A Study of the Peptide Hormone Oxytocin and of Prolylleucylglycinamide by ^{15}N NMR

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Abstract: We have performed an extensive ^{15}N NMR study in water of the peptide hormone oxytocin and of L-prolyl-L-leucylglycinamide, the peptide with the sequence of the C-terminal tripeptide of oxytocin. The chemical shifts of all nitrogen nuclei in the two peptides have been determined. These shifts are shown to be very similar to those of *N'*-acetylamino acids in dimethyl sulfoxide. Deviation from this correlation may be related to conformational restrictions or lack of exposure to solvent. Spin-lattice relaxation times (T_1's), nuclear Overhauser enhancements (NOEs), and one-bond N–H coupling constants are reported. Dipolar relaxation dominates the observed T_1's, and the ^{15}N relaxation measurements are consistent with isotropic motion of the tocin ring with a correlation time similar to that determined previously from ^{13}C NMR measurements. Paramagnetic metal ions can be particularly troublesome in such ^{15}N studies, and a technique for their removal is described.

Introduction

Nuclear magnetic resonance studies of the nuclei present in peptides have predominantly concerned ^{13}C and protons, while the nitrogen nucleus has been relatively neglected. Of the two common isotopes of nitrogen, the more abundant ^{14}N nucleus is unsuitable for most NMR investigations because of its quadrupole relaxation properties, while the ^{15}N nucleus is difficult to study because of its low natural abundance[2] and its low magnetogyric ratio, which make it hard to detect compared to ^{13}C or protons. Recent advances in two areas have made studies of ^{15}N NMR in peptides far more feasible.

New NMR spectrometers with greater electronic sensitivity and larger sample volumes have greatly increased the obtainable ratios of signal to noise. The ability to synthesize sufficient quantities of peptides rapidly and economically for these studies, particularly using solid-phase peptide synthesis,[3] makes available quantities of material much greater than can be readily obtained from biological sources, and provides a means of isotopic labeling. Such labeling is crucial for the assignment of chemical shifts described here, and for the determination of heteronuclear coupling constants described elsewhere.[4]

A few peptides have been examined by ^{15}N NMR[5-16] but only one peptide of biological origin, the aluminum analogue of ferrichrome, alumichrome, has been the subject of comprehensive ^{15}N NMR studies in which chemical shifts,[5] coupling constants,[5,6] and relaxation times[7] have been reported for solutions of the peptide in organic solvents.

Taking advantage of the developments mentioned above, we have undertaken a detailed study of the peptide hormone oxytocin and its C-terminal tripeptide L-prolyl-L-leucylglycinamide (PLG), the proposed melanocyte-stimulating hormone release-inhibitory factor (Figure 1).[17] Oxytocin is a nonapeptide pituitary hormone with the first six residues cyclized to form the tocin ring by a disulfide between the half-cystyl residues in positions 1 and 6. Conformations have been proposed for this peptide in dimethyl sulfoxide (Me_2SO) solution; in water there appears to be a dynamic interconversion between several conformations.[18]

It might be expected that both ^{15}N chemical shifts and T_1's would be sensitive to various features of the conformations of oxytocin and PLG. In particular, amide hydrogen bonding, an important determinant of solution structure(s), might be reflected in these values. In order to interpret meaningfully the ^{15}N chemical shifts in various compounds, a suitable set of values from model compounds is obviously necessary. We report here that values from *N'*-acetylamino acids in Me_2SO are usefully correlated with those of peptide ^{15}N amides in aqueous solution.

The values of T_1's of ^{15}N peptide nitrogens are expected to be dependent on the flexibility or segmental motion of the peptide backbone and on the overall rotational correlation time of the molecule.[2,19] An important consideration for the interpretation of T_1's is whether the relaxation process is predominantly dipolar, with the attached amide proton acting as the relaxing nucleus. The extent of dipolar relaxation is most effectively monitored by measuring the magnitude of the nuclear Overhauser enhancement (NOE).[19] These measurements of ^{15}N T_1's are an important adjunct to previous studies of relaxation of carbons at the α positions in oxytocin,[20] because of the different molecular locations and vectors of motion of the various nuclei.

