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Cis/Trans Conformational Equilibrium across the Cysteine[6]–Proline Peptide Bond of Oxytocin, Arginine Vasopressin, and Lysine Vasopressin[1]

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Abstract: The peptide hormones oxytocin, arginine vasopressin (AVP), and lysine vasopressin (LVP) consist of a macrocyclic hexapeptide ring to which a tripeptide side chain is attached via a cysteine^6–proline peptide bond. Previous studies have provided evidence only for the trans conformation across the cysteine^6–proline peptide bond. ^{1}H NMR results are presented which establish that a small, but significant, fraction of oxytocin, AVP, and LVP, in both their native disulfide and reduced dithiol forms, exists in the cis conformation. Even though most of the resonances of the cis isomer are obscured by much more intense resonances of the trans isomer, it was possible to identify all the amino acids of the cis isomer, to determine their sequence, and to establish the conformation across the cysteine^6–proline peptide bond using connectivities in two-dimensional COSY, TOCSY, and ROESY spectra. Exchange cross peaks between resonances of the trans isomer and those assigned to the cis isomer in ROESY spectra of the peptides in CD_3OH solution together with the results of magnetization transfer experiments in H_2O solution provide the final, unequivocal proof of the existence of the cis isomers. In aqueous solution, the proportion of the cis isomer is 10% for oxytocin, 9% for AVP, and 8% for LVP. The proportions of the cis isomer for the reduced forms of oxytocin and AVP are 10% and 6%, respectively. These results suggest there are no specific interactions between the tripeptide side chains and macrocyclic hexapeptide rings which stabilize the cis or trans isomers of oxytocin, AVP, or LVP. However, the temperature dependence of the chemical shifts of the resonances for the amide protons is consistent with a higher proportion of structured forms for the hexapeptide rings of the cis isomers.

Introduction

The neurohypophyseal peptide hormones oxytocin, arginine vasopressin (AVP), and lysine vasopressin (LVP) are nonapeptides, with a macrocyclic hexapeptide ring portion and a tripeptide tail. The hexapeptide ring is closed by a disulfide bond between cysteine residues at positions 1 and 6. These hormones mediate such processes as lactation, uterine contraction, vasoconstriction, and antidiuretic functions. Synthesized principally within the hypothalamus, they are stored in neurosecretory granules as noncovalent neurophysin–hormone complexes which dissociate by dilution upon secretion into the blood.

Chart I

Cys-Tyr-X-Gln-Asn-Cys-Pro-Y-Gly-NH_2

	X	Y
oxytocin	Ile	Leu
AVP	Phe	Arg
LVP	Phe	Lys

Since their isolation and synthesis in the early 1950's,[2,3] considerable effort has been made to characterize their conformational

(1) Presented, in part, at the 32nd Experimental Nuclear Magnetic Resonance Spectroscopy Conference, St. Louis, Missouri, April 1991.

(2) (a) du Vigneaud, V.; Ressler, C.; Trippett, S. *J. Biol. Chem.* **1953**, *205*, 949–957. (b) du Vigneaud, V.; Ressler, C.; Swan, J. M.; Roberts, C. W.; Katsoyannis, P. G.; Gordon, S. *J. Am. Chem. Soc.* **1953**, *75*, 4879–4880.

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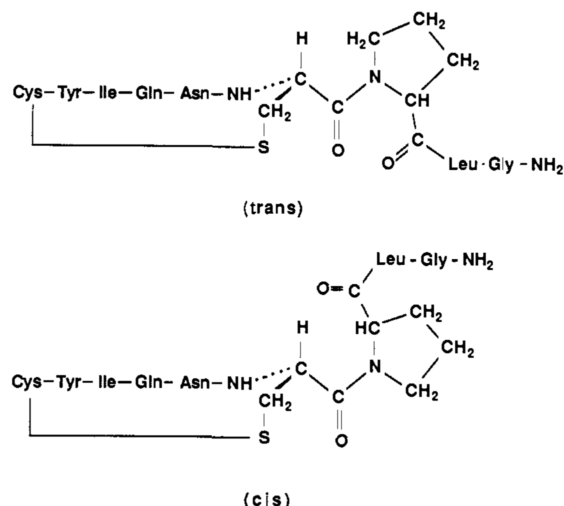

Figure 1. Structural formulas of oxytocin showing the cis and trans conformations across the cysteine[6]–proline peptide bond.

and dynamic behavior.[4,5] The neurophysin–hormone system also has been studied extensively because it provides a particularly useful model with which to analyze the noncovalent association of a peptide and a protein.[6] The ultimate goal of many of these studies, including numerous NMR studies of the hormones free in solution and in association with neurophysin, has been to relate solution conformation to biological activity. However, in spite of the many reported studies, the conformations of these peptides which are biologically active at their hormone receptors are not known.

It was established soon after their discovery that both the macrocyclic hexapeptide ring portion and the tripeptide tail are required for maximum biological potency.[7] For example, tocinamide and pressinamide, the cyclic hexapeptide moieties of oxytocin and vasopressin, respectively, with a primary amide group replacing the tripeptide side chain, are significantly less active than the parent hormones.[7a,b] The tripeptide side chains are connected to the macrocyclic hexapeptides via cysteine[6]–proline peptide bonds, which are considered to exist completely in the trans conformation[8] (Figure 1) on the basis of ^{1}H and ^{13}C NMR results.[9,10] In the only report to date of cis isomers, cis/trans isomerism was detected in 600-MHz ^{1}H NMR spectra of the oxytocin analogs (sarcosyl[7])–oxytocin and (*N*-methylalanyl[7])–oxytocin, in which proline is replaced by N-substituted amino acids.[11] The apparent absence of the cis isomer of the parent peptide hormones is rather surprising since the cis and trans conformations of proline peptide bonds are often similar in energy and thus are both generally populated for proline-containing small peptides.[12] Usually the cis isomer is the minor species, although Dyson et al. have reported relative concentrations of the cis isomer greater than 50% for several proline-containing hexapeptides.[12b]

In our high-field ^{1}H NMR studies of oxytocin, AVP, and LVP and their reduced dithiol analogs in aqueous solution and in methanol, we consistently observe a few minor resonances with intensities some 4–10% of those which have been assigned to the trans conformations. Minor resonances have also been observed by others, who attributed them to impurities. In view of the importance of these peptides in biology and as model systems for noncovalent peptide–protein interactions, we have investigated the origin of these minor resonances by one- and two-dimensional ^{1}H NMR methods. In this paper, we present unequivocal evidence that a small, but significant, fraction of oxytocin, AVP, and LVP, in both their native disulfide and reduced dithiol forms, exists in the cis conformation across the cysteine[6]–proline peptide bond. We also present evidence which suggests that the relative population of structured forms of the macrocyclic hexapeptide ring is higher for the cis isomers.

