Meeting Am. Soc. Microbiol. **1983**, 190. We thank Prof. Switzer for bringing this information to our attention.

Registry No. 1, 4099-85-8; **1** (5-phosphorodichloridate), 87372-46-1; PRPP, 7540-64-9; PRPP synthetase, 9015-83-2; r-5-P, 4300-28-1; ribokinase, 9026-84-0; D-ribose, 50-69-1; UMP, 58-97-9; r-5-P·2Na, 18265-46-8; r-5-P·Ba, 15673-79-7; RuBP·2Ba, 82130-67-4; sodium orotate, 154-85-8; O-5-P, 2149-82-8; O-5-P-pyrophosphorylase, 9030-25-5; O-5-P-decarboxylase, 9024-62-8; PAN, 25014-41-9; AMP·2Na, 4578-31-8; PRPP·4Na, 87372-47-2.

Side Chain Conformations of Oxytocin and Vasopressin Studied by NMR Observation of Isotopic Isomers[1a]

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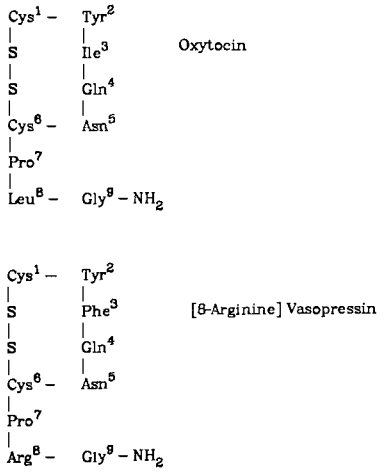
Received March 28, 1983

Abstract: The side chain conformations of several residues of oxytocin and [8-arginine]vasopressin are compared by using measurements of the circumjacent vicinal couplings of $^1H^\alpha$, $^{13}C'$, and $^{15}N'$ to β protons and stereospecific β deuteration in series of 18 specifically designed and synthesized isotopic isomers. The conformation(s) of half-cystyls-1 and -6 and tyrosyl-2 is (are) markedly similar when comparing the two peptides, and other residues show only small differences. Conformational classes of side chain are identified. It is concluded that most of the side chain conformations are largely uninfluenced by the differences in the primary structures of the two peptides.

In attempting to understand more fully the time-dependent or dynamic conformations of peptides in solution, we have concentrated our attention on determining the distribution of rotamers about individual torsion angles in oxytocin and [8-arginine]vasopressin (AVP). In examining these angles and their rotamer



states, we have measured multiple homo- and heteronuclear coupling constants about the angles. This approach is well calibrated for staggered rotamers about the χ^1 angle in amino acids[2] and has been used to refine the solution conformation of valinomycin[3] with the assumption that rigid torsion angles pertain for the backbone in that case. In addition, we have applied this approach to the χ^1 angles of the half-cystyl bridge in oxytocin.[4] We present here data for the half-cystyl bridge in AVP and for several other side chains in both oxytocin and AVP.

In these peptides, the two C^α–C^β torsion angles, χ^1's, of the cystine bridge are torsion angles in the 20-atom ring structure. Other χ^1's determine the relative orientations of their respective side chains, and it had been generally assumed that rotations about these C^α–C^β bonds are relatively free.

Previous experimental and theoretical studies of the conformations of oxytocin and AVP in aqueous solution have suggested that an equilibrium exists between several conformers of the ring and of the side chains (reviewed in ref 4–6). Their precise conformational characterization presents a considerable challenge, because the NMR spectra are complex, because there are multiple possible solutions for derived geometries from measured NMR values, and because a completely general approach to derivation of dynamic structures of relatively flexible molecules is not yet at hand.[7] In this paper we show that the multiple circumjacent coupling constants about the respective C^α–C^β bonds of several residues are concordant with averaging among staggered rotamers. In addition, we compare the derived apparent free energies of rotamers of several residues of oxytocin and AVP, appropriate model peptides, and free amino acids. Our conclusions are based on measurement of circumjacent vicinal couplings between protons on C^β and the C^α substituents $^1H^\alpha$, $^{13}C'$, and $^{15}N'$ and use of stereospecific deuteration of protons on C^β in 18 isotopic isomers of oxytocin and AVP that were synthesized by using standard techniques.

In conformational terms the results show that the C^α–C^β torsion angles of the half-cystyl residues of AVP are eclipsed and predominantly fixed, similar to the situation previously found in oxytocin.[4] Probes in the C-terminal acyclic tripeptide indicate that noncovalent interactions with the cyclic portion are quite minimal. Differences in side chain conformation between oxytocin and AVP are few. At this level of detail, the conformations of