Experimental Section

Labeled and unlabeled oxytocins were prepared as previously described.[21] The isotopic isomers are more fully described in ref 4. These isomers are part of a series, designated "OR". Those isomers used for this work contain the following substitutions with ^{15}N-containing residues, as well as other deuterium and ^{13}C substitutions: OR-1, 1-[$^{15}N'$,α-^2H]cysteine; OR-2, 6-[$^{15}N'$,α-^2H]cysteine and 8-[$^{15}N'$]-leucine; OR-3, 9-[$^{15}N'$]glycine; OR-4, 5-[$^{15}N'$,$\beta 2$-^2H]asparagine and 8-[$^{15}N'$,α,γ,δ-$^2H_3$]leucine; and OR-5, 2-[$^{15}N'$]tyrosine. All amino acids (other than glycine) are of L configuration unless otherwise specified. [$^{15}N'$]tyrosine, [$^{15}N'$]glycine, [ϵ-^{15}N]glutamine, and $^{15}NH_4NO_3$ were from Merck Sharpe and Dohme. [δ-^{15}N]Asparagine was from Isotope Labelling Corp.; *N'*-acetyltyrosine was from Cyclo Chemical Corp.; *N'*-acetylglutamine and *N'*-acetylasparagine were from Vega-Fox Biochemicals. [$^{15}N'$]Leucine was from Koch Inc. *S*-*p*-Methylbenzyl[$^{15}N'$,α-^2H]cysteine,[22] [$^{15}N'$,α,γ,δ-$^2H_3$]leucine,[23] and [$^{15}N'$,$\beta 2$-^2H]asparagine[24] were prepared in this laboratory. All amino acids used in peptide synthesis were converted to their *N'*-*tert*-butoxycarbonyl derivatives by standard methods.[25] Chloroform and acetic acid were Mallinckrodt analytical grade, nitric acid and Me_2SO were J. T. Baker analytical grade, and distilled, deionized water was used throughout.

^1H NMR spectra were measured at 220 MHz on a Varian/Nicolet Technology HR/TT-220 spectrometer using continuous-wave or pulse/Fourier-transform modes. ^{15}N spectra were obtained on a Bruker WH-180 spectrometer operating at 18.24 MHz at Bruker Instruments, or on an extensively modified Bruker HX-90 spectrometer operating at 9.12 MHz and equipped with a multinuclear detection scheme[26] at The Rockefeller University. Data for T_1's were obtained on the latter instrument by the progressive saturation method, and relaxation times were computed using the NTCFT data reduction subroutines.[27] Reported errors are the estimated standard deviations. Nuclear Overhauser enhancements were measured by acquiring spectra with and without proton decoupling. Sample tem-

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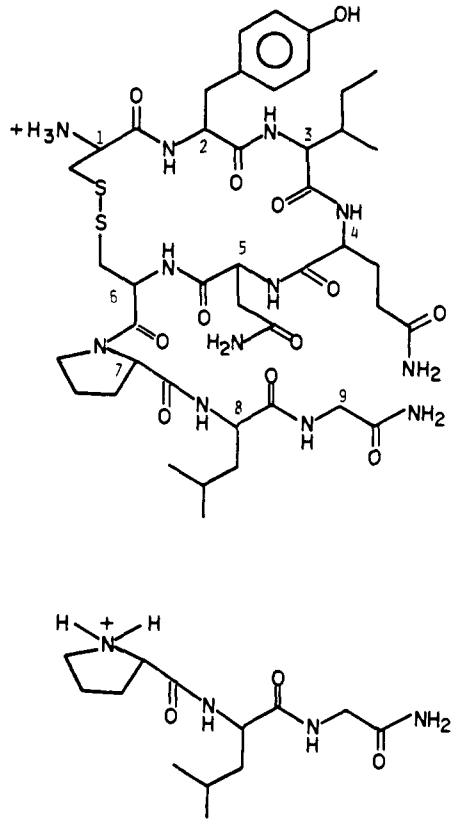

Figure 1. Structures of oxytocin and prolylleucylglycinamide in their cationic forms. Numerals near the C^{α}'s of oxytocin indicate the residue number.

perature was not significantly affected by decoupling as determined by a thermocouple close to the sample tube. The ratio of integrals of the signals with and without decoupling was used to calculate the NOE.

For spectroscopic examination at natural abundance, 1.5 g of oxytocin was dissolved in 15 mL of water, and 1 g of PLG in 20 mL; the pH of each solution was adjusted to 4.0. These samples were run in 25-mm sample tubes on the WH-180 spectrometer. When enriched isomers were examined, 25–35 mg of material was dissolved in about 0.5 mL of water and the pH adjusted to 4.0. These samples were examined on the HX-90 in 5-mm NMR tubes, inserted in 10-mm sample tubes, or in glass microcell inserts in 10-mm NMR tubes (approximate i.d. 8 mm; volume 0.4 mL).

When preparing aqueous solutions free of metal ions, about 50 mg of each isomer of oxytocin was separately dissolved in 2 mL of water and the pH adjusted to 6.5. To each solution, 2 mL of 0.1 M 8-hydroxyquinoline (Aldrich) in chloroform was added.[28] The chloroform phase rapidly turned yellow indicating some formation of metal ion/chelate complex in the organic phase. These solutions were mechanically shaken for 30 min, the chloroform layer was removed, and the aqueous phase was washed three times with 2 mL of chloroform. The resulting solution was then diluted to 20 mL with 1 M acetic acid and lyophilized. Greater than 90% of each peptide was recovered, purity was checked by thin layer chromatography,[21] and ^1H NMR was unaffected by these treatments.