Experimental Section

Sample Preparation. AVP and LVP were used as received from Bachem: oxytocin (Bachem) solutions were generally treated with Dowex 1-X8 anion exchange resin (Baker) in the hydroxide form to remove acetate ion which was present as the counterion in some oxytocin samples. No impurities were detected by reverse-phase liquid chromatography on a C_{18} column with a water–acetonitrile mobile phase and UV detection. Also, HPLC traces supplied with the peptides showed no impurity peaks. Most ^{1}H NMR spectra were measured for 5–10 mM solutions of the peptides in 90% H_2O/10% D_2O. Some measurements were also made on the peptides in D_2O or CD_3OH solutions. In experiments where the water resonance was eliminated by the WATR (Water Attenuation by Transverse Relaxation) method,[13] sufficient NH_2OH or NH_4Cl was added to give a final concentration of 0.25–0.30 M and the pH was adjusted to 3 (NH_2OH) or 7 (NH_4Cl). For all pH measurements, the pH meter was calibrated with aqueous standard solutions at pH 4.00 and 7.00 (Fisher Scientific); for D_2O and CD_3OH solutions, the pH meter reading is reported as pH*. The pH of D_2O or 90% H_2O/10% D_2O solutions was adjusted with DCl and NaOD in 99.9% D_2O or 90% H_2O/10% D_2O, respectively. The solution of oxytocin in CD_3OH (99% D, Cambridge Isotope Labs) was adjusted to a pH meter reading of 3.01 using small amounts of concentrated DCl in D_2O dissolved in CD_3OH.

The reduced dithiol forms of the peptides were prepared in situ by reduction with excess deuterated dithioerythritol. The procedure involved adjustment of the pH of a solution of the native disulfide form to 7.4 and then bubbling with argon to remove oxygen. Excess deuterated dithioerythritol (MSD Isotopes) was added and the solution was transferred to a glovebag. Under a nitrogen atmosphere in the glovebag, NH_2OH solution which had been degassed by bubbling with argon was added and the pH of the solution was adjusted to 3.0. An aliquot of the solution was transferred to an NMR tube and the tube was capped; after removal of the NMR tube from the glovebag, the cap was wrapped with parafilm to provide a further barrier to oxygen. There was no evidence of reoxidation to the native disulfide form during the course of the NMR experiments.

NMR Spectroscopy. ^{1}H NMR spectra were measured at 500 MHz with a Varian VXR-500S spectrometer. Unless noted otherwise, spectra were measured at 23 ± 1 °C. Chemical shifts are reported relative to 2,2-dimethyl-2-sila-5-pentanesulfonate (DSS). The variable-temperature unit was calibrated using the chemical shifts of ethylene glycol and methanol. The temperature coefficients of the amide proton chemical shifts were measured for at least five different temperatures in 5-deg steps. In cases of resonance overlap, a temperature coefficient was calculated only if the chemical shift of the resonance could be measured for at least three different temperatures.

Most ^{1}H NMR measurements were made on pH 3.0 solutions of the peptides in 90% H_2O/10% D_2O so that resonances could be observed for

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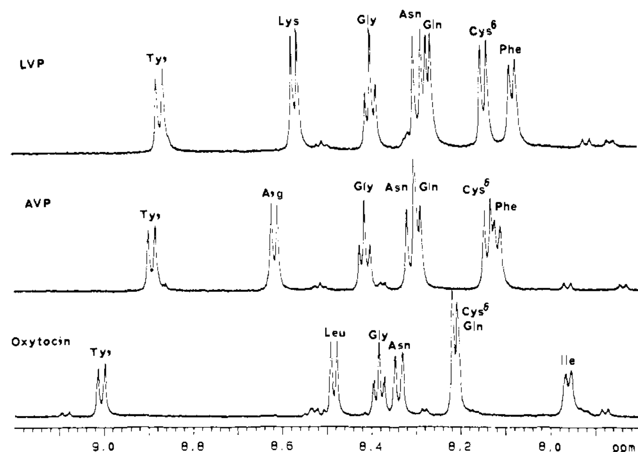

Figure 2. The backbone amide proton region of the 500-MHz ^{1}H NMR spectra of 10 mM oxytocin, 8 mM AVP, and 7 mM LVP in 90% H_2O/10% D_2O/0.25 M NH_2OH at pH 3.0. Resonances of the trans isomer are assigned. Spectra were measured by the single pulse method, with suppression of the H_2O resonance by presaturation. A spectral width of 5000 Hz was used and 64 transients were coadded.