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Table I. Isotopic Isomers of Protected Amino Acids Used in Peptide Isomers

amino acid[a]	isotopic substitution	references to synthesis
cysteine	$\beta 2,\beta 3\text{-}^2H_2$	4, 9
	$^{15}N',\alpha\text{-}^2H$	4, 9
	$^{13}C',\alpha\text{-}^2H$	4, 9
	$\alpha,\beta 3\text{-}^2H_2$	4, 9
tyrosine	$\beta 2,\beta 3\text{-}^2H_2$	9, 10, *b*
	$^{15}N'$	*c*
	$^{13}C',\alpha,\delta,\epsilon\text{-}^2H_5$	9, 10, 12, *b*
	$\alpha,\beta 3\text{-}^2H_2$	13, *b*
phenylalanine	$^{13}C'$	*c*
	$^{15}N'$	*c*
	$\beta,\delta,\epsilon,\xi\text{-}^2H_7$	*d*, 11 (pp 2164–2165)
glutamine	$\gamma\text{-}^2H_2$	14, 15, 16, 17, *e*
asparagine	$\beta 3\text{-}^2H$	18, 19, *f*
	$^{15}N',\beta 3\text{-}^2H$	18, 19, *f*, *g*
	$\beta\text{-}^2H_2$	19
leucine	$\alpha,\delta\text{-}^2H_2$	2
	$^{15}N',\alpha,\gamma,\delta\text{-}^2H_3$	2
	$^{13}C',\alpha,\gamma,\delta\text{-}^2H_3$	2
arginine	$\gamma,\delta\text{-}^2H_4$	*h*

[a] The protecting groups used were as described in ref 8; *tert*-butoxycarbonyl was used throughout for N' protection; *p*-methylbenzyl was the side chain protection for cysteine and nitro for arginine. All amino acids used were L. Resolution used standard methods of ref 11, unless indicated. [b] Resolution used carboxypeptidase on trifluoroacetyl-DL-tyrosine. Reference 11, p 236. [c] From Merck Sharpe & Dohme, Inc. [d] Perdeuteriobenzyl bromide was prepared by photobromination of perdeuteriotoluene from Merck and subsequently reacted with diethyl acetamidomalonate. [e] Substitution of γ protons by deuterium proceeded to only about 80% on a single exchange, and a second treatment was required to produce [γ-(96%)$^2H_2$]glutamic acid. [f] Under the conditions used by us, using *Proteus vulgaris* as a source of aspartase, a mixture of the possible isotopic isomers of combination of α, $\beta 2$, and $\beta 3$ positions was formed. NMR (acetone, of the *tert*-butoxycarbonyl derivative of asparagine) δ 1.45 (s, 9 H), 2.73 (m, 0.09 H), 2.80 (br m, 0.51 H), 4.43 (d, 0.49 H); cf. unsubstituted material 1.45 (s, 9H), 2.79 (octet, 2 H), 4.43 (t, 1 H). From this analysis, it was concluded that the most likely composition was $\beta 3\text{-}^2H{:}\alpha,\beta 3\text{-}^2H_2{:}\beta 2\text{-}^2H \simeq 0.45{:}0.45{:}0.1$. Full synthetic details and NMR spectra are in the supplementary material. [g] $^{15}NH_4Cl$ used as the nitrogen source. [h] [$\gamma\text{-}^2H_2$]Glutamine was converted to γ-cyano-α-L-(carbobenzoxyamino)[$\gamma\text{-}^2H_2$]butyric acid, ref 20a, and then to [$\gamma,\delta\text{-}^2H_4$]ornithine by deuteration using Raney nickel and hydrolysis with DCl, ref 20. Conversion to nitroarginine used standard methods, ref 11, p 1849.

the peptides in aqueous solution are very similar and little influenced by the changes (isoleucyl-3 → phenylalanyl-3; leucyl-8 → arginyl-8) between oxytocin and AVP. The implications of these findings for the structure–activity relationships and for earlier proposals of conformational differences in these peptides are discussed.

Experimental Section

Isotopic Isomers. The methods of peptide synthesis and characterization for oxytocin and AVP have been previously described.[8] Table I summarizes the isotopic isomers of amino acids used in the various peptide isomers. Footnotes and references identify the sources of the methods of synthesis of the amino acids and, where appropriate, the methods of resolution and protection. Isotopic isomers of oxytocin, AVP, and L-prolyl-L-leucylglycinamide (PLG) are summarized in Table II. Code numbers in this table are used in the subsequent discussion.

Strategy of Isotopic Substitutions. There are six homo- and heteronuclear vicinal couplings between the two β protons present in most side chains and the C^α substituents ^1H, $^{13}C'$, and $^{15}N'$. A conservative strategy for the NMR observation of a particular residue's β protons and their couplings to the C^α substituents is to replace protons with deuterons in other residues whose resonances fall close to those under investigation. Enrichment of $^{13}C'$ and $^{15}N'$ in separate isotopic isomers, often accompanied by replacement with deuterium of $^1H^\alpha$, leads to spectra readily

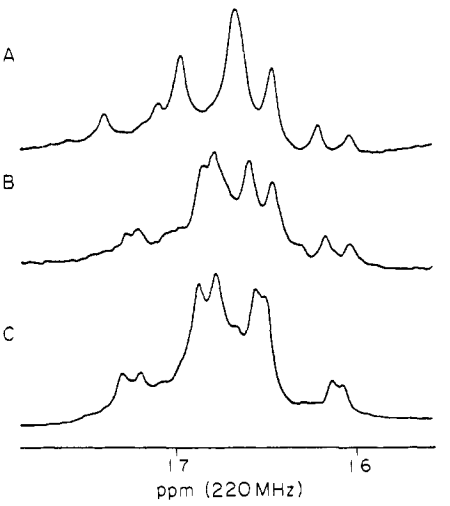

Figure 1. The 220-MHz ^1H NMR spectra of the region between 1.555 and 1.789 ppm for three oxytocin isomers, showing the β protons of leucyl-8 coupled to (A) α proton (OR-1), (B) $^{15}N'$ (OR-4), and (C) $^{13}C'$ (OR-5), with simplification from γ deuteration in all cases.