Chemical shifts of ^{15}N nuclei are reported here in parts per million downfield from an external reference of 5 M $^{15}NH_4NO_3$ in 2 M HNO_3. Conversion constants for this reference compared to others are available.[11,29-31] ^1H NMR spectra were referenced to sodium [2,2,3,3-$^2H_4$]-3-trimethylsilylpropionate (TSP).

Rotational correlation times were calculated as follows. If the molecular tumbling is isotropic, the spin-lattice time, T_1, is related to a single correlation time, τ_c, by[2]

$$1/T_1 = \langle 1/r^6 \rangle (N\hbar\gamma_N^2\gamma_H^2)\tau_c[\mathbf{f}(\omega_H - \omega_N) + 3\mathbf{f}(\omega_N) + 6\mathbf{f}(\omega_H + \omega_N)]/10 \quad (1)$$

where $\mathbf{f}(\omega) \equiv 1/(1 + \omega^2\tau_c^2)$, N = the number of ^1H nuclei directly bonded to the ^{15}N nucleus, $\gamma_N = -2711\ s^{-1}\ G^{-1}$, $\gamma_H = 26\,752\ s^{-1}\ G^{-1}$, ω_H and ω_N are the spectrometer frequencies of ^1H and ^{15}N nuclei, respectively, in rad s^{-1}, and ω_N is taken to be negative. If r is taken to be 1.04 Å and $N = 1$, then eq 1 reduces to

$$1/T_1 = 4.6223 \times 10^8\ \tau_c\{\mathbf{f}(\omega_H - \omega_N) + 3\mathbf{f}(\omega_N) + 6\mathbf{f}(\omega_H + \omega_N)\} \quad (2)$$

If extreme narrowing is manifest—i.e., $(|\omega_H| + |\omega_N|)\tau_c \ll 1$—then the term in braces in eq 2 approaches a constant, 10, and τ_c is inversely proportional to T_1. Although the extreme narrowing condition approximately holds for examples in this paper, that term was not taken to be constant. Reported values of τ_c were calculated by use of eq 2 from values of T_1 by iteration.

The effects of variations of r on determined τ_c's are mentioned in the Discussion.

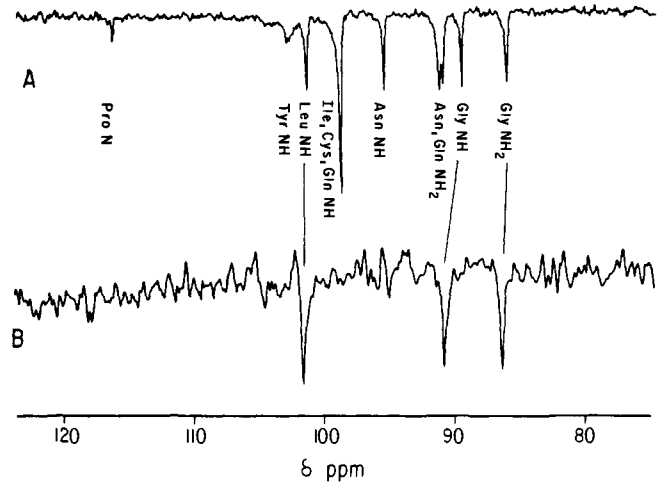

Figure 2. ^{15}N NMR spectra at 18.14 MHz accumulated using 0.81-s cycle time and a 70° 55-μs pulse. Shifts are relative to 5 M $^{15}NH_4NO_3$ in 2 M NHO_3. Assignments are described in text. (A) Oxytocin, 100 mg/mL H_2O, pH 4.0, 22 000 accumulations. (B) PLG, 50 mg/mL H_2O, pH 4.0, 2000 accumulations.

Table I. ^{15}N NMR Chemical Shifts[a]

residue	oxytocin[b]	Pro-Leu-Gly-NH_2[c]	N-acetyl amino acid[d]	amino acid[e]
Cys^1 (NH_3^+)	17.0			
Gly^9 (NH_2)	85.8	85.9		
Gly^9 (NH)	89.3	90.4	89.4[f]	
Asn^5 (NH_2)	91.1, 90.8		89.2	90.4
Gln^4 (NH_2)			88.6	89.4
Asn^5 (NH)	95.3		101.6	
Cys^6 (NH)	98.7		99.0 (SH)[g]	
Gln^4 (NH)	98.7		102.1	
Ile^3 (NH)	98.7		99.0[f]	
Leu^8 (NH)	101.2	101.3	101.7[f]	
Tyr^2 (NH)	102.6		101.8	
Pro^7 (N)	116.1	31.7 (NH_2^+)		

[a] Shifts are relative to 5 M $^{15}NH_4NO_3$ in 2 N HNO_3; assignments are described in the text. [b] 100 mg/mL in H_2O, pH 4.0. [c] 50 mg/mL in H_2O, pH 4.0. [d] 1–2 M in Me_2SO. [e] Amino acid 0.07 M in H_2O at pH 4.0. [f] From ref 9. [g] N-Acetylcysteine, ref 9.