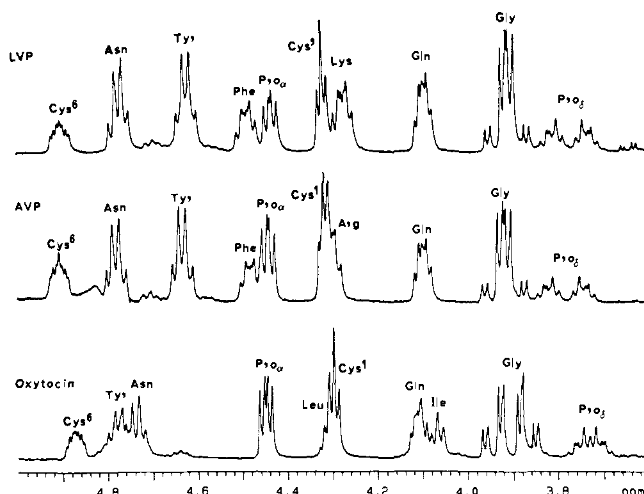

Figure 3. The $C_\alpha H$ proton region of the 500-MHz ^{1}H NMR spectra of oxytocin, AVP, and LVP in 90% H_2O/10% D_2O/0.25 M NH_2OH at pH 3.0. Resonances of the trans isomer are assigned. Spectra were measured by the CPMG pulse sequence with a transverse relaxation period ($2\tau n$) of 0.25 s to achieve complete and selective elimination of the water resonance at 4.77 ppm.

the amide protons. One-dimensional spectra were measured with suppression of the water resonance by presaturation or complete and selective elimination with the WATR method.[13] To eliminate the water resonance by the WATR method, NH_2OH or NH_4Cl was added to reduce the transverse relaxation time of the water protons and the one-dimensional spectrum was measured by the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence ($90°$–(τ–$180°$–τ)$_n$–acquisition)[14,15] with a sufficiently long delay ($2\tau n$) between the 90° pulse and acquisition for complete elimination of the water resonance by transverse relaxation.[13] Two-dimensional COSY (correlated spectroscopy),[16] TOCSY (total correlation spectroscopy),[17] and ROESY (rotating-frame Overhauser enhancement spectroscopy)[18] spectra were measured with elimination of the water resonance by the WATR method.[19,20] COSY and TOCSY spectra were measured with modified pulse sequences which include a period for elimination of the water resonance by transverse relaxation.[19] ROESY spectra were measured by the standard ROESY pulse sequence, with elimination of the water resonance by transverse relaxation during the mixing period.[20] The inversion-transfer pulse sequence of Robinson et al.[21] was used to observe the transfer of magnetization from the trans to the cis isomer by rotation around the Cys⁶–Pro peptide bond. One- and two-dimensional ^{1}H NMR spectra of oxytocin in CD_3OH were measured with suppression of the hydroxyl resonance by presaturation. Two-dimensional spectra were measured using spectral widths of 5000 Hz in both dimensions. In all 2048 data points were acquired in t_2 and either 32 or 64 transients were coadded at each of 256 t_1 increments with zero filling to 2048 points. Gaussian or shifted sinebell apodization was applied in both dimensions. Phase-sensitive spectra were acquired using the method of States et al.[22] Additional experimental parameters are given in the figure legends.

Results and Discussion

The cis and trans conformations across the X–proline peptide bond are often similar in energy and may both be populated under physiological conditions. Since interconversion between the cis and trans isomers by rotation around the C–N partial double bond is slow on the NMR time scale, resonances for both isomers can be observed in the spectra of proline-containing peptides if spectral resolution is sufficient. The first example of cis–trans isomerism across the X–proline peptide bond in nonoligomeric short peptides was reported for the side chain peptide of oxytocin (S-Bzl-Cys-Pro-Leu-Gly-NH_2).[9a–c] Resonances for both isomers were observed in ^{1}H and ^{13}C NMR spectra of DMSO solutions of this peptide, at a trans to cis ratio of about 3 to 2.[9a] However, the cis isomer was not detected in ^{13}C NMR spectra of the parent hormone in DMSO or in other solvents, even though cis and trans conformations of proline-containing peptides can often be distinguished on the basis of the ^{13}C chemical shifts of the proline carbons.[23] We also have not observed the expected resonances for the cis isomer in ^{13}C NMR spectra of AVP. We have, however, established the existence of the cis isomer of oxytocin, AVP, and LVP by ^{1}H NMR.

The amide proton and C_α proton regions of the 500-MHz ^{1}H NMR spectra of oxytocin, AVP, and LVP in 90% H_2O/10% D_2O are shown in Figures 2 and 3, respectively. The spectra in Figure 2 were measured by the standard single pulse sequence with suppression of the water resonance by presaturation; those in Figure 3 were measured by the CPMG pulse sequence with elimination of the water resonance by the WATR method so that resonances at the chemical shift of the water resonance (4.77 ppm) could be observed.[24] Both the amide proton and $C_\alpha H$ regions of the spectra consist of a number of intense resonances, which have been assigned to peptides having the trans conformation across the Cys⁶–Pro peptide bond,[25] together with a small number of much less intense resonances. By using results from two-dimensional ^{1}H NMR experiments, we have established that the minor resonances are from peptides having the cis conformation across the Cys⁶–Pro peptide bond, and not from degradation products or synthetic impurities in which one or more amino acid residues are missing from the sequence. The NMR evidence for assignment of the minor resonances to the cis isomers will be illustrated with results for the oxytocin system.

The assignment procedure involved first identification of the specific amino acids giving rise to the minor resonances, then

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(22) States, D. J.; Haberkorn, R. A.; Ruben, D. J. *J. Magn. Reson.* **1982**, *48*, 286–292.

(23) Jardetzky, O.; Roberts, G. C. K. *NMR in Molecular Biology*; Academic Press: New York, 1981; pp 162–163.

(24) Although the concentration of water protons is >10^4 times that of the $C_\alpha H$ protons, the water resonance at 4.77 ppm is completely and selectively eliminated, making it possible to observe resonances for all of the $C_\alpha H$ protons.

