300-MHz Nuclear Magnetic Resonance Study of Oxytocin in Aqueous Solution: Conformational Implications

([4-leucine]oxytocin)

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ABSTRACT The 300-MHz nuclear magnetic resonance spectrum of the peptide hormone, oxytocin, in water, was obtained. Extensive nuclear magnetic resonance spin-decoupling experiments, including those involving irradiation of the α -proton resonances under the water peak, studies using partially deuterated hormone derivatives, and nuclear magnetic resonance studies of some precursor peptides to oxytocin, permitted us to assign most of the proton resonances of the hormone unambiguously. The J_{α} values and temperature dependence of the chemical shift of the peptide amide and carboxamide protons of oxytocin were also obtained. These studies indicate that, in aqueous solution, oxytocin is a flexible molecule possessing several different conformations. Corresponding studies of [4-leucine]oxytocin were also made, with similar results and conclusions.

Current interest in the structure-activity relationships of the neurohypophyseal hormone, oxytocin, has led to the proposal of a number of possible energetically favorable conformations for this molecule in dimethylsulfoxide solution (1-3). Conformations have also been proposed for deamino-oxytocin and for several analogs of oxytocin (1,4). Since it is not known whether oxytocin performs its biological functions in the body in an aqueous or a nonaqueous environment, it is also important to investigate its conformational possibilities when it is dissolved in water.

Oxytocin is a nonapeptide with the following sequence:

The cyclic hexapeptide portion is closed by a disulfide bridge. Whereas the α - and β -proton resonances can be seen and assigned in deuterium oxide (D_2O) solution, the amide proton resonances are exchanged with deuterium and do not appear. In water (H_2O), the solvent peak obscures the α -proton peaks, making assignment of the peptide NH peaks extremely difficult. Feeney *et al.* (5) measured the peptide NH to α -proton coupling constants ($J_{N\alpha}$) of these peaks but did not attempt to assign them. Recently, Glickson *et al.* (6) reported assignments for these resonances, using the method reported by Von Dreele *et al.* (7) in which a dimethylsulfoxide solution is diluted with water, and vice versa, while the chemical shift of each peak is plotted. Unfortunately, this method can lead to ambiguities as the peaks overlap and "cross over."

We have been able, by frequency sweep homonuclear spin-decoupling at 300 MHz, to irradiate the α -proton resonances

under the water peak and to assign the peptide NH resonances from the resultant spin-decoupling. In this paper we present our assignments for oxytocin and for [4-leucine]oxytocin and the conformational implications deduced from the nuclear magnetic resonance (NMR) spectra of these two molecules. Except in the case of the lowest field peak, our values of JN_{α} are the same within experimental error as those reported by both Feeney *et al.* (5) and Glickson *et al.* (6). However, our decoupling experiments indicate different assignments for the peptide amide protons from those proposed by Glickson *et al.* (6).

EXPERIMENTAL

Preparation of Peptides. Oxytocin was prepared by standard methods (8) and was purified by partition chromatography on Sephadex G-25 (9, 10) followed by gel filtration (11) on Sephadex G-25. [4-Leucine]oxytocin was a generous gift of Dr. V. du Vigneaud or was prepared by published methods (12). [1-Hemi- $[\alpha, \beta, \beta^2\text{H}_3]$ -L-cystine]oxytocin and [6-hemi- $[\alpha, \beta, \beta^2\text{H}_3]$ -L-cystine]oxytocin (Spatola, A. F., Cornelius, D. A., Blomquist, A. T., and Hruby, V. J., manuscript in preparation) and [7- $[\alpha, \beta, \beta, \gamma, \gamma, \delta, \delta^2\text{H}_7]$ -L-proline]oxytocin were prepared by Dr. A. F. Spatola and were generously provided to us for these studies. The $[\alpha, \beta, \beta, \gamma, \gamma, \delta, \delta^2\text{H}_7]$ -L-proline was provided by Dr. A. T. Blomquist. The peptides Pro-Leu-Gly-NH₂, Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂, and Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂ were prepared in the form protected at the amino terminus by standard methods (8) followed by removal of the terminal benzyloxycarbonyl group with 2.5 N HBr in acetic acid and neutralization of the peptide salt with Rexyn 201 resin (OH form).