yielding the heteronuclear couplings. In several cases, one of a pair of prochiral β protons of a residue has also been stereospecifically replaced by deuterium in a separate isomer, so that the prochiral protons may be independently identified.[2,4]

For the two peptide hormones slightly different strategies of β deuteration were used. For oxytocin, the set of three residues, half-cystyl-1, tyrosyl-2, and half-cystyl-6 were permuted through the various combination of two residues with β deuteration and one β protonated, with appropriate C^α substitution.[21] For AVP, the β protons from half-cystyl-1, tyrosyl-2, phenylalanyl-3, asparaginyl-5, and half-cystyl-6 and the δ protons of arginyl-8 overlap considerably,[22] and for observation of the first two residues, the asparaginyl-5 β's and arginyl-8 δ's were consistently deuterated. Permutations were then taken among the C^α-substituted half-cystyl-1 and tyrosyl-2 residues, with phenylalanyl-3 and half-cystyl-6 β deuterated. In separate isomers, with perdeuteration of other protons normally resonant in this region, we incorporated ^{13}C and ^{15}N isotopic isomers of half-cystyl-6 (PR-6 and -7), combinations of isotopic isomers of phenylalanyl-3 and asparaginyl-5 (PR-2 and -8), and a combination of natural half-cystyl-6 and phenylalanyl-3 (PR-1). This last isomer was used to resolve unequivocally the assignments of this region.[22]

NMR Spectroscopy. Most proton spectra were measured on a Varian HR-Nicolet Technology Corp. TT-220 spectrometer by using pulse–Fourier transform techniques. In a few cases, spectra were also obtained at 300 MHz on a Nicolet Magnetics NT-300 W or at 600 MHz on the MPC 600 spectrometer at Carnegie-Mellon University[23] by using the frequency-swept correlation mode.[24] Sample concentrations were in the range 1–30 mg/mL, pH/pD was 4.0, and the temperature was 22–25 °C

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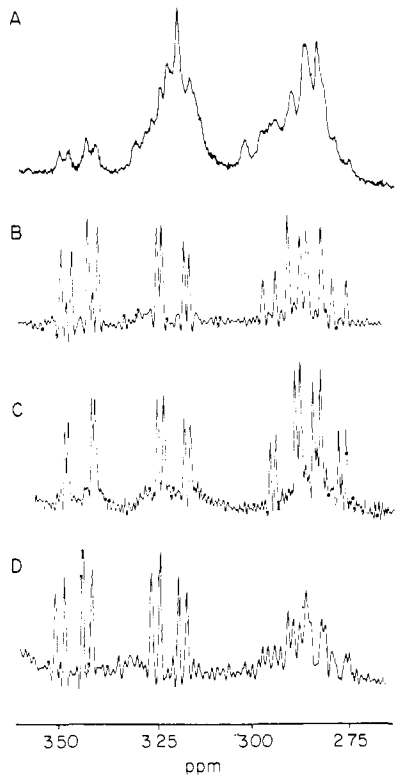

Figure 2. The 220-MHz ^1H NMR spectra of the region between 2.7 and 3.6 ppm for AVP (A) and three isotopic isomers with the β protons of half-cystyl-1 (left-hand side) and tyrosyl-2 (right-hand side) coupled to $^{13}C'$ (1) and $^1H^\alpha$ (2) in isomer PR-5 (B), to $^{15}N'$ (1) and $^{13}C'$ (2) in isomer PR-4 (C), and to $^1H^\alpha$ (1) and $^{15}N'$ (2) in isomer PR-3 (D). (B)–(D) are resolution enhanced (see ref 25).

unless otherwise indicated. Analysis of NMR data and necessary calculations were carried out as previously described.[4]

Results

Oxytocin. In Figure 1 the 220-MHz spectra of the β protons of the leucyl residue, reflecting coupling to their vicinal $^1H^\alpha$, $^{13}C'$, and $^{15}N'$, are shown, with isomers OR-1, -4, and -5 illustrating typical results. The appropriate couplings are summarized by residue in Table III for this and the other residues subsequently discussed. Spectra from other isomers are available in supplementary material (see paragraph at end of paper regarding supplementary material). Prochiral β protons were independently identified by stereospecific deuteration for half-cystyls-1 and -6, tyrosyl-2, and asparaginyl-5.

AVP. In Figure 2, the 220-MHz resolution-enhanced[25] spectra of three isomers of AVP are shown, illustrating the signals from the β protons of half-cystyl-1 and tyrosyl-2 in isomers PR-3, -4, and -5, and comparing the isomers with the spectrum from natural material. Spectra of additional isomers are available in the supplementary material. The appropriate couplings for this and other residues of AVP are summarized in Table III. Prochiral β protons were independently identified by stereospecific deuteration for half-cystyls-1 and -6, tyrosyl-2, phenylalanyl-3, and asparaginyl-5.

L-Prolyl-L-leucylglycinamide (PLG). Values for the vicinal couplings of the isotopic isomers of PLG of Table II are summarized in Table III.