Results and Discussion

The ^{15}N NMR spectra of aqueous solutions of oxytocin and PLG are shown in Figure 2. Values of the shifts and their assignments are shown in Table I. Assignment of the ^{15}N resonances of oxytocin was accomplished by site-specific ^{15}N labeling of the peptide amide nitrogens of Cys^1, Tyr^2, Asn^5, Cys^6, Leu^8, and Gly^9 in five isotopic isomers of oxytocin. Two isomers (OR-2 and OR-4) contain two nitrogen labels, and these two isomers share a site of substitution, the 8-leucine position. Of the remaining unidentified ^{15}N nuclei, the prolyl nitrogen may be assigned to the 116.1-ppm resonance on the basis of com-

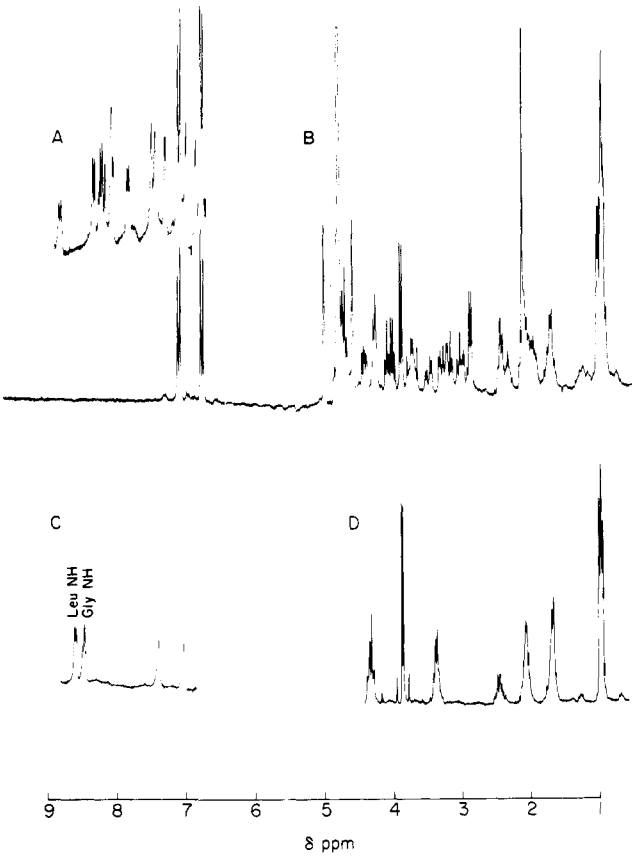

Figure 3. ^1H NMR spectra at 220 MHz. (A) CW spectrum of oxytocin at 100 mg/mL in H_2O at pH 4.0. (B) FT spectrum of oxytocin at 100 mg/mL in D_2O at pD 4.0. (C) CW spectrum of prolylleucylglycinamide at 50 mg/mL in H_2O at pH 4.0. (D) FT spectrum of prolylleucylglycinamide at 50 mg/mL in D_2O at pD 4.0. Shifts are relative to internal TSP.

parison of its extreme shift with that observed in other prolyl-containing peptides (Table I).[32] Such a shift is expected for a nitrogen directly bonded to three carbons.[33]

There are three shifts to be assigned from carboxamide nitrogens; two of side chains of glutamine and asparagine, and one of the C-terminal glycinamide. This last nitrogen is assigned to the resonance at 85.8 ppm, on the basis of comparison with the spectrum and assignments, subsequently discussed, of PLG. Resonances from the two side-chain carboxamide nitrogens are assigned to the signals at 91.1 and 90.8 ppm on the basis of their similarity in shift to resonances from these groups in the corresponding amino acids in water and from *N'*-acetylamino acids in Me_2SO (Table I). No individual assignments are made for these two carboxamides.

Two nitrogen nuclei remain unassigned to resonances, those of Ile^3 and Gln^4. The unassigned resonances fortuitously fall together at a line centered at 98.7 ppm (Figure 2). This peak has an integral three times that of the individual resonances in the 85–103-ppm region:[34] it is known from isotopic labeling that the resonance from Cys^6 is at this position,[35] and therefore it can be concluded that the Ile^3 and Gln^4 resonances are also at this frequency.