NMR Methods. The three linear precursors were dissolved in D₂O (99.8% D) to a concentration of about 10% w/v. Oxytocin and its analogs were dissolved first in D₂O (99.8% D) to a concentration of about 10% w/v, then lyophilized and redissolved to the same concentration in H₂O. The pH was adjusted to between 3.5 and 3.9 for all samples with HCl and NaOH or DCl and NaOD.† Spectra were run on a Varian Associates HR-300 NMR spectrometer operating in either field-sweep or frequency-sweep mode. Homonuclear spin-decoupling was performed in the frequency-sweep mode with a Varian SC-8525-2 Decoupler. Temperatures were controlled by a Varian variable temperature accessory and were known

Abbreviations: NMR, nuclear magnetic resonance; TSS, 2,2,3,3-tetradeutero-3-(trimethylsilyl)-propionic acid sodium salt.

‡ No correction has been made for pD. For pD values, $pD = pH + 0.40$ (13).

FIG. 1. Spectrum of oxytocin, pH 3.8, 28°C. (a) [6-Hemi-[$\alpha,\beta,\beta\text{-}^2\text{H}_3$]-L-cystine]oxytocin; (b) [7-[$\alpha,\beta,\beta,\delta,\delta,\gamma,\gamma\text{-}^2\text{H}_7$]-L-proline]-oxytocin; (c) [1-hemi-[$\alpha,\beta,\beta\text{-}^2\text{H}_3$]-L-cystine]oxytocin. (Bottom scan is oxytocin with no deuterium substitution.)

within $\pm 2^\circ\text{C}$. The chemical shifts of the amide proton resonances were measured at various temperatures and at various pH values. A drop of *t*-butanol-*d* was used as reference. The *t*-butanol had no measurable effect on the nmr spectrum of the samples. The *t*-butyl resonance is temperature invariant relative to 2,2,3,3-tetradeutero-3-(trimethylsilyl)-propionic acid sodium salt (TSS) and occurs at 1.247 δ relative to TSS. Peak positions are measured from the *t*-butyl peak and reported relative to TSS.

RESULTS

The composite spectrum of oxytocin in D_2O and in H_2O is shown in Fig. 1. Through spin-decoupling the α -proton resonances were all assigned by irradiating the β -proton resonances of the sample in D_2O . By raising the temperature of the sample, the HDO peak was shifted upfield, enabling assignment of the three lowest field α -proton peaks which were under the solvent peak at ambient temperature. Unequivocal assignment of the proline resonances and the half-cystine-1 and half-cystine-6 resonances was obtained by comparison with the spectra of [7-[$\alpha,\beta,\beta,\delta,\delta,\gamma,\gamma\text{-}^2\text{H}_7$]-L-proline]oxytocin, [1-hemi-[$\alpha,\beta,\beta\text{-}^2\text{H}_3$]-L-cystine]oxytocin, and [6-hemi-[$\alpha,\beta,\beta\text{-}^2\text{H}_3$]-L-cystine]oxytocin, as seen in Fig. 1.

The order of the α -proton peaks relative to each other closely approximates the order in dimethylsulfoxide, and their positions are also much the same. Two changes, however, are to be noted. The half-cystine-1 α -proton resonates in aqueous solution next to the leucine-8 α -proton at 4.29 δ , considerably downfield from its position in dimethylsulfoxide. The isoleucine-3 α -proton, unmistakable due to its doublet structure when no longer coupled to its peptide NH, is on the high-field side of the glutamine-4 α -proton peak in contrast to its position to low field of the glutamine-4 α -proton in dimethylsulfoxide. The glycine α -protons in H_2O show up in a clear AB pattern.

The β -proton resonances hold only one surprise when compared to the dimethylsulfoxide solution of oxytocin. In aqueous solution, the β -proton peaks of half-cystine-1 have moved clearly downfield from those of half-cystine-6 and tyrosine-2. The tyrosine-2 β -proton peaks are centered at 3.11 δ , while the half-cystine-6 β -proton resonances, also

centered at 3.11 δ , are more widely separated into two distinct halves: 3.24 δ and 2.98 δ .

The peptide NH peaks were assigned in H_2O by irradiating the frequencies measured for the α -proton resonances in D_2O and observing the spin-decoupling produced. When the α -proton of tyrosine-2 was irradiated, no spin-decoupling was observable, but the tyrosine-2 NH peak could be assigned by elimination. This broad peak sharpens upon lowering of the pH and undergoes a reduction in intensity as the center of the H_2O peak is approached by the irradiating frequency. The assignments we obtained for the peptide NH protons differ, for the most part, from those suggested by Glickson *et al.* (6), who used another method for assignment.