In Table IV the derived populations of staggered rotamers are presented for residues other than cysteine by using the standard values previously obtained. Where appropriate, tests of the validity of this analysis are also shown in Table IV. These are the sum of individually derived populations and the ratios of R factors for discriminating the assignments of the prochiral β protons. Both these tests have been previously described.[2,4]

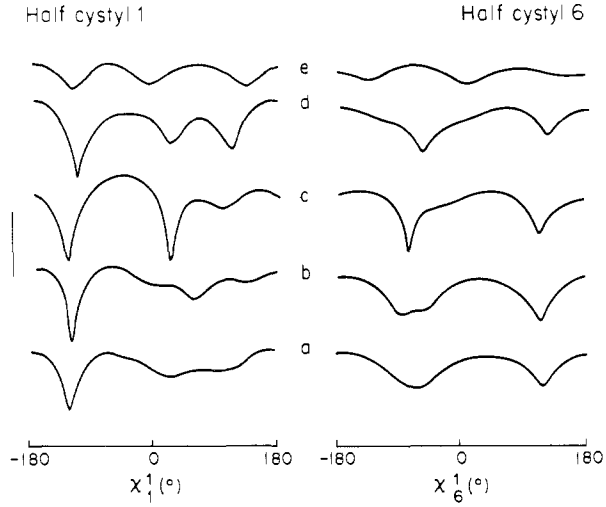

Figure 3. Loci of log R (fit of observed and calculated ^3J's) as a function of χ^1 for various combinations of the couplings of the two β protons of half-cystyl-1 in AVP (left) and half-cystyl-6 (right) to the α substituents. In each, log R is calculated as a function of χ^1 for (a) the six circumjacent couplings (see Table IV), (b) the pair to $^1H^\alpha$, (c) the pair to $^{15}N'$, (d), the pair to $^{13}C'$, and (e) the same set as (a) with the assignments of $\beta 2$ and $\beta 3$ reversed. The solid vertical bar on the left is one base-ten log unit (i.e., a decade of R).

In Figure 3, the degrees of fit between observed and calculated values of couplings, assuming that the appropriate χ^1 is fixed,[4] are shown for the half-cystyls-1 and -6 of AVP.

Discussion

We discuss first the results for individual torsion angles.

Half-Cystyl Residues 1 and 6. The conformational details of χ^1_1 and χ^1_6 in oxytocin have been previously reported and discussed.[4] Plots of fits[4] of calculated to observed couplings (R) for a fixed angle in the range −180 to +180° are shown in Figure 3 for AVP. For half-cystyl-1 it is readily apparent that in AVP, as in oxytocin, a fixed angle of −120° is strongly supported. On the other hand, a simple fixed angle analysis is inadequate for half-cystyl-6 in both molecules. The observed couplings in AVP are very similar to those in oxytocin, and for each peptide there are two substantial minima (Figure 3a, right) around +120 and −80°. In oxytocin[4] the minimum at +120° was distinguishably lower, but only at the 90% confidence level; in AVP the two minima are statistically indistinguishable. Further investigations of these χ^1_6's are under way to identify more specifically their partition functions. The observation of similar values in AVP, compared with oxytocin, establishes that self-consistent and satisfactory measurements of these relatively small couplings can be obtained by using a number of synthetic isotopic isomers for individual measurements.

Tyrosyl-2. We have obtained a complete set of circumjacent couplings from the C^α substituents of tyrosyl-2 to the H^β's and identified the prochiral H^β's by stereospecific substitution. This analysis, available for both oxytocin and AVP, is therefore comparable in level of detail to that obtained for the half-cystyl residues. As one can see in Table III, the coupling values about χ^1 for tyrosyl-2 are very similar, comparing oxytocin and AVP. Without further analysis of data, we may then readily conclude that these χ^1_2's of the peptides are behaving similarly in the two peptides. On the assumption that staggered rotamers exist, it is also possible to calculate the small energy differences between the rotamer states, and these may also be compared for the two peptides. The largest differences in these free energies comparing oxytocin and AVP is then less than 30 cal mol^{-1},[26] quite insig-

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Table II. Isotopic Isomers of Oxytocin, AVP, and L-Prolyl-L-leucylglycinamide Synthesized and Used in This Report