(25) We have assigned the resonances of the trans isomers of oxytocin, AVP, and LVP by a combination of one- and two-dimensional ^{1}H NMR experiments. Our assignments agree with previous assignments made for similar solution conditions.[4]

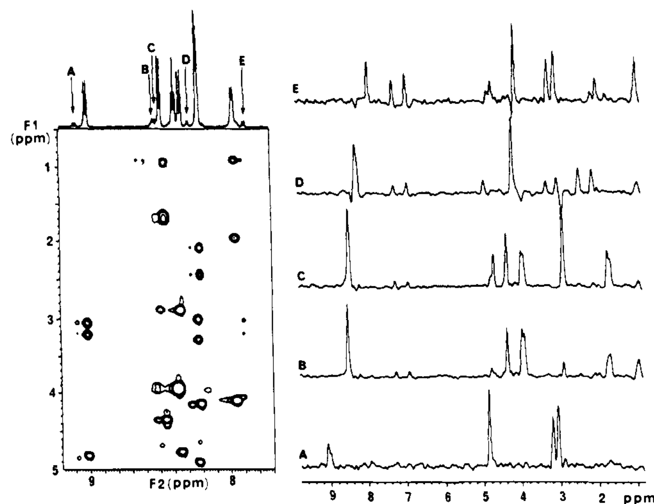

Figure 4. The NH-$C_\alpha H$ region of the TOCSY spectrum of 10 mM oxytocin in 90% H_2O/10% D_2O/0.25 M NH_2OH at pH 3.0. Data were acquired in the phase-sensitive mode and only positive contours have been plotted. A total delay of 0.26 s was used in the CPMG portion of the modified TOCSY pulse sequence[19] to achieve elimination of the water resonance. A spin-locking period of 0.070 s was used. Traces are taken parallel to the *F*1 axis at the chemical shifts (*F*2) of the amide proton resonances of the cis isomer.

elucidation of the sequence of these amino acids in the peptide, and finally determination of the conformation across the Cys[6]–Pro peptide bond. Although this is now a fairly standard procedure for assigning resonances of peptides,[26] its application to this problem is complicated because the resonances being assigned are of low intensity relative to those of the trans isomer and most are obscured by the more intense resonances, as illustrated by the spectra in Figures 2 and 3. Indeed, almost all the resolved resonances for the cis isomers are shown in Figures 2 and 3; those regions of the spectra where the other resonances would be observed are considerably more congested and cis isomer resonances are obscured by overlap.

Although the resonances of the trans isomers are assigned in Figures 2 and 3, the minor resonances cannot be assigned to specific amino acids simply on the basis of chemical shift similarities.[27] The minor resonances in the amide portion of the spectrum of oxytocin in Figure 2 were assigned to specific amino acids using amide proton to side chain proton scalar connectivities obtained from TOCSY spectra. A portion of the TOCSY spectrum of oxytocin, together with the amide region of the one-dimensional spectrum plotted across the top, is shown in Figure 4. Also plotted in Figure 4 are traces taken parallel to the *F*1 frequency axis at the indicated chemical shifts of the minor amide resonances on the *F*2 axis. The traces contain some, if not all, of the resonances expected for the following seven amino acids: trace (A) tyrosine; (B) glycine and leucine; (C) asparagine; (D) glutamine; and (E) isoleucine and cysteine. It should be noted that, since these amino acid spin systems are identified through connectivities to their amide protons, the cysteine identified in trace E cannot be the N-terminal residue; as shown below it is Cys[6] of the cis isomer. It also should be noted that the extra resonances in some of the traces, e.g. the resonances at 4.0 and 4.4 ppm in trace C, are from the tails of more intense cross peaks of the trans isomer.

The presence of a minor abundance proline residue was established using the upfield portion of the TOCSY spectrum (Figure 5), based on the unique chemical shift pattern for the proline spin system. This can be seen most clearly in the trace taken at 4.72 ppm, the chemical shift of the *cis*-Pro $C_\alpha H$ resonance on the *F*2 frequency axis. Observation of this trace was possible

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(27) For example, substantial differences are reported for the chemical shifts of the NH and the $C_\alpha H$ protons of the cis and trans isomers of a large number of penta- and hexapeptides.[12b]

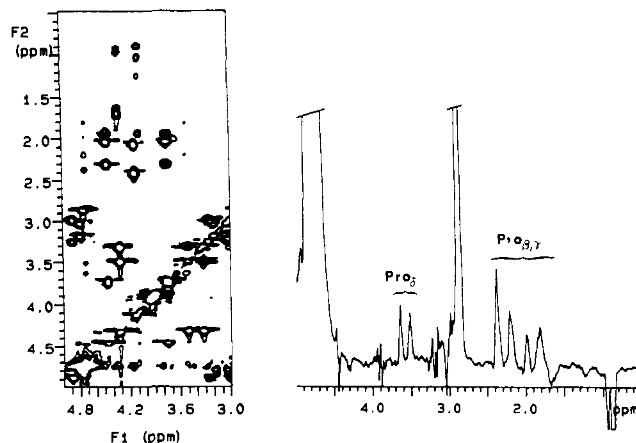

Figure 5. The 3.0–5.0 ppm (*F*2) region of the TOCSY spectrum of oxytocin in Figure 4. The trace was taken at 4.72 ppm (*F*2) and contains resonances of proline of the cis isomer.

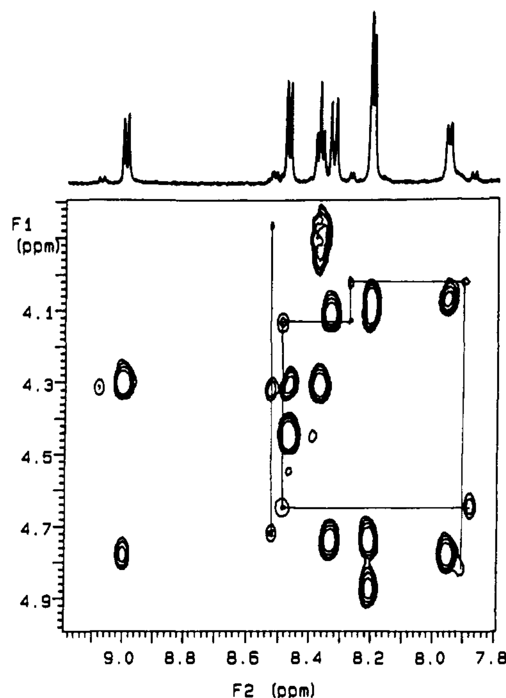

Figure 6. The NH-$C_\alpha H$ region of the phase-sensitive ROESY spectrum of 10 mM oxytocin in 90% H_2O/10% D_2O/0.25 M NH_2OH at pH 3.0. The amide proton region of the one-dimensional spectrum, measured by the single-pulse method, is plotted across the top. The water resonance was completely eliminated during the 0.25 s spin-locking-period in the standard ROESY pulse sequence. The spin lock (2.6 kHz) was generated using a series of 30° pulses (on for 5 μs, off for 36.5 μs). The 2D spectrum was symmetrized before plotting.

because of complete and selective elimination of the water resonance by the WATR method.

