Conformational Mobility of the Pyrrolidine Ring of Proline in Peptides and Peptide Hormones as Manifest in Carbon 13 Spin-Lattice Relaxation Times*

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SUMMARY
The spin-lattice relaxation times (T1) of carbon 13 in natural abundance were determined for proline, N-acetylprolineamide, glycylproline, cyclo(triprol), and a series of proline-containing peptide hormones. The data are interpreted in terms of rapid interconversion of proline between various ring-puckered forms. The nature of the puckering depends upon the type of group attached to proline. In proline, the β, γ, and δ carbon atoms are appreciably more mobile than the α carbon atom, suggesting rapid interconversion between a number of ring-puckered forms. In melanocyte-stimulating hormone release-inhibiting hormone (Pro-Leu-Gly-NH2) and its dimethylamido analog, the γ carbon atom has the greatest mobility, suggesting a rapid endo-exo interconversion at this position. In Gly-Pro and in acetyl-Pro-NH2, the cis and trans conformers of proline have very similar T1 values, indicating very little dependence of the dynamic proline ring conformation on the peptide bond; in both isomers, the proline ring interconverts rapidly between half-chair conformers puckered at Cβ and Cγ. In thyrotropin-releasing hormone (<Glu-His-Pro-NH2), the proline ring conformation is similar to that in Gly-Pro, and independent of the state of ionization of the histidine residue. Oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2), lysine-vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH2), [Ile5]angiotensin II (Asp-Arg-Val-Ile-His-Pro-Phe), and luteinizing hormone-releasing hormone (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) all have proline in the trans conformation about the peptide bond, and the δ carbon atom has a mobility comparable to that of the α carbon atom. The latter reflects severe steric constraints at C6 due to the neighboring residues. In oxytocin and angiotensin II, the β carbon atom is considerably more mobile than Cα or Cδ. In luteinizing hormone-releasing hormone, the proline ring apparently undergoes rapid interconversion between half-chair forms puckered at Cβ and Cγ, whereas in lysine-vasopressin, the mobility of Cγ is greatest.

The conformation of the pyrrolidine ring in proline and proline-containing peptides has been extensively investigated by x-ray crystallography (1-7), model building (8), minimum energy (9-12) calculations, as well as by proton magnetic resonance (13-16). The 1H NMR method can provide experimental information concerning the conformation in solution and conformational equilibria, but it is considerably hampered by the complexity of the spectra. An alternative method of studying the conformational mobility of proline in solution is the measurement of carbon 13 spin-lattice relaxation times (17, 18). We have examined a number of proline-containing peptides and peptide hormones, and find the pyrrolidine ring to be more mobile than previously thought. In general, the γ carbon atom is the most mobile and the α carbon atom is the least mobile. Differences in the mobility of the various carbon atoms relative to their analogs in proline monomer are proposed to be due to the presence of the contiguous residues.

MATERIAL AND METHODS
Spectra of L-proline (Seikagaku Kyogo Co., Tokyo, Japan; 100 mg per ml in D2O) were taken at pH meter readings of 1.0, 6.4, and 11.3. Spectra of Gly-Pro (Bachem Fine Chemicals, Marine del Rey, Calif.; 170 mg per ml) were taken in D2O at “pH” values of 1.0, 5.0, and 11.0 (“pH” = pH meter reading + 0.4). Acetyl-

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† Abbreviations for amino acids and peptides follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature in (1972) Biochem. J. 126, 773-780. All optically active amino acids are of the L configuration. Other abbreviations used are: D2O, deuterium oxide; T1, spin-lattice relaxation time; N, number of directly attached protons.
The preparation and experimental methods for Pro-Leu-Gly-NH\(_2\) (melanocyte-stimulating hormone release-inhibiting factor) and Pro-Leu-Gly-N(CH\(_2\))\(_2\) have been described (17).** <Glu-His-Trp-Ser-Tyr-Glu-Arg-Pro-Gly-NH\(_2\) (luteinizing hormone-releasing hormone), a gift from Dr. H. McGregor, Wyeth Laboratories, Philadelphia, Penn., was examined at \(pH 5.3\) (100 mg per ml of \(D_2O\)). Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (\([Ile^5]\)angiotensin II), a gift from Dr. A. C. M. Paiva, São Paulo, Brazil, was run at \(pH 4.5\) (100 mg per ml of \(D_2O\)). Cys-Tyr-Ile-Glu-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH\(_2\) (luteinizing hormone-releasing hormone) was run at \(pH 9.9\) (100 mg per ml of \(D_2O\)). D2O was used to obtain \(T_1\) measurements. For the peptide hormones, \(pH\) was adjusted using CD\(_3\)COOH or solutions of NH\(_2\)OH in D\(_2O\).**