isomer no.	residue no.	residue name	angle investigated	nuclei coupled	isotopic substitution for: observation	isotopic substitution for: simplification or other purpose	^3J value, Hz
				Oxytocin			
OR-1	1	Cys	χ^1	^{15}N'–	^{15}N'	α-^{2}H	
				Hβ2			−3.4[a]
				Hβ3			−2.0
	2	Tyr				β-$^2H_2$	
	6	Cys				β-$^2H_2$	
	8	Leu	χ^1	Hα–		γ,δ-$^2H_2$	
				Hβ2			10.4
				Hβ3			5.1
OR-2	1	Cys				β-$^2H_2$	
	2	Tyr				β-$^2H_2$	
	6	Cys	χ^1	^{15}N'–	^{15}N'	α-^{2}H	
				Hβ2			−2.3
				Hβ3			−3.9
	8	Leu			^{15}N'		
OR-3	1	Cys	χ^1	^{13}C'–	^{13}C'	α-^{2}H	
				Hβ2			3.1
				Hβ3			5.0
	2	Tyr				β-$^2H_2$	
	4	Gln	χ^1	Hα–		γ-$^2H_2$	
				Hβ2			*b*
				Hβ3			*b*
	5	Asn	χ^1	Hα–Hβ2		β3-^{2}H	8.1
	9	Gly			^{15}N'		
OR-4	1	Cys				β-$^2H_2$	
	2	Tyr				β-$^2H_2$	
	5	Asn	χ^1	^{15}N'–			
				Hβ3	^{15}N'	β2-^{2}H	−2.4
	6	Cys	χ^1	^{13}C'–	^{13}C'	α-^{2}H	
				Hβ2			2.0
				Hβ3			2.3
	8	Leu	χ^1	^{15}N'–	^{15}N'	α,γ,δ-$^2H_3$	
				Hβ2			−1.8
				Hβ3			−4.3
OR-5	1	Cys				β-$^2H_2$	
	2	Tyr	χ^1	^{15}N'–			
				Hβ2			−3.1
				Hβ3			−2.1
	6	Cys				β-$^2H_2$	
	8	Leu	χ^1	^{13}C'–	^{13}C'		
				Hβ2		α,γ,δ-$^2H_3$	3.4
				Hβ3			1.2
OR-6	1	Cys	χ^1			α,β3-$^2H_2$	*c*
	2	Tyr				β-$^2H_2$	
	6	Cys	χ^1			α,β3-$^2H_2$	*c*
	9	Gly				α2-^{2}H	
OR-7	2	Tyr	χ^1			α,β3-$^2H_2$	*c*
	2	Pro				20% U[d] ^{13}C	
OR-8	1	Cys				β-$^2H_2$	
	2	Tyr	χ^1	^{13}C'–	^{13}C'		
				Hβ2			3.0
				Hβ3			4.4
						β-$^2H_2$	
	8	Leu				α,δ-$^2H_9$	
				AVP			
PR-1	1	Cys				β-$^2H_2$	
	2	Tyr				β-$^2H_2$	
	3	Phe	χ^1	Hα–			
				Hβ2			5.5
				Hβ3			8.9
	5	Asn				β-$^2H_2$	
	6	Cys	χ^1	Hα–			
				Hβ2			3.1
				Hβ3			10.5
	8	Arg				γ,δ-$^2H_4$	
PR-2	1	Cys				β-$^2H_2$	
	2	Tyr				β-$^2H_2$	
	3	Phe	χ^1	^{13}C'–	^{13}C'		
				Hβ2			2.3
				Hβ3			3.3
	5	Asn	χ^1	^{15}N'–	^{15}N'	β2-^{2}H	−2.6
				Hβ2			
	6	Cys				β-$^2H_2$	
	8	Arg				γ,δ-$^2H_4$	

Table II (*Continued*)

isomer no.	residue no.	residue name	angle investigated	nuclei coupled	isotopic substitution for: observation	isotopic substitution for: simplification or other purpose	^3J value, Hz
PR-3	1	Cys	χ^1	Hα–			
				Hβ2			5.4
				Hβ3			4.7
	2	Tyr	χ^1	^{15}N'–	^{15}N'		
				Hβ2			−3.1
				Hβ3			−1.8
	3	Phe				β-^{2}H$_2$, ring-^{2}H$_2$	
	5	Asn				β-^{2}H$_2$	
	6	Cys				β-^{2}H$_2$	
	8	Arg				γ,δ-^{2}H$_4$	
PR-4	1	Cys	χ^1	^{15}N'–	^{15}N'	α-^{2}H	
				Hβ2			−2.0
				Hβ3			−3.5
	2	Tyr	χ^1	^{13}C'–	^{13}C'	α-^{2}H	
				Hβ2			3.0
				Hβ3			4.4
	5	Asn				β-^{2}H$_2$	
	6	Cys				β-^{2}H$_2$	
	8	Arg				γ,δ-^{2}H$_4$	
PR-5	1	Cys	χ^1	^{13}C'–	^{13}C'		
				Hβ2			5.8
				Hβ3			2.8
	2	Tyr	χ^1	Hα–			
				Hβ2			6.7
				Hβ3			8.1
	3	Phe				β-^{2}H$_2$, ring-^{2}H$_5$	
	5	Asn				β-^{2}H$_2$	
	6	Cys				β-^{2}H$_2$	
	8	Arg				γ,δ-^{2}H$_4$	
PR-6	1	Cys				β-^{2}H$_2$	
	2	Tyr				β-^{2}H$_2$	
	3	Phe				β-^{2}H$_2$, ring-^{2}H$_5$	
	5	Asn				β-^{2}H$_2$	
	6	Cys	χ^1	^{15}N'–	^{15}N'	α-^{2}H	
				Hβ2			−2.3
				Hβ3			−4.5
	8	Arg				γ,δ-^{2}H$_4$	
PR-7	1	Cys				β-^{2}H$_2$	
	2	Tyr				β-^{2}H$_2$	
	3	Phe				β-^{2}H$_2$, ring-^{2}H$_5$	
	6	Cys	χ^1	^{13}C'–	^{13}C'	α-^{2}H	
				Hβ2			2.1
				Hβ3			1.8
	8	Arg				γ,δ-^{2}H$_4$	
	9	Gly			^{15}N'		
PR-8	1	Cys				β-^{2}H$_2$	
	2	Tyr				β-^{2}H$_2$	
	3	Phe	χ^1	^{15}N'–	^{15}N'		
				Hβ2			−3.5
				Hβ3			−2.0
	5	Asn		Hα-Hβ2		β3-^{2}H	7.7
	6	Cys				β-^{2}H$_2$	
	8	Arg				γ,δ-^{2}H$_4$	
PR-9	1	Cys	χ^1			β3,α-^{2}H$_2$	*c*
	2	Tyr	χ^1			β3,α-^{2}H$_2$	*c*
	3	Phe				β-^{2}H$_2$	
	5	Asn				β-^{2}H$_2$	
	6	Cys				β-^{2}H$_2$	
	8	Arg				γ,δ-^{2}H$_4$	
PR-10	1	Cys				β-^{2}H$_2$	
	3	Phe	χ^1			β3,α-^{2}H$_2$	*c*
	4	Gln	χ^1			α-^{2}H$_2$	
				Hα–			
				Hβ2			5.4
				Hβ3			8.6
	5	Asn				β-^{2}H$_2$	
	6	Cys	χ^1			β3,α-^{2}H$_2$	
	7	Pro				U-^{2}H$_6$	
	8	Arg	χ^1	Hα–		γ,δ-^{2}H$_4$	
				Hβ2			4.5
				Hβ3			9.0