In spectra of unlabeled oxytocin it was observed that two resonances, those of Tyr^2 at 102.6 ppm and Cys^1 at 17.0 ppm, were considerable broader than others. The Cys^1 resonance was observed to be about 50 Hz wide and of opposite sign to the other resonances. A similar observation was made using isotopic isomer OR-1. It was considered highly likely that these effects might arise from the presence of small quantities of paramagnetic ions in solution. Similar effects have been observed in ^{15}N studies of glycylglycine.[10] Extraction using hydroxyquinoline (see Experimental Section) of aqueous solutions of oxytocin containing enrichments of ^{15}N in Cys^1 (OR-1) or Tyr^2 (OR-5) was performed, and spectra of these solutions gave resonances of about 1-Hz line width, comparable to those observed for resonances of other residues in unlabeled (Figure 2) and labeled oxytocins.[36]

Table II. Comparison of the ^1H NMR Shifts of the Common Residues in Prolylleucylglycinamide and Oxytocin[a]

residue	atom	PLG[b]	oxytocin[c]
Pro	α	4.40	4.45
	β	2.42, 2.65	2.26, 2.65
	γ	2.05	2.01
	δ	3.40	3.73
Leu	α	4.34	4.32
	β	1.62, 1.68	1.63, 1.71
	γ	1.70	1.70
	δ	0.91, 0.94	0.92, 0.94
Gly	α	3.88, 3.95	3.86, 3.93

[a] Shifts are in parts per million relative to internal TSP. [b] Concentration 50 mg/mL in D_2O at pD 4.0. [c] Concentration 100 mg/mL in D_2O at pD 4.0.

The spectral assignment of ^{15}N resonances for PLG was performed using off-resonance proton decoupling. The assignments of the proton chemical shifts in PLG have not been previously reported and are shown in Table II. The proton spectra of PLG and oxytocin in water and in D_2O are shown in Figure 3. The proton assignments of the amide protons of PLG in water may be simply made from their degree of multiplicity—a triplet to the glycyl peptide amide, a doublet to leucyl, and two singlets at about 7 ppm to the glycyl carboxamide protons.[37]

In $^{15}N\{^1H\}$ experiments, single-frequency proton decoupling at about 8.5 ppm (^1H) near the peptide amide proton resonances produces a triplet centered at 85.9 ppm (^{15}N) and three singlets at 31.7, 90.4, and 101.3 ppm. Under these experimental conditions the triplet can only arise from the C-terminal carboxamide nitrogen, and the 31.7-ppm resonance from the N-terminal prolyl nitrogen. The carboxamide assignment was confirmed by proton decoupling at 7.0 ppm (^1H) of the ^{15}N spectrum. The glycyl and leucyl resonances are assigned to 90.4 and 101.3 ppm on the basis of comparisons with model compounds (Table I). Such assignments are further supported by the comparison of these shifts to those of oxytocin, in which absolute assignments of $^{15}N'$ of the leucyl and glycyl residues are available from chemical labeling (Table I).

In Figure 4 we show a comparison between the ^{15}N chemical shifts of peptide amides in water and *N*-acetylamino acids in Me_2SO. The peptide results include those for oxytocin and PLG from this work and relevant data reported by others.[13,15,16] Data for this figure were restricted to internal primary L-amino acid residues and glycyl residues in which the angle (C^{α}_i–N'_i–C'_{i-1}–C^{α}_{i-1}) is either assumed to be or known to be trans (180°). In addition to those residues, we have included shifts of the peptide nitrogens in the C-terminal glycinamide residues of oxytocin and PLG, since the replacement of a C-terminal carboxyl with a carboxamide is expected to give those peptide nitrogen nuclei environments similar to those of residues incorporated within a peptide chain. In Figure 4, if all the shifts of residues were identical with those of the corresponding amino acid derivatives in Me_2SO, then all points should fall on the solid line of −45° slope. Although this is not the case, many points fall very close to this line. Dashed lines in Figure 4 are at ±1 ppm from the solid line and indicate a range which may be expected to cover deviations resulting from experimental errors arising from the use of different reference compounds or variations in bulk susceptibility,[38] from experimental variability due to pH or ionic strength among the

aqueous samples, and from other minor effects on the chemical shifts, such as ring current shifts.

The similarity of the ^{15}N chemical shifts in peptides in water and of their corresponding *N'*-acetylamino acids in Me_2SO suggests a substantial likeness of the electronic environments at the ^{15}N nuclei.