The carboxamide proton resonances were assigned from comparison of the spectra of the series Pro-Leu-Gly-NH₂, Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂, and Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂, in which pairs of new carboxamide proton resonances are added consecutively along the series. In addition, comparison with the spectrum of [4-leucine]oxytocin, where the glutamine carboxamide group is absent, confirmed the glutamine assignments. By irradiating one carboxamide peak and observing which other peak was reduced in area by a transfer of saturation, we determined the pair of resonances for the glycine carboxamide protons. The transfer of saturation could not be used to pair up the glutamine and asparagine peaks in oxytocin because they occur too close to one another. However, this technique was used successfully for the asparagine and glycine carboxamide resonances in [4-leucine]-oxytocin.

The temperature dependence of the chemical shift of the peptide and carboxamide NHs was studied between 12 and 52°C. The coupling constants did not change over this temperature range. The values of $d\delta/dT$ for oxytocin are shown in Table 1. As a comparison, the chemical shift of *N*-methylacetamide over this temperature range was found to be -9.5×10^{-3} ppm/degree. The positions of the amide resonances were plotted between pH 2.8 and 5.1. The positions of the peaks changed very little, all of them moving between 0.03 and 0.06 ppm upfield in going from low to high pH.

The spectrum of [4-leucine]oxytocin (Fig. 2) allowed confirmation of the assignment of the glutamine-4 resonances

Fig. 2. Spectrum of [4-leucine]oxytocin, pH 3.9, 28°C.

in oxytocin. In the α -proton region, the leucine-4 α -proton resonance almost exactly replaces the glutamine-4 α -proton peak in position, although the multiplicity is clearly different. In the peptide NH region, the glutamine peak is obviously absent, with the leucine-4 resonance appearing remarkably upfield at about 7.62 δ under an asparagine carboxamide peak. In fact, it was only at lower temperatures that the leucine-4 peptide NH peak could be seen. Assignment of the carboxamide peaks and their pairing up has been described. Table 1 includes the values of $J_{N\alpha}$ and $\delta\delta/dT$ for [4-leucine]oxytocin.

DISCUSSION

The difference in chemical shifts and in the relative position of the half-cystine-1 α - and β -proton resonances in aqueous solution compared to dimethylsulfoxide solution may be indicative of a difference in conformation in that part of the molecule. It should be noted that the circular dichroism studies (14–16), which suggest a right-handed screw sense and a dihedral angle of approximately 90° for the disulfide bridge, were conducted in aqueous solutions. Although these criteria have been used in proposing conformations for oxytocin in dimethylsulfoxide, the changes in both the half-cystine-1 and half-cystine-6 resonances of oxytocin in water suggest that this may have been an erroneous assumption.

The change in position and the spread of the tyrosine β -proton peaks may point to some change in the orientation of the tyrosine-2 ring. Further changes in conformation of the residue side-chains are seen in the decreased superposition of the glutamine γ and proline β -proton resonances in water, and in the fact that whereas the asparagine and glutamine carboxamide peaks are separated by about 0.5 ppm in dimethylsulfoxide, they are separated by about 0.7 ppm in water.

Differences in peptide backbone conformation, aside from those already mentioned involving the half-cystine residues, are evidenced in the α -proton region by the switch in position between the isoleucine-3 and glutamine-4 peaks. Most indicative of backbone conformational change is the change in the peptide NH region of the spectrum. The coupling constants, while not remarkably different from those measured in dimethylsulfoxide, do differ in that in aqueous solution, $J_{N\alpha}$ for tyrosine-2, glutamine-4, and half-cystine-6 have decreased while the vicinal couplings in isoleucine-3 and asparagine-5 have increased.

Carbon-13 NMR studies (refs. 17–19; Brewster, A. I. R. and Hruby, V. J., unpublished results) indicate that a *trans* peptide bond from half-cystine-6 to proline-7 connects the tripeptide side-chain to the ring. That this side-chain has a different conformation in aqueous solution is suggested by the decreased value of $J_{N\alpha}$ for leucine-8, by the greater difference between the chemical shifts of the glycine-9 α -proton peaks, and by the separation of 0.3 ppm between the glycine-9 carboxamide resonance. These latter were coincident in the dimethylsulfoxide solution of oxytocin (3). In studies of the tripeptide side-chain alone in dimethylsulfoxide, the glycine-9 carboxamide resonances appeared separately in both the unblocked and *N*-benzyloxycarbonyl-blocked prolyl-leucyl-glycyl-amide. When the cysteine residue was added to the NH₂ terminus, these two resonances became nearly coincident (20) and remained so as each amino acid was added consecutively.