Table II (*Continued*)

isomer no.	residue no.	residue name	angle investigated	nuclei coupled	isotopic substitution for observation		simplification or other purpose	^3J value, Hz
				L-Prolyl-L-leucylglycinamide				
PLG-1	2	Leu	χ^1	$^1H\alpha$–	$^1H^{\alpha}$–	α,δ-$^2H_2$		
				Hβ2	$H^{\beta 2}$			9.7
				Hβ3	$H^{\beta 3}$			5.7
PLG-2	2	Leu	χ^1	$^{15}N'$–	$^{15}N'$–	α,γ,δ-$^2H_3$		
				Hβ2	$H^{\beta 2}$			−2.1
				Hβ3	$H^{\beta 3}$			−4.1
PLG-3	2	Leu	χ^1	$^{13}C'$–	$^{13}C'$–	α,γ,δ-$^2H_3$		
				Hβ2	$H^{\beta 2}$			3.9
				Hβ3	$H^{\beta 3}$			2.3

[a] Three-bond ^{15}N–^1H vicinal couplings assumed negative throughout. [b] Not completely analyzable due to chemical shift overlap. Sum of couplings available from observation of H^{α}. See the text. [c] Stereospecific deuteration of one β proton permitted direct assignment of the prochiral β protons of this residue in this isomer. [d] U = uniformly labeled.

nificant compared to thermal fluctuation at room temperature.

Phenylalanyl-3 (AVP) and Isoleucyl-3 (Oxytocin). The values of the six circumjacent couplings from the C^{α} substituents to the H^{β}'s in phenylalanyl-3 are similar to those of the adjacent Tyr-2 (Table III). A simple interpretation of this observation is that both residues are relatively free, and that rotamer states reflect both the simple primary effect of the peptide backbone on the side chain via steric exclusion and also the influence of solvation. The single observed coupling in isoleucyl-3 of oxytocin provides little information about the behavior of this side chain, but it is compatible with a conformation similar to that of phenylalanyl-3 in AVP.

Glutaminyl-4. In oxytocin, the chemical shifts of the β protons are equal at all fields employed, and no analysis of the apparent coupling to H^{α} is possible, even though γ deuteration does indeed simplify the spectrum to a doublet. In AVP, on the other hand, sufficient separation of the β proton shifts gives $^1H^{\alpha}$–$^1H^{\beta}$ couplings consistent with averaging. The sum of couplings $^3J(^1H^{\alpha}$–$^1H^{\beta 2})$ + $^3J(^1H$–$^1H^{\beta 3})$ for AVP is equal to that observed in oxytocin.

Asparaginyl-5. Since the separate isotopic isomers used containing β3-^2H and [$^{15}N'$,β3-^2H] were mixtures with respectively α,β3-$^2H_2$ and $^{15}N'$,α,β3-$^2H_2$ isomers (Table I, footnote *d*), the resulting spectra are somewhat complex. For the β3-^2H isomers in oxytocin (OR-3), a triplet is observed for the β2 proton with the outer line positions being field (220–600 MHz) and temperature (4–61 °C) independent. These outer lines are separated by 8.1 Hz. For AVP, the equivalent separation is 7.7 Hz. From spectral analyses of results at different fields, the central line from the α,β^3-$^2H_2$ isomer is chemically shifted 0.0067 ppm from its β^3-^2H isomer in each peptide. For the $^{15}N'$-substituted asparaginyl isomer, the transitions of the β proton are all equally split by couplings at 2.4 Hz in both peptides. The incomplete set of circumjacent couplings H^{α}–$H^{\beta 2}$, 8.1, $^{15}N'$–$H^{\beta 2}$, −2.4, and H^{α}–$H^{\beta 2}$ + H^{α}–$H^{\beta 3}$, 14.4, is characteristic of rotational isomerism about the α–β bond with high population occupancy at $\chi' = -60$ and +180° and significantly reduced occupancy at +60° in oxytocin. These proton–proton coupling results for oxytocin are comparable to those reported for the asparaginyl residue in [Ala^2]oxytocin.[27]

Arginyl-8 (AVP) and Leucyl-8 (Oxytocin). These residues, in the center of the tripeptide tail pendent to the ring, would be expected to be particularly sensitive to any noncovalent tail–ring interaction. Comparisons of other properties of leucyl residues in oxytocin to those in the respective tripeptides by ^{15}N NMR[28] or in derivatives by hydrogen exchange[29] have shown no evidence for noncovalent interactions in aqueous solution.