A semiquantitative explanation of this correspondence may be found in the previously reported shifts in water or Me_2SO of *N*-acetyl $(Gly)_n$ for $n = 1, 2$, and 3.[11] The substitution effect of the acetyl group compared to glycyl is about −5.5 ppm in Me_2SO[11] (Ac-Gly compared to Ac-Gly-*Gly and Ac-Gly-*Gly-*Gly) and the value is nearly counteracted by the solvent effect of the transition between Me_2SO and water of about +4.4 ppm found for Ac-Gly-Gly.[11]

There are four cases in Figure 4 which fall well outside the range of the dashed lines. Two of these points arise from tripeptides used in model studies, and two from residues in oxytocin. Two large shift deviations are observed in model compounds containing glycine, for Leu-Gly-Gly and for Val-Gly-Gly. The ^{15}N shifts of the Gly^2 residues in these peptides have been ascribed to the effect of the neighboring Leu or Val residue.[8,15] These substitution effects were clearly observed in free tripeptides and dipeptides in water,[15] but are not observed for analogous *N*-acetyl dipeptides in Me_2SO or trifluoroacetic acid,[11] or observed here for the glycine nitrogen in the Leu-Gly sequences of oxytocin and PLG. The conditions under which the shift effects of Val or Leu on a glycyl nitrogen are observed are therefore somewhat unclear. Chemical shifts of nitrogens of glycyl residues in a large open-chain polymer $(\beta$-Ala-Sar-Gly$)_n$ in water[13] or in *cyclo*-(Gly-Pro-Gly-D-Ala-Pro)[16] are not unusual.

Two other ^{15}N shifts in Figure 4 are also well away from the expected correlation, being displaced upfield from the model compound. They are associated with Gln^4 and Asn^5 in oxytocin, and are the only two shift values in oxytocin and PLG that are substantially different from the values of the appropriate *N*-acetylamino acids in Me_2SO. The most probable cause of deviations from the expected values is from differences in solvation and hydrogen bonding associated with the peptide group. It has been proposed[29] that in amides downfield nitrogen shifts occur when there is either a hydrogen bond formed by proton acceptance at the carbonyl group or a hydrogen bond formed by proton donation of the amide proton. These shifts may arise because either hydrogen bond can increase the degree of double-bond character of the nitrogen-carbonyl carbon bond, and thus change the effective electron density at the nitrogen nucleus.[39] Supporting evidence for this qualitative explanation is available from studies of the effects of solvents on alumichrome,[5] polysarcosine (in which only the carbonyl acceptor role is possible),[13] and *N*-acetyl oligoglycines.[11] It follows that a reasonable explanation of the upfield displacement of the shifts of Asn^5 and Gln^4 compared to their *N*-acetylamino acids in Me_2SO arises from the lack of hydrogen bonding, whether intramolecularly or by solvent, to the N'_5 and N'_4 and/or to the carbonyl groups with which these peptide nitrogens are associated.[40]

Another possible explanation of the deviations of the shifts for Asn^5 and Gln^4 is that these amide nitrogens are participants in bonds that are unusually strained, or that they may be fixed in conformations that are highly dissimilar to those of other peptide units in oxytocin. It is well known that the N'-C^α torsion angles for Asn^5 and Gln^4 are apparently quite dissimilar to those of other residues in the tocin ring of oxytocin.[18] In other residues, the vicinal proton-proton coupling constants, $^3J(H'$-$H^\alpha)$, fall in the range 5.5-7.2 Hz, a set of values consistent with, but not proof of, averaging about the respective torsion angles,[41,42] whereas the equivalent values for Asn^5 and Gln^4 are 8.6 and 4.3 Hz, respectively. These last values have been interpreted[18,42] in terms of fixed torsion angles, ϕ, associated with these two residues.

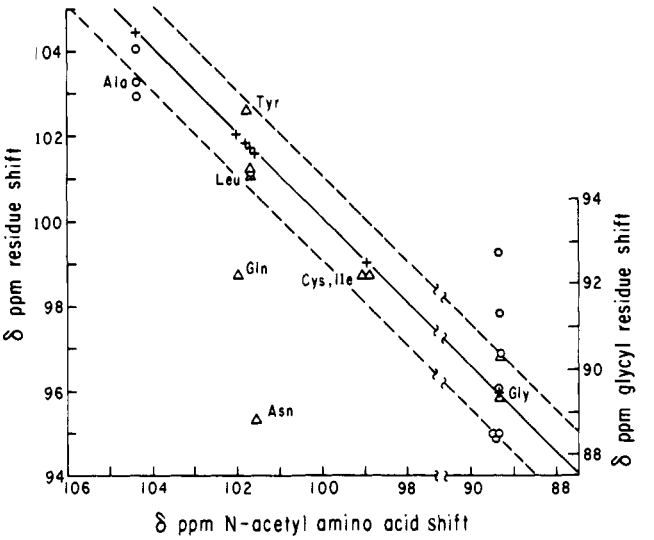

Figure 4. Plot of ^{15}N shifts of peptide nitrogen nuclei of *N'*-acetylamino acids in Me_2SO (along the abscissa) against the shifts of corresponding amino acid residues in peptides whose ^{15}N NMR spectra have been reported in H_2O. Crosses indicate the hypothetical points at which the two shifts would be equal, and the solid line is drawn through these points. Circles are data from other reports (see ref 13, 15, and 16). Triangles are for data reported here. The dashed lines are at ±1 ppm from the solid line (see text). Shifts have been corrected to external 5 M $^{15}NH_4NO_3$ in 2 N HNO_3. The right-hand scale applies only to the glycyl residues.