The presence of cysteine[1] and the sequence of the amino acids identified from the TOCSY spectra were established using connectivities obtained from ROESY spectra. The amide NH-$C_\alpha H$ portion of the ROESY spectrum for a pH 3 solution of oxytocin is shown in Figure 6. Also plotted across the top is the amide NH portion of the one-dimensional spectrum. The ROESY spectrum is dominated by intense NH_i-$C_\alpha H_{(i-1)}$ and NH_i-$C_\alpha H_i$ NOE cross peaks for the trans isomer. The weaker cross peaks are for NH_i-$C_\alpha H_{(i-1)}$ and NH_i-$C_\alpha H_i$ NOE connectivities of the cis isomer. The cross peak at 4.32 (*F*1) and 9.08 (*F*2) was assigned to the Cys[1] $C_\alpha H$-Tyr NH NOE connectivity on the basis of the above assignment of the NH resonance at 9.08 ppm to tyrosine and the assumption that the chemical shifts of the Cys[1] $C_\alpha H$ resonances will be similar for the cis and trans isomers. The expected cross peak for the Tyr NH-Tyr $C_\alpha H$ NOE connectivity

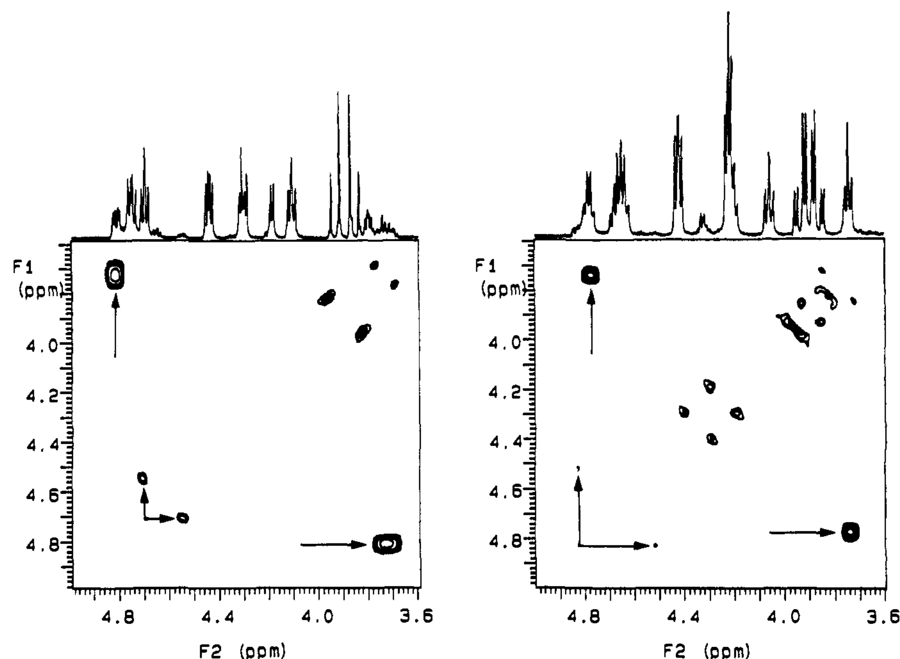

Figure 7. (Left) The α-proton region of the phase-sensitive ROESY spectrum of 8 mM oxytocin in D_2O solution containing 0.3 M NH_4Cl at pH* 6.85. Only negative contours are plotted. The HOD resonance was selectively eliminated during the 0.4 s spin-locking period. The spin lock (1.8 kHz) was generated using a series of 30° pulses (on for 5.8 μs, off for 52.2 μs). The one-dimensional spectrum measured by the CPMG pulse sequence ($2\tau n$ = 0.36 s) is plotted across the top. (Right) The α-proton region of the phase-sensitive ROESY spectrum of 10 mM reduced oxytocin in 90% H_2O/10% D_2O/0.25 M NH_2OH at pH 3.0. Only negative contours are plotted. The water resonance was eliminated during the 0.25 s spin-locking period. The spin lock (2.6 kHz) was generated using a series of 30° pulses (on for 5 μs, off for 36.5 μs).

at 4.82 (*F*1) and 9.08 ppm (*F*2) is not detected in Figure 6; however, it should be noted that, for the trans isomer, this cross peak is also much weaker than that for the Cys^1 C_αH-Tyr NH NOE. The cross peak at 4.82 (*F*1) and 7.91 ppm (*F*2) is for the Tyr C_αH-Ile NH NOE connectivity. The connectivities traced in Figure 6, starting from this cross peak, together with the Cys^1-Tyr connectivity, establish the sequence Cys-Tyr-Ile-Gln-Asn-Cys. There is the expected break in connectivities at Cys^6-Pro because proline lacks an NH proton. However, starting with the Pro C_αH-Leu NH cross peak at 4.72 (*F*1) and 8.15 ppm (*F*2), the sequence Pro-Leu-Gly can be established.

The Cys^6-Pro sequential connectivity was established on the basis of ROESY cross peaks between the Cys^6 C_αH and Pro C_αH protons. At pH 3, the chemical shift difference between these two resonances (0.07 ppm) is so small that their weak ROESY cross peaks are obscured by the much stronger diagonal peaks. However, the chemical shift of the Cys^6 C_αH resonance is sensitive to pH, reflecting titration of the NH_3^+ moiety of Cys^1. Thus, by increasing the pH* of a D_2O solution of oxytocin to 6.85, the Cys^6 C_αH resonance was shifted upfield by ~0.1 ppm, allowing observation of the Cys^6 C_αH-Pro C_αH NOE cross peak. A portion of the ROESY spectrum is plotted in Figure 7a. Since NOE cross peaks are negative while diagonal peaks are positive in ROESY spectra,[18] only the negative contours are plotted in Figure 7. The sequential connectivity of Cys^6 to Pro is established by the pair of cross peaks at 4.56 (*F*1), 4.73 ppm (*F*2) and 4.73 (*F*1), 4.56 ppm (*F*2) between Cys^6 C_αH and Pro C_αH.