In oxytocin and PLG the leucyl residues have circumjacent couplings quite similar to those of the free amino acid, leucine.[2] For example, in Table IV the sums of the derived unequivocally identified populations[2] is 0.98 for oxytocin and 0.81 for PLG. The difference between these experimental values and the value expected if only the three staggered rotamers are populated, 1.0, is small, especially for oxytocin. As an alternative to examining the sum of these elements, *R*-factor analysis can be applied. The calculated *R* factors for the rotamer model for the β2 and β3 proton assignments in Table IV are 0.041 (oxytocin) and 0.055 (PLG), while the reversed assignments yield *R* factors of 0.442 and 0.329, respectively. The ratios of these *R* factors confirm that the possibility of misassignments can be rejected at the 0.005 level of significance.

The observed couplings of Table III for leucyl residues are quite precisely determined, certainly better than 0.1 Hz, except for the $^{13}C'$–$H^{\beta 3}$ value in oxytocin. At first sight, the calculated populations in Table IV differ to a much greater degree than might be expected. Such discrepancies might arise from inaccurate data, from use of an inappropriate model and method of calculation, or from mischoice of standard values for trans and gauche states. As a test of this last case, we may, as an alternative to the independent use of the various measured couplings, calculate the values of the heteronuclear trans and gauche standard states from the populations derived from H^{α}–H^{β} couplings. The results are shown in Table V. With the exception of $^3J_g(^{13}C'$–$H^{\beta})$ and $^3J_t(^{13}C'$-$H^{\beta})$ calculated from the oxytocin data, the results are quite consistent with previously reported values (reviewed in ref 2). The most likely explanation, then, for inconsistency of the ^{13}C–H trans and gauche couplings relates to the low magnitude and precision (±0.5 Hz) of $^3J(^{13}C'$–$H^{\beta 3})$. If so, the available data are totally consistent with averaging over staggered rotamers, as are measurements of temperature dependence.[21]

In AVP, the observable H^{α}–H^{β} couplings in PR-8 are similar to the proton couplings in the leucyl residue in oxytocin and in free arginine, and if it is assumed that staggered rotamers pertain, a similar set of orientations about C^{α}–C^{β} is derived (Table IV).

We now discuss these results for individual torsion angles, considering the place of this technical approach in conformational analysis, comparing the conformations of oxytocin and AVP, and contrasting these results with previous conformational studies of these molecules.

We have demonstrated that NMR measurements on a series of specifically designed and synthesized isotopic isomers of oxytocin and AVP can provide stereochemical assignments for prochiral β protons and can differentiate side chains that are relatively rigid (half-cystyls) from those undergoing rotational isomerism. In the case of relatively rigid conformations the value of the dihedral angle can be obtained, and in the case of staggered rotamers statistical weights or populations can be determined. The approach used here for the nine-residue peptides oxytocin and AVP of incorporating isotopic substituents through direct total synthesis may not be easily applied to larger peptides and proteins. The analysis of circumjacent couplings may still be feasible for large molecules, however, because of recent developments in NMR

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Table III. Summary of Vicinal Coupling Constants about the C^{α}–C^{β} Bonds of Various Residues of Oxytocin and AVP in D_2O[a]

nucleus coupled to β protons	residue name and no. in oxytocin	coupling constant, Hz β2	coupling constant, Hz β3[b]	isomer used and/or reference[c]	residue name and no. in AVP	coupling constant, Hz β2	coupling constant, Hz β3	isomer used and/or reference
$^1H^{\alpha}$	Cys-1	5.0 (4.8)[d]	5.7 (4.8)	4, 23	Cys-1	4.7 (4.6)[d]	5.4 (4.6)	PR-3
$^{15}N'$		−3.4 (−4.0)	−2.0 (−1.5)	4, OR-1		−3.5 (−4.0)	−2.0 (−1.6)	PR-4
$^{13}C'$		3.1 (3.6)	5.0 (5.8)	4, OR-3		2.8 (3.4)	5.8 (5.8)	PR-5
$^1H^{\alpha}$	Tyr-2	7.9 (7.9)	6.9 (7.1)	21	Tyr-2	8.1 (7.8)	6.7 (7.2)	PR-5
$^{15}N'$		−2.1 (−2.1)	−3.1 (−3.3)	OR-1		−1.8 (−2.1)	−3.1 (−3.2)	PR-3
$^{13}C'$		4.4 (4.8)	2.1 (2.2)	OR-8		4.4 (4.9)	3.0 (2.2)	PR-4
$^1H^{\alpha}$	Ile-3		6.5	21	Phe-3	8.9 (8.2)	5.5 (5.7)	PR-1
$^{15}N'$						−2.0 (−2.1)	−3.5 (−3.6)	PR-8
$^{13}C'$						3.3 (3.7)	2.2 (2.2)	PR-1
$^1H^{\alpha}$	Gln-4	7.0	7.0[e]	21, OR-3	Gln-4	8.6	5.4	PR-10
$^1H^{\alpha}$	Asn-5	8.1[f] (8.0)	6.3 (6.2)	OR-3	Asn-5	7.7[f] (7.6)	6.7 (6.6)	PR-2
$^{15}N'$		−2.4 (−2.3)		OR-4		−2.4 (−2.3)		PR-2
$^1H^{\alpha}$	Cys-6	9.6 (9.6)	3.7 (4.0)	4, 21	Cys-6	10.5 (9.6)[g]	3.1 (4.0)	PR-1
$^{15}N'$		−2.3 (−1.9)	−3.9 (−4.0)	4, OR-2		−2.3 (−1.9)	−4.4 (−4.0)	PR-6
$^{13}C'$		2.0 (2.9)	2.3 (4.0)	4, OR-4		1.8 (2.9)	2.1 (4.0)	PR-7
$^1H^{\alpha}$	Leu-8	10.4 (11.0)	5.1 (5.0)	OR-1	Arg-8	9.0	4.5	PR-10
$^{15}N'$		−1.8 (−1.8)	−4.3 (−4.1)	OR-4				
$^{13}C'$		3.4 (3.2)	1.2 (1.4)	OR-5				