It therefore may be concluded that the anomalous values of chemical shift for the nitrogens of these two residues are indeed indicators that these residues are unusual compared to other residues in oxytocin. Further experimentation is required, however, to discern the molecular features from which such shifts arise, and to explain these shifts in theoretical terms.[43]

The general agreement between the ^{15}N shifts of peptide amide nitrogen nuclei for residues of several peptides in aqueous solutions and those for the corresponding *N*-acetylamino acid in Me_2SO leads us to suggest that the values of the shifts observed with these model compounds are a good indication of those expected for amino acid residues in a peptide in water provided that conformational restriction is not great and that both the N-H and C=O components of the peptide linkage to the neighboring residues are exposed to solvent.

The prolyl nitrogen in oxytocin has a shift several parts per million to lower field than for the corresponding *N*-acetyl compound in Me_2SO. There are no other ^{15}N data for this residue as a component of a peptide examined in aqueous solution, but similar shifts have been reported for two peptides examined in a variety of nonaqueous solvents.[9,16]

Examination of the resonances of common residues of PLG and oxytocin in Tables I and II and Figures 2 and 3 shows a substantial similarity between the two. The proton spectra, including coupling constants, are quite similar for the two, as are the ^{13}C resonances.[44] The ^{15}N data are then consistent with other NMR data in indicating similar conformations for PLG and the C-terminal tripeptide segment of oxytocin in water.

The measurement of the direct coupling constant, $^1J(^{15}N'$-$H')$, has been facilitated by the ^{15}N enrichments in the isotopic isomers of oxytocin. The absolute values of this coupling constant for Tyr^2, Asn^5, Cys^6, Leu^8, and Gly^9 peptide amide nitrogen to proton are 94, 92, 93, 93, and 95 Hz, respectively. A value of 93 Hz had been previously reported for Tyr^2 in oxytocin.[45] These values all fall in the region expected for a peptide bond linkage.[2]

We have measured the T_1 relaxation times for Asn^5, Cys^6, and Leu^8 amide nitrogen nuclei in two isotopic isomers of

oxytocin (OR-2 and OR-4), each of which has an ^{15}N label on Leu^8. The T_1's measured were 0.55 ± 0.02 s for Asn^5 and Cys^6 and 0.60 ± 0.02 s for Leu^8. Since we have observed full NOEs for these measurements, we can conclude that the relaxation mechanism is dipolar via the directly bonded proton and that motional narrowing is in effect.[2,19,46] The conditions for such motional narrowing are briefly mentioned in the Experimental Section. If isotropic molecular motion and a N-H bond length of 1.04 Å[47] are assumed, correlation times of 406 and 370 ps, respectively, can be deduced from the Asn^5 or Cys^6 datum and from the Leu^8 datum. The τ_c's determined from ^{13}C T_1's of $^{13}C^\alpha$ nuclei are 450, 440, and 320 ps, respectively, for Asn^5, Cys^6, and Leu^8 C^α-H^α vectors.[48]

The above τ_c values for the residues in the tocin ring are approximately equal, determined from the relaxation of either nucleus, and greater than the values for nuclei in the Leu^8 residue of the tripeptide tail. The ratio of τ_c (Asn, Cys)/τ_c(Leu) is 1.1 from the ^{15}N and 1.4 from the ^{13}C data. These ratios are apparently significantly different, based on estimates of experimental error, though the possibility of systematic errors precludes a judgment as to whether the τ_c's associated with the ring residues or the Leu^8 or both cause the variations in the ratios. The two most likely causes of the difference in ratio are inequalities in the effective motional freedoms of $^{13}C^\alpha$-H^α and $^{15}N'$-H' vectors in the same residue, Leu^8, or variations in the bond lengths $^{13}C^\alpha$-H^α or $^{15}N'$-H'. In the case of different motional freedoms, the data are consistent with the motional freedom of $^{15}N'$-H' (ring) being greater than $^{13}C^\alpha$-H^α (ring) and/or $^{13}C^\alpha$-H^α (Leu^8) being greater than $^{15}N'$-H' (Leu^8). The latter seems plausible, reflecting a gradient of mobility along the C'_7-N'_8-C^α_8-C'_8 chain of the tripeptide.

In considering different bond lengths, a contraction of 0.027 Å in N'-H' of Leu^8 compared to ring amides would be sufficient to account for the observed differences of T_1's if an isotropic correlation time of 320 ps is assumed. Such a changed bond length is within the range of those reported,[47] but its possible origin is not obvious.