As in lysine vasopressin in aqueous solution (7), the tyrosine-2 peptide NH was found to be rapidly exchanging with the solvent at a rate exceeding the rate of relaxation of the NH proton. This was demonstrated by the decrease in area of this peak when the H₂O peak was irradiated. Raising the pH from 3.8 to 4.5 resulted in severe broadening of this resonance, showing that its exchange rate is pH dependent. As in lysine

TABLE 1. $J_{N\alpha}$ and $\delta\delta/dT$ of oxytocin and [4-leucine]oxytocin

AMINO ACID RESIDUE		$J_{N\alpha}$ Hz		CHEMICAL SHIFT AT 28°C		$-\delta\delta/dT$ $\times 10^3$ ppm	
OXYTOCIN	(4-LEU) OXYTOCIN	OXYTOCIN	(4-LEU) OXYTOCIN	OXYTOCIN	(4-LEU) OXYTOCIN	OXYTOCIN	(4-LEU) OXYTOCIN
TYR-2	TYR-2	6.5	7 $\pm$ 1	8.99	9.05	6.5	6.5
LEU-8	LEU-8	6.5	6.5	8.44	8.42	9.5	11.5
GLY-9	ASN-5	5.5	6 $\pm$ 1	8.35	8.35	7	8
ASN-5	GLY-9	8	6	8.32	8.34	5	8
GLN-4	half CYS-6	4	6.5	8.20	8.13	5.5	6
half CYS-6	ILE-3	7	5	8.20	7.89	5.5	7
ILE-3	LEU-4	6	4	7.95	7.62	7	8
ASN	ASN	c a r b o x a m i d e		7.60	7.61	6.5	6.5
GLN				7.55		6	
GLY	GLY			7.36	7.40	4.5	5
GLY	GLY			7.02	7.07	6	6
ASN	ASN			6.86	6.93	6	6
GLN				6.81		5	

vasopressin (7), and following the studies of Sheinblatt on triglycine (21), the rapid pH-dependent exchange of the tyrosine-2 NH can be due to the NH_3^+ group present in the next amino-acid residue, half-cystine-1.

All the amide proton resonances showed significant changes of chemical shift with temperature variation under our conditions. Although *N*-methylacetamide in water has a $d\delta/dT$ of -9.5×10^{-3} ppm/degree and three peptide NHs as well as two carboxamide protons in oxytocin had values of $d\delta/dT$ of -5.5×10^{-3} ppm/degree or less, all amide proton resonances exhibited significant chemical-shift dependence on temperature. Although we do not exclude the possible presence of intramolecular hydrogen bonds in aqueous solution of oxytocin, we cannot predict their presence or the amino-acid residues participating in hydrogen bonds, should there be any.

The spectrum of [4-leucine]oxytocin does not differ markedly from that of oxytocin in aqueous solution, the only significant difference being in the position of the leucine-4 peptide NH resonance. Although this resonance has a different chemical-shift position and a different $d\delta/dT$ from that of the glutamine which it replaces, its $J_{\text{N}\alpha}$ value remains the same.

CONCLUSIONS

Whereas in dimethylsulfoxide solution, the ^1H NMR studies of oxytocin suggested the presence of an intramolecular hydrogen bond as well as some sort of constraint on the glycine-9 carboxamide and thus allowed certain energetically favorable conformations which agreed with these observations to be proposed, the ^1H NMR study of oxytocin in aqueous solution leads to the conclusion that the molecule is extremely flexible, in agreement with previous studies utilizing deuterium nmr relaxation methods (22, 23) and conformational energy calculations (24). The values of $J_{\text{N}\alpha}$ are intermediate, suggesting an averaging over several values rather than a single value for any particular amino-acid residue. Although the amide protons do have differing dependences of chemical shift on temperature, none appears to indicate intramolecular hydrogen bonding. It remains very likely, however, that oxytocin does prefer somewhat different conformations in water from those in dimethylsulfoxide. The flexibility of oxytocin is perhaps corroborated by the close similarity of the spectrum of [4-leucine]oxytocin, a molecule with considerably different biological properties (25, 12).

We conclude that both oxytocin and [4-leucine]oxytocin are flexible molecules in aqueous solution and that if they perform their biological function in an aqueous environment, then there are additional influences to their attaining and

maintaining the particular conformation(s) that may be necessary for their biological activity.

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