As can be seen from the structures in Figure 1, an NOE will be observed between the Cys^6 C_αH and Pro C_αH protons only for the cis isomer. Thus, not only are the Cys^6 C_αH-Pro C_αH cross peaks in Figure 7 evidence for the Cys^6-Pro sequential connectivity, they also establish that the peptide bond has the cis conformation. The much more intense pair of cross peaks at 3.75 (*F*1), 4.82 ppm (*F*2) and 4.82 (*F*1), 3.75 ppm (*F*2) in Figure 7a is from the NOE between the Cys^6 C_αH and Pro $C_\delta H_2$ protons of the more abundant trans isomer. The other weaker pair of cross peaks at 3.82 (*F*1), 4.00 ppm (*F*2) and 4.00 (*F*1), 3.82 ppm (*F*2) is from the NOE between the two protons of the glycinamide methylene group of the trans isomer.

Reduction of the disulfide bond also causes the Cys^6 C_αH and Pro C_αH resonances of the cis isomer to shift further apart, making the Cys^6 C_αH-Pro C_αH NOE cross peaks visible at pH 3, as shown in Figure 7b. The one-dimensional spectrum across the top of Figure 7b shows that the *cis*-Pro C_αH resonance, which is completely obscured in the one-dimensional spectrum of the native disulfide form of oxytocin by the Asn C_αH resonance of the trans isomer (Figure 3), is shifted downfield to 4.84 ppm where it appears as a shoulder on the *trans*-Cys^6 C_αH resonance. The sequential connectivity of Cys^6 to Pro in the reduced dithiol form of *cis*-oxytocin is established by the pair of cross peaks at 4.84 (*F*1), 4.53 ppm (*F*2) and 4.53 (*F*1), 4.84 ppm (*F*2).

The final, unequivocal proof that the weak resonances in Figures 2 and 3 are from the cis isomer of oxytocin is provided by chemical exchange connectivity of the weaker resonances and those of the trans isomer. In Figure 8 is shown a portion of the two-dimensional phase-sensitive ROESY spectrum for an 8 mM solution of oxytocin in CD_3OH at pH* 3.0 and 23 °C. The major and minor resonances were assigned by the methods described above.[28] In the phase-sensitive ROESY experiment, cross peaks which result from transfer by chemical exchange are of the same sign as the diagonal peaks while those from NOE transfer are of opposite sign.[29] Thus, only the positive contours, which comprise the diagonal peaks and the exchange cross peaks, are shown. Exchange cross peaks are observed for the Asn, Cys^6, Leu, and Gly amide protons. In Figure 9 magnetization-transfer data are presented for 8 mM oxytocin in H_2O at pH 3.0 and 72 °C. In this experiment, the Cys^6-NH resonance for the trans isomer was inverted, followed by a variable mixing time and then data acquisition. The resonance assigned to Cys^6-NH of the cis isomer is plotted as a function of the mixing time in Figure 9. The dependence of the resonance intensity on mixing time proves the chemical exchange connectivity of the strong resonance assigned to Cys^6-NH of the trans isomer and the much weaker resonance assigned to Cys^6-NH of the cis isomer. It is interesting to note that interchange between the cis and trans isomers by rotation

(28) Assignment of resonances was aided by the temperature dependence of NH chemical shifts. For example, the highly overlapped cis and trans resonances in Figure 8 were resolved at some temperatures.

(29) Neuhaus, D.; Williamson, M. *The Nuclear Overhauser Effect in Structural and Conformational Analysis*; VCH Publishers, Inc.: New York, 1989; pp 212.

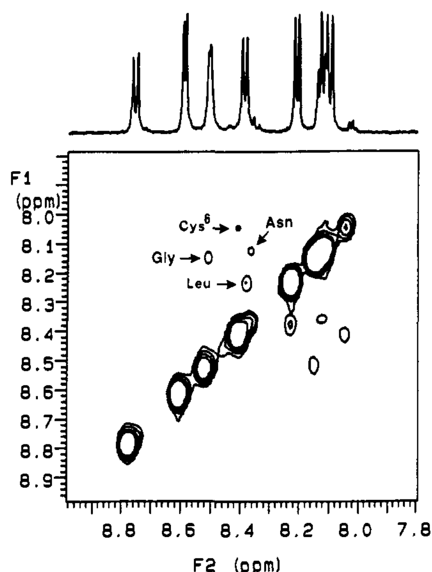

Figure 8. The amide proton region of the phase-sensitive ROESY spectrum of oxytocin in CD_3OH at pH* 3.0 and 23 °C. A 0.3 s spin-locking period was used. The corresponding region of the one-dimensional spectrum is plotted across the top. Both spectra were measured with suppression of the OH resonance by presaturation. Only positive contours are plotted. The amide protons giving rise to the exchange cross peaks are identified.

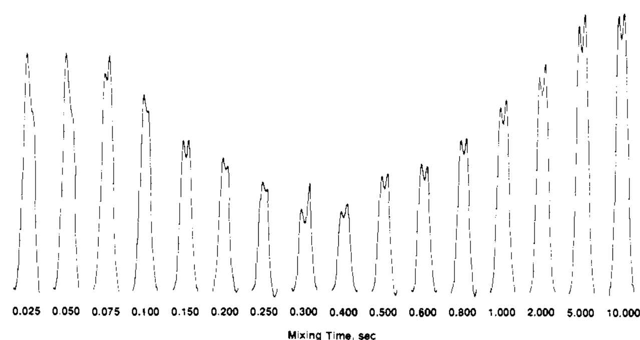

Figure 9. Intensity of the resonance assigned to Cys^6-NH of the cis isomer of oxytocin as a function of the length of the mixing time following inversion of the resonance assigned to Cys^6-NH of the trans isomer in an inversion-transfer experiment. 8 mM oxytocin in H_2O at pH 3.0 and 72 °C.

around the Cys^6–Pro peptide bond is somewhat faster in CD_3OH solution.