[a] For conditions, see Experimental Section. [b] Stereochemical assignments of β2 and β3 protons are derived from data analysis (ref 2 and 4) and confirmed by stereospecific deuteration (see the text). [c] Isomers are detailed in Table I. [d] Values in parentheses are those calculated as best fitting the model derived from all the available couplings for this residue. In the case of half-cystyls, values shown are calculated from fixed angles; for other residues, the values are calculated from averaged rotamer populations. [e] β2 and β3 protons are chemical shift equivalent, and the reported values are half the sum. [f] See footnote *d* in Table I. [g] For half-cystyl-6 of AVP, the calculated values given are for $\chi_6^1 \simeq 120°$, as for oxytocin. See the text.

techniques. For example, the need for extensive deuteration in order to resolve resonances can be reduced by use of very high field spectrometers (e.g. ref 31) or 2-D *J*-resolved NMR[32] or their combination (e.g., ref 33); the need for ^{15}N enrichment can be substantially reduced by use of polarization transfer techniques.[34]

The observed couplings appear to fall qualitatively into three classes, (A) cases involving considerable averaging among three rotameric states, (e.g., tyrosyl-2), (B) cases involving interconversion predominantly between rotamers at $\chi^1 = -60$ and 180° (leucyl-8 in oxytocin), and (C) cases where the weight of evidence suggests that χ^1 is fixed. In oxytocin and AVP, residues 2–5 fall into the first class (A), leucyl-8 and possibly arginyl-8 in AVP fall into the second (B), and the half-cystyl residues are in the third (C). There are apparently no significant differences between oxytocin and AVP in C^{α}–C^{β} conformations, and a possible explanation is that in these peptides side chains are involved in interactions with the local peptide backbone and with the solvent but not with other side chains.

Various hypotheses have been put forward concerning indirect methods for determining the "bioactive" conformation(s) of peptide hormones (for a recent review, see ref 35). It is possible that one of the ensemble of solution conformations may be close to the bioactive conformation, and if so, solution studies could provide useful guidance in design of active analogues. Such has been the case for somatostatin.[37] Alternatively, the solution structure of a hormone may be very flexible,[38] and binding to the receptor then determines bioactive conformation, as is probably the case for enkephalins.[39] With regard to side chain conformations, it appears that oxytocin and AVP fall into this latter category, as has been previously suggested.[40]

Other authors have suggested conformational differences between oxytocin and AVP that might be of relevance to the bioactive conformation. These data included dialysis studies to determine overall molecular size,[41] ^{13}C T_1's to determine segmental flexibility,[42] proton chemical shifts to measure interactions with aromatic side chains[43] and proton vicinal coupling constants for investigation of dihedral angles.[44] Some of these details have been used in models of the bioactive conformation.[45] In the area of dialysis studies,[41] the small difference in apparent overall molecular size is consistent with the interpretation of ^{13}C T_1's in aqueous solution[42] in terms of a more flexible tripeptidic tail in AVP, occupying a slightly larger molecular volume. Our results for leucyl-8 in oxytocin, in comparison with prolylleucylglycinamide, seem to exclude any major noncovalent interaction between the tripeptidic section and the peptide ring and suggest that the small differences observed previously arise from different solvation of the prolylleucylglycinamide and prolylarginylglycinamide entities.

The complete assignment of AVP's β proton resonances, confirming previous tentative assignments,[22] and the similar values of circumjacent couplings for tyrosyl-2 in AVP and oxytocin exclude any solution model in which residue 2 is in a significantly different conformational state in the two peptides, a difference that had been previously suggested.[43,45] Although models of a bioactive conformation for AVP including aromatic interactions between tyrosyl-2 and phenylalanyl-3 are not excluded by this solution study, it is evident that there is no major interaction of

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Table IV. Populations Calculated from the Coupling Constants of Table III, for Three Staggered Rotamers[a]

nucleus coupled to β protons	residue name and no.	p_I[b]	p_{II}	p_{III}	Σp[c]	R[d]
		Oxytocin				
$^1H^\alpha$, $^{15}N'$, $^{13}C''$	Tyr-2	0.484	0.392	0.124	1.13	6.48
		0.433	0.467	0.100		
		0.541	0.365	0.094		
$^1H^\alpha$	Ile-3		0.360[e]			
$^1H^\alpha$	Gln-4			0.197[f]		
$^1H^\alpha$, $^{15}N'$	Asn-5	0.502	0.338	0.161		4.06[g]
				0.200[e]		
$^1H^\alpha$, $^{15}N'$, $^{13}C'$	Leu-8	0.715	0.228	0.057	0.986	8.46
		0.830	0.167	0.003		
		0.762	0.246	0[h]		
		AVP				
$^1H^\alpha$, $^{15}N'$, $^{13}C'$	Tyr-2	0