB with $(\phi, \psi) = (-60°, -30°)$ is also shown in (C).

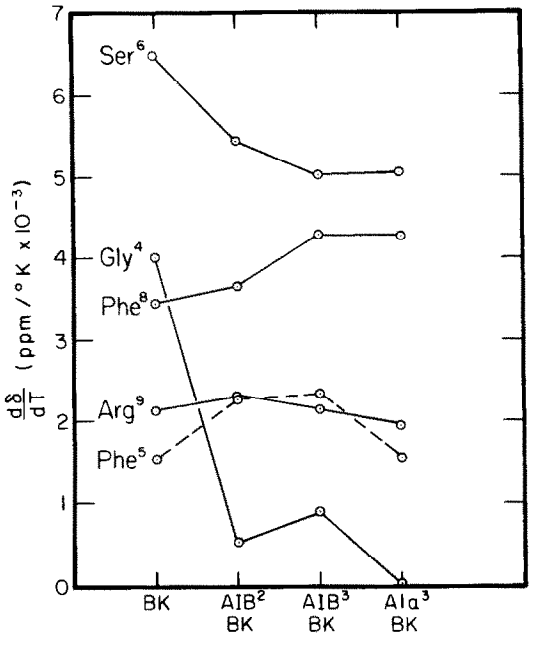


Fig. 9. Effects of AIB/Pro substitutions on the temperature dependence of the amide proton shifts, $d\delta/dT$, in AIB^2-bradykinin and AIB^3-bradykinin. Ala^3-bradykinin also shows a reduction in $d\delta/dT$. Data from Ref. 48 were obtained in DMSO-d_6.

conformations, several lines of evidence suggest the presence of a folded structure near the C-terminal portion of the peptide. ^1H NMR studies indicate that the Gly^4 proton resonances are sensitive to titration of the C-terminal carboxyl group [39]. CD evidence in support of a γ turn bridging Pro^7 was noted above. NMR evidence in support of a type II β turn structure involving the Pro^2-Pro^3-Gly^4-Phe^5 residues has also been obtained in dimethyl sulfoxide (DMSO) [40–42]. Alternatively, model peptides containing the Pro-Pro sequence have been shown to adopt a 3_{10} helical structure containing two overlapping type III β turns in solution [43].

To evaluate the significance of this conformational preference further, the analog α-aminoisobutyric acid (AIB), which theoretically favors regions in the Ramachandran plots with $(\varphi, \psi) = (-60°, -30°)$ or $(+60°, +30°)$ [44, 45], was substituted for each of the proline residues of bradykinin. Hence, one of the stable conformations of AIB containing peptides will exhibit (ϕ, ψ) values similar to those anticipated for *cis'* proline, with the introduction of a relatively small steric constraint—the pro-R methyl group (Fig. 8). The ability of AIB to substitute for both the Pro^2 and Pro^3 residues of bradykinin would further provide specific support for the presence of a type III β turn [46, 47]. The introduction of AIB residues at positions 2 and/or 3 resulted in upfield shifts for the Gly^4 NH amide proton resonance as well as in a reduced temperature dependence $d\delta/dT$, of the Gly^4 amide proton shift (Fig. 9) [48, 49]. These AIB substitutions are therefore consistent with stabilization of a type III β turn or, in the case of AIB^3-bradykinin, a type I turn involving Pro^2-AIB^3-Gly^4-Phe^5. Similar conformational conclusions follow from an analysis of the CD spectra of AIB substituted bradykinin analogs [49]. However, the activities of the AIB^2- and AIB^3-substituted bradykinin analogs are very low, suggesting that this conformation does not correspond closely with the receptor bound form of the peptide. Substitution of AIB for Pro^7 resulted in many significant chemical shift perturbations throughout the peptide, suggesting a significantly altered conformation. However, AIB^7-bradykinin is the most active position 7 analog of the peptide that has been tested to date [50]. The observation that an analog with an alkyl (methyl) substituent in the pro-R position had significant activity led to further evaluation of analogs containing D-amino acids at position 7, with D-Phe^7 yielding the first analog with significant bradykinin antagonist activity that has been tested [2].

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