Using evidence from 2D-NMR experiments of the type described above for oxytocin, the existence of a minor species having the cis conformation across the Cys^6–Pro peptide bond has also been established for both the native disulfide forms of AVP and LVP and the reduced dithiol forms of oxytocin and AVP. The proportions of cis isomer are given in Table I, the chemical shifts of the amide proton resonances for the cis and trans isomers are given in Table II,[30] and the chemical shifts of the resonances for some of the carbon-bonded protons of the cis isomers are reported in Table III.

Although the conformations of the neurohypophyseal peptide hormones have been studied by several spectroscopic[4,9,10] and computational[5] methods, with the objective of elucidating relationships between solution conformation and biological activity, their biologically active conformations have not been established. The general conclusions from these studies are that both the macrocyclic hexapeptide ring and the tripeptide side chain are highly flexible in aqueous solution, with considerable librational motion, but the numbers of conformers contributing to the overall dynamic conformations are small.[5,10] The results in Table I show that small, but significant, fractions of oxytocin, AVP, and LVP exist with the Cys^6–Pro peptide bond in the cis conformation. Since previous studies have considered only those forms having the trans conformation across the Cys^6–Pro peptide bond, it will be of interest to compare the solution conformations of the cis and trans isomers.

Characterization of the solution conformational properties of the cis isomers by NMR will be difficult because they are present at such low relative concentrations and their resonances are obscured by those of the more abundant trans isomers. However, some indication of their conformational properties can be obtained from the distribution between the cis and trans forms (Table I) and the temperature dependence of the chemical shifts of the amide protons (Table IV). The fact that the population of the cis isomer is small and essentially the same for the native disulfide form of oxytocin AVP, and LVP in aqueous solution suggests that there is no unique interaction between the tripeptide tail and the macrocyclic hexapeptide ring of oxytocin, AVP, or LVP which specifically stabilizes either the cis or trans isomer. The lack of any significant change in the population of the cis isomer when the hexapeptide ring is broken by reduction of the disulfide bond provides further evidence for this conclusion. However, the temperature dependence of the chemical shift of the amide protons (Table IV) suggests that a higher population of the macrocyclic hexapeptide ring of the cis isomers exists as structured species.

The resonances for the amide protons of the trans isomer and all those which can be resolved for the cis isomer shift upfield with temperature. An upfield shift of an amide proton resonance with increasing temperature is usually attributed to the breaking of an increasing fraction of its hydrogen bonds.[10,32] The majority of the $\Delta\delta/\Delta T$ values in Table III are in the -6 to -10×10^{-3} ppm/K range, including those for both the cis and trans isomers of the reduced dithiol forms of oxytocin and AVP, consistent with open structures and solvent–amide proton hydrogen bonds. The most notable exceptions are those of the Asn and Cys^6 amide protons of the native disulfide forms of the peptides, which indicate that the disulfide bond imposes constraints on the conformation of the hexapeptide ring. Previous solvent saturation transfer studies of the accessibility of the peptide amide protons of the trans isomer to the aqueous solvent environment also suggest that, in oxytocin,

Table I. Cis–Trans Equilibrium across the Cysteine[6]–Proline Peptide Bond of Neurohypophyseal Peptide Hormones

	% cis[a]	
peptide	native disulfide form	reduced dithiol form
oxytocin[b]	10	10
oxytocin[c]	10	10
oxytocin[d]	6	
arginine vasopressin[b]	9	6
arginine vasopressin[c]	9	6
arginine vasopressin[d]	4	
lysine vasopressin[b]	8	
lysine vasopressin[c]	8	

[a] Percentages were obtained from integrated intensities of amide proton resonances. Errors are estimated to be ±1–2%. [b] At pH 3 and 23 °C in 90% H_2O/10% D_2O. [c] At pH 3 and 23 °C in 90% H_2O/10% D_2O + 0.25 M NH_2OH. [d] At pH* 3 and 23 °C in CD_3OH.

(30) The amide proton chemical shifts in Table II were obtained from spectra of the peptides in 90% H_2O/10% D_2O/0.25 M NH_2OH solution. The amide proton chemical shifts in 90% H_2O/10% D_2O solution, i.e. in the absence of NH_2OH, differ from these values by an average of −0.007 ppm.

(31) Because the solution contains a water proton exchange reagent, care should be exercised in extracting additional information about the conformation of the peptide backbone or side chains from spectra measured with elimination of the water resonance by the WATR method. It is interesting to note however that the populations of the *cis* isomer in Table I are independent of the presence of the water proton exchange reagent NH_2OH.

(32) Since intramolecular hydrogen bonds are broken less than intermolecular solute–solvent hydrogen bonds as the temperature is increased, $\Delta\delta/\Delta T$ for an intramolecular hydrogen bond is of the order of 0 to -3×10^{-3} ppm/K, while that for a solute–solvent hydrogen bond is in the range of −6 to −10 $\times -10^{-3}$ ppm/K. Temperature coefficients in the intermediate range may indicate long noncoplanar hydrogen bonds or conformational equilibrium between intramolecular and solute–solvent hydrogen-bonded environments, or partial shielding of the proton from solvent.[10]

Table II. Chemical Shifts of the Amide Protons of the Cis and Trans Isomers of the Neurohypophyseal Peptide Hormones[a,b]

	oxytocin		reduced oxytocin		AVP		reduced AVP		LVP	
	trans	cis	trans	cis	trans	cis	trans	cis	trans	cis
Tyr	9.006	9.081	8.741		8.889	8.870	8.649		8.865	
Ile, Phe	7.961	7.92	8.102		8.114	7.837	8.159		8.077	7.862
Gln	8.213	8.282	8.365		8.292	8.372	8.219		8.263	
Asn	8.339	8.49	8.512		8.307		8.422		8.287	
Cys^6	8.213	7.879	8.293	8.035	8.136	7.960	8.224	8.016	8.140	7.914
Leu, Arg, Lys	8.464	8.514	8.380	8.600	8.616		8.478	8.703	8.563	
Gly	8.383	8.534	8.387	8.538	8.411	8.512	8.394	8.507	8.393	8.503

[a] At pH 3 and 23 °C in 90% H_2O/10% D_2O + 0.25 M NH_2OH. [b] ppm vs DSS.