**Some errors have been corrected in this text.**

**RESULTS AND DISCUSSION**

The \(T_1\) values of the carbons in proline and proline-containing peptides multiplied by \(N\), the number of directly attached hydrogen atoms, are given in Table I. The products \(N\alpha\) are indicative of the motion of the individual carbon atoms—either over-all rotation of the molecule, intramolecular motion, or both (21, 22). The larger the \(N\alpha\) value, the greater is the mobility of that carbon atom. Internal motion must take place at a rate significantly greater than the rate of over-all rotation if it is to be manifest in the \(T_1\) values, as discussed recently by Allerhand and Komoroski (22). We shall use the \(N\alpha\) values within the pyrrolidine ring of proline in a variety of environments as indicators of the relative degrees of intracyclic motion.

**Proline Monomer**

An analysis of crystal structures of proline-containing compounds showed that \(\alpha\), \(\beta\), \(\delta\), and \(N\) atoms are nearly coplanar and that the \(\gamma\) carbon atom was displaced, either above or below the plane formed by the other 4 atoms (6, 9) (the endo conformation being that in which the displaced carbon is syn to the carboxyl group, i.e. above the plane formed by the other...
4 atoms; the evo conformation being that in which the displace-
ded carbon atom is below the plane and thus near to the
carboxyl group). However, in DL-proline hydrochloride, an x-ray
analysis study showed that the ring was puckered at the a carbon
atom, which deviated about 0.5 A from the best plane formed by
the 4 remaining atoms (2). Minimum energy conformational calcu-
lations (9) have indicated that any 1 of the atoms can be appreciably
out of the plane formed by the other 4 atoms. Similar conclusions
have been drawn for cyclo-
peptide by Beckett et al. (23) from data gathered by electron
diffraction, Raman, infrared, entropy, and specific heat mea-
urements. They proposed that the cyclopeptide ring confor-
mation is not of a definite type, and that the ring pucker rotates
around the ring as though a static pucker ring were rotating.
This process is known as pseudorotation.

Substitution in the ring creates a barrier to pseudorotation
and the energy minima were found to correspond to a fixed
"envelope" or to a fixed "half-chair" (24). In general, only
these static conformations have been considered in 1H nuclear
magnetic resonance studies because of the difficulties involved
in considering a number of puckered conformations of similar
energy (25). Abraham and McLaughlin (26, 27) analyzed the
1H NMR spectrum of trans-4 hydroxyproline and found that
this compound exists in the envelope conformation with Cγ out
of the plane formed by the other 4 atoms. The spectrum of cis-4
hydroxyproline revealed that this compound adopts a
highly puckered envelope conformation with Cβ out of the
plane.

Pyrrylidine rings, by analogy with tetrahedrosuran and
cyclopeptide, are expected to interconvert rapidly between ring-
puckered conformations (25). The 1H NMR spectrum of proline
in basic aqueous solution (15) was interpreted in terms
of fast interconversion between the Cγ endo and evo confor-
mations. The flexibility of pyrrylidine rings has been suggested
by Mitsui et al. (2) in their x-ray study of DL-proline hydrochloride.
They found that Cβ, Cδ, and particularly Cγ had an unusually
large vibrational amplitude in the direction perpendicular to the
best plane of the pyrrylidine ring.

Our 1H data (Table I) show that the α carbon atom of proline,
compared to the β, γ, and δ carbon atoms, has the shortest T1;
the variation in T1 as a function of "pH" is similar to that ob-
served by Saito and Smith (28) for lysine and glycine. The
NT1 values for the β, γ, and δ carbon atoms differ from one
other by only about 10%, but this difference is maintained
at all the "pH" values examined. A number of explanations
are possible for the different NT1 values around the ring.