In conclusion, the ^{15}N NMR spectra of peptides in aqueous solution can be readily examined and well-resolved spectra of peptides as complex as oxytocin can be observed. Much useful information can be obtained from this approach even though it is still at an early stage of development. The correlation of the chemical shifts of component residues with the shifts of N'-acetylamino acids in Me_2SO appears to provide a useful way of investigating a number of molecular features: which residues have relatively fixed backbone conformations; which amide protons are involved in hydrogen bonding; and which peptide linkages have groups that are shielded from exposure to solvent. With the aid of ^{15}N enrichments, the measurements of relaxation times are greatly facilitated and can be used to gain information on molecular flexibility and perhaps N-H bond distances.

These studies support some general details of the conformation of oxytocin in aqueous solution suggested by other techniques. The ^{15}N chemical shifts are compatible with the exposure of most of the peptide linkages to the solvent. The shifts of the Gln^4 and Asn^5 peptide nitrogen nuclei may be an additional confirmation that these residues have relatively restricted backbone conformations, or are limited in the exposure of their backbone to solvent. The correlation times for the motion of the tocin ring derived from ^{15}N data are close to those previously deduced from ^{13}C data and give support to the hypothesis that the tocin ring is rigid on a time scale of 10^{-10} s. The tail tripeptide seems to be relatively more mobile than the ring, with a conformation similar to that of the free tripeptide, PLG.

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used.
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Intramolecular Water Bridge between the 2'-OH and Phosphate Groups of RNA. Cyclic Nucleotides as a Model System

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Abstract: The investigation of the ^1H NMR spectra of cNMPs in aqueous and mixed solvents shows that the 2'-OH proton is protected from exchange with bulk water. In the presence of low salt, 0.05 M, the free energy of stabilization of the 2'-OH is about 1.5 kcal and the activation energy for exchange is about 7 kcal. The protection of the 2'-OH proton against exchange cannot be attributed to an intermolecular association. The ^1H NMR results are consistent with there being an intramolecular water bridge between the 2'-OH and the phosphate group. A similar water bridge has been proposed for RNA to explain the ^1H NMR results obtained for a variety of RNA samples. The energies determined for the cNMP intramolecular water bridge can be cautiously extended to RNA.

Introduction

The backbones of RNA and DNA differ only in the substituent at the 2' position of the ribose and yet the conformational properties of the two polymers are quite different.[1,2] DNA double helices are normally found in the B form with a 2'-endo ribose conformation, but can adopt a number of other conformations in the appropriate experimental conditions. In contrast to the variety of conformations found for DNA, RNA is only found to exhibit ordered, helical conformations of the A family of helices in which the ribose is in the 3'-endo conformation.[1,2] While it has been known for some time that the 2'-OH group of RNA is responsible for these differences in the properties of RNA and DNA, the role of the 2'-OH has remained unclear. Recently, we proposed that the 2'-OH of RNA forms an *intra*molecular water bridge with the adjacent 3'-phosphate, as shown in Figure 1.[3] This proposed water bridge accounts for ^1H NMR results which showed that the exchange rate of the 2'-OH of RNA samples in aqueous solution is anomalously slow and that the resonance position of the 2'-OH proton in polymers is shifted about 1.5 ppm to lower field than that observed for mononucleosides. This hydrogen-bonding scheme involving a "bound" water molecule may also explain, at least in part, the differences between RNA and DNA.

Attempts to test the proposed hydrogen bonding scheme and to gain information about the strength of the hydrogen bonding involved have been hampered by the limited range of conditions in which the 2'-OH resonance of RNA samples can be observed.[3,4] For example, the 2'-OH resonance of denatured RNA cannot be observed, as disruption of the helical structure results in rapid exchange of the 2'-OH proton with water.[3] To overcome some of these limitations, we searched for other molecules which might exhibit a water-2'-OH-phosphate interaction similar to that proposed for RNA. Specifically, we looked for molecules with a stereochemical arrangement of the ribose hydroxyl group and an oxygen atom of a neighboring phosphate group that might mimic that of the A form RNA helix.

The 3',5' cyclic nucleotides have a rigid ribose conformation which is similar to that of RNA[5] and appear to be suitable for our purpose. The ribose conformation is a modification (3'-endo, 4'-exo) of the 3'-endo form found for RNA and the distance between the 2'-OH oxygen atom and the nearest phosphodiester oxygen atoms is about 0.36 nm, compared with 0.48 nm in the A form of RNA.[6] The ^1H NMR data on cyclic nucleotides presented here support the notion that 3',5'-cNMPs do form an intramolecular water bridge similar to that proposed for RNA. The unique properties of the cNMPs allow the further characterization of the nature of the hydrogen-bonding interactions responsible for the slow exchange of, and unexpectedly large downfield chemical shift of, the 2'-OH proton in aqueous solution.

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