Table III. Chemical Shifts of Carbon-Bonded Protons of the Cis Isomers of Oyxtocin, AVP, and LVP[a-c]

amino acid residue	proton	oxytocin	AVP	LVP
Cys^1	$C_\alpha H$	4.31[d]		
Tyr	$C_\alpha H$	4.822[e]		
	$C_\beta H$	3.02, 3.16[e]		
X[g]	$C_\alpha H$	3.979[d]	4.57[d]	4.60[d]
	$C_\beta H$		3.00, 3.34[e]	3.01, 3.34[e]
	$\phi_{2',6'}$		7.243[d]	7.242[d]
Gln	$C_\alpha H$	4.13[e,f]	4.10[e]	
	$C_\beta H$, $C_\gamma H$	2.06, 2.41[e]	2.07, 2.31[e]	
Asn	$C_\alpha H$	4.64[d]	4.70[d]	4.70[f]
Cys^6	$C_\alpha H$	4.64[d]	4.66[d]	4.65[d]
	$C_\beta H$	2.98, 3.17[e]	2.94, 3.02[e]	2.93, 3.03[e]
Pro	$C_\alpha H$	4.72[e,f]	4.72[e]	4.70[d]
	$C_\beta H$, $C_\gamma H$	1.82, 1.98, 2.19, 2.36[e]	1.98, 2.39, 2.18[e]	1.93, 2.33, 2.07[e]
	$C_\delta H$	3.50, 3.63[e]	3.50, 3.62[e]	3.51, 3.64[e]
Y[h]	$C_\alpha H$	4.31[e,f]	4.32[f]	
Gly	$C_\alpha H$	3.91[e]	3.92[e,f]	3.89[e]

[a] Due to overlap by more intense resonances of the trans isomers, chemical shifts could not be determined for some protons of the cis isomers. The values in this table were determined either directly from one-dimensional spectra, measured by the CPMG pulse sequence, or from TOCSY or ROESY spectra. Values measured from two-dimensional spectra are less precise due to lower digital resolution. [b] At pH 3.0 in 90% H_2O/10% D_2O + 0.25 M NH_2OH. [c] ppm vs. DSS. [d] Determined from a one-dimensional spectrum. [e] Determined from a TOCSY spectrum. [f] Determined from a ROESY spectrum. [g] X = Ile for oxytocin, Phe for AVP and LVP. [h] Y = Leu for oxytocin, Arg for AVP, and Lys for LVP.

Table IV. Temperature Coefficients ($\Delta\delta/\Delta T$) of the Amide Proton Chemical Shifts[a,b]

	oxytocin		reduced oxytocin		AVP		reduced AVP		LVP	
NH	trans	cis	trans	cis	trans	cis	trans	cis	trans	cis
Tyr	−6.0	−7.1	−6.5		−6.8	−7.4	−6.0		−5.9	
Ile, Phe	−7.0		−7.1		−7.2	−5.4	−6.8		−5.9	−4.6
Gln	−6.4	−6.8	−9.9		−6.8	−7.3	−5.9		−5.6	
Asn	−4.9		−7.6		−4.6	−5.7	−6.0		−3.9	
Cys^6	−4.6	−2.9	−7.1	−6.5	−4.5	−3.8	−5.5	−5.9	−4.0	−2.7
Leu, Arg, Lys	−9.4	−6.4	−7.6	−8.2	−9.4	−8.0	−8.7	−7.7	−8.1	
Gly	−7.8	−8.4	−8.0		−8.0	−7.9	−7.3	−8.2	−7.1	−6.7

[a] At pH 3 in 90% H_2O/10% D_2O. [b] In units of ppm/K × 10^3.

the Cys^6 amide proton is significantly shielded from solvent while the Asn amide proton may be shielded to a small extent.[33] It is of particular interest to note in Table IV that the $\Delta\delta/\Delta T$ values for the chemical shifts of the Cys^6 amide proton are significantly less for the cis than the trans isomer and for *cis*-oxytocin and *cis*-LVP, in particular, are in the range observed for amide protons which are either in an intramolecular hydrogen bond or shielded from solvent.[32] In either case, these results suggest that the conformational properties of the cis and trans forms are different, with the macrocyclic hexapeptide ring of the cis isomer existing in a higher population of structured species. Elucidation of the specific details of the conformations of the cis isomers will be difficult because of their low abundance and spectral overlap by resonances of the trans isomer.

Summary and Conclusions

Evidence has been presented for the existence of both the cis and trans isomers of oxytocin, AVP, and LVP with respect to the conformation across the cysteine⁶–proline peptide bond. For all three peptides, the trans isomer is the more abundant, both in the native disulfide and reduced dithiol forms of the peptides. The proportion of the cis isomer is essentially the same for the three peptides in the native disulfide form, which indicates there are no specific interactions between the tripeptide side chain and the macrocyclic hexapeptide ring which provide extra stabilization of either the cis or trans isomer of oxytocin, AVP, or LVP. However, the temperature dependence of the amide proton chemical shifts suggests that the macrocyclic hexapeptide rings exist in a higher population of structured forms for the cis isomer. Interchange between the trans and cis isomers by rotation around the Cys^6–Pro peptide bond is faster in CD_3OH solution than in aqueous solution. The possibility that the cis isomer might play a role in the biological potency of oxytocin, AVP, or LVP warrants further investigation.

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