The first possibility is simply that all carbon atoms in proline
are not subject to the same relaxation mechanisms. We have
verified that all the carbon atoms relax via dipole-dipole coupling
to protons by measuring an over-all intensity enhancement of 3
due to the Overhauser effect (29). Thus T1 is given by (21)

\[
\frac{1}{T_1} = \frac{1}{T_{10}} = \frac{N \gamma^2}{r^2} \left[ f(\omega_H - \omega_C) + 3f(\omega_C) + 6f(\omega_H + \omega_C) \right]
\]

where N is the number of directly attached hydrogens, f(\omega) =
\(\tau_r/(1 + \omega^2 \tau_r^2)\), \(\tau_r\) is an effective correlation time for reorientation
of a particular carbon moiety, \(\omega_C\) and \(\omega_H\) are the resonance
frequencies of 13C and 1H, and r is the distance between the
proton and the vC nucleus. For carbons bearing protons, we
can neglect nearest neighbor protons because of the r^6 de-
pendence of the dipolar relaxation mechanism.

Equation 1 assumes isotropic tumbling such as that which
would occur with a molecule of high symmetry. Our observa-
tion of unequal T1's for the pyrrylidine carbon atoms of proline
could be due either to over-all rotational anisotropy (30, 31), or
rapid intramolecular ring puckering. Although over-all aniso-
trropic motion can be important for highly asymmetric molecules
such as proline monomers, we believe the major contribution for
proline in all the compounds considered here arises from rapid
ring puckering for the reasons described below. Levy et al. (31)
have found that the relaxation times of protonated ring carbon
atoms in five-membered heterosaromatic compounds show
moderate to small differences as a result of anisotropic tumbling
mainly because there are no C—H bonds exactly aligned with
the preferred molecular rotational axis coincident with a sub-
stitute-ring bond. Proline could rotate about an axis co-
cident with the Cα carboxyl carbon bond (Fig. 1), thus partly
explaining the observed differences in T1 values (i.e. α < β, 6,
and γ). Substitution at the N atom would decrease the im-
portance of this type of rotation, and yet in acetyl-Pro-NH2
(εido infra) we observe a difference in ring carbon T1 values
qualitatively similar to that found in proline itself. More
convincing evidence comes from preliminary T1 data (Table 1)
for cyclo(Proα) which has C3 symmetry and therefore has a
reduced possibility for anisotropic over-all motion of the proton
residues.2 We therefore interpret the T1 values observed in
proline and proline-containing peptides in terms of contribution
from rapid interconversion between various ring-puckered
forms. In proline, the T1 value of the α carbon atom reflects
the rate of over-all molecular motion, whereas the NT1 values
of the β, γ, and δ carbon atoms reflect rapid conformational
interconversions of these carbon atoms, with the γ carbon atom
undergoing the largest variation of its position relative to the
α carbon atom.

Proline in NH2-terminal Positions

Pro-Leu-Gly-NH2 and Pro-Leu-Gly-N(CH3)+1—T1 measure-
ments have been reported for all carbon atoms in these com-
ounds (17) and it was found that the α carbon atoms of proline
and leucine had similar T1 values. In all cases, the β and δ
carbon atoms of the pyrrylidine ring had identical T1 values
and that of the γ carbon atom was significantly longer. Thus,
the effect of substitution at the carboxyl group is to exaggerate
the greater mobility of the γ carbon atom with respect to the

others. The dynamic equilibrium is now more toward rapid endo-endo conversion at the γ carbon atom. The effect is less pronounced in Pro-Leu-Gly-N(C1)H2. This may be due to the inability of this compound to form the β turn suggested to occur in the unmethylated compound (32). As this turn is thought to be stabilized by hydrogen bonding between an amido hydrogen of glycine and the carbonyl oxygen of proline, its presence would result in a change in the steric environment of proline. The much shorter T1 values for Pro-Leu-Gly-NH2 in (CD3)2SO may be due to aggregation (17) or to a decreased tumbling rate due to the greater viscosity of (CD3)2SO (44). The relative T1 values indicate that it also serves to diminish the preference for rapid motion of the γ carbon atom of proline. This phenomenon is not observed in the dimethyl derivative—the T1 values in both solvents are comparable. Thus, methylation of the amide group of glycine eliminates the tendency of Pro-Leu-Gly to aggregate, implying that the amide hydrogen are involved in the aggregation process.

Proline in COOH-terminal Positions

Gly-Pro—N-acetylated proline residues (X-Pro) exhibit cis-trans peptide bond isomerism, as most clearly shown in solution by 13C NMR spectra (33-41). Cis isomers usually represent 25 to 40% of the total population in dipeptides. We have determined the T1 values of the carbon atoms of both the cis and the trans isomers in Gly-Pro (Table I). In both the cis and the trans isomers, the β and γ carbon atoms had identical NT1 values which were significantly longer than those of the δ or α carbon atoms. This raises the possibility that the time-averaged ring conformation includes a substantial proportion of the half-chair conformations in which the β and γ carbon atoms undergo more motion than other carbon atoms. This type of conformation has been observed in substituted cyclopentane rings (25).

Proline in Nonterminal Positions

Acetyl-Pro NH2—This compound serves as a model for proline in a nonterminal position in a peptide which is not sterically constrained by adjacent residues. We have determined the T1 values for both cis and trans isomers and found the same order of T1 values as in Gly-Pro. The β and γ showed similar NT1 values and these were longer than those of the δ and α carbon atoms, leading us to conclude that the ring conformation is best described as a rapidly interconverting set of half-chair conformers.

Thyrotropin-releasing Hormone—The T1 values for this hormone, <Glu-His-Pro-NH2, have been reported in D2O and (CD3)2SO (18, 43). In the present study, data were taken at "pH" 4.2 and "pH" 9.9 to enable evaluation of the effect of changing the charge of an adjacent residue (pK = 6.2 for His in <Glu-His-Pro-NH2) on the T1 values of the carbon atoms of proline. This compound showed the presence of both cis (14%) and trans (86%) isomers about the X-Pro bond (18); the relative populations were independent of "pH." The T1 values could be measured only for the trans isomer due to the small amplitude of the cis resonances. At both "pH" values, the T1 values of the γ and β resonances are the longest. We conclude that proline in <Glu-His-Pro-NH2 behaves essentially like that in acetyl-Pro-NH2 and Gly-Pro, and that changing the charge on the histidine residue has a negligible influence on the time-averaged proline ring conformation.

Oxytocin, Lysine-Vasopressin, [Ile4]Angiotensin II, Luteinizing Hormone-Releasing Hormone—We have measured the T1 values of all of the carbon atoms in these compounds (42) and have found the X-Pro bond in these hormones to be in the trans conformation in aqueous medium (36, 40). The α and δ carbon atoms of the proline residue in these hormones show similar NT1 values, the β and γ carbon atoms have longer NT1 values. It was not possible to measure T1 values for the γ carbon atom of the proline residue in oxytocin and [Ile4]angiotensin II due to partial overlap with other resonances. In these three hormones, the very similar NT1 values of the α and δ carbon atoms reflect the steric constraints imposed on the pyrrolidine ring of proline by the peptide backbone. A similar result was reported for the proline of gramicidin S in methanol (22). Lysine vasopressin, oxytocin, [Ile4]angiotensin II and luteinizing hormone-releasing hormone are the first examples in which the T1 value of the δ carbon atom of proline is half of that of the β carbon atom. Only the β (and γ) carbon atoms in these compounds retain more mobility than the α carbon atom; the δ carbon atom apparently encounters the same steric restrictions as the α carbon atom.

CONCLUSION

The T1 values of the pyrrolidine ring carbon atoms of proline may be interpreted in terms of rapid interconversion between various ring-puckered conformations. In proline monomer the β, γ, and δ carbon atoms are more mobile than the α carbon atom, with the γ carbon atom undergoing the greatest alteration of position. A proline residue substituted at the carboxyl carbon has NT1 values in the order α < β = δ < γ, possibly as a consequence of rapid endo-exo interconversion at the γ carbon atom, as has been observed by 1H NMR.5 Substitution at the N atom of proline results in equal T1 values for the β and γ carbon atoms; this is thought to be the result of a conformational equilibrium in which half-chair interconversions are predominant. Similar conclusions result from studies on acetyl-Pro-NH2 and <Glu-His-Pro-NH2. Steric constraints imposed on nonterminal proline residues in oxytocin, lysine-vasopressin, luteinizing hormone-releasing hormon, and [Ile4]angiotensin II result in equal NT1 values for the α and δ carbon atoms. These are the most extreme cases in which mobility of the pyrrolidine ring is restricted to the β and γ carbon atoms. The conformation of the pyrrolidine ring of proline is thus a time average of a series of classical puckered conformers, which is highly dependent on the presence and nature of contiguous moieties.

REFERENCES

10. It should be pointed out that rapid on the 1H NMR scale is approximately 10^4 sec^-1, whereas on the time scale of 13C spin-lattice relaxation times it is about 10^8 sec^-1.
Conformational Mobility of the Pyrrolidine Ring of Proline in Peptides and Peptide Hormones as Manifest in Carbon 13 Spin-Lattice Relaxation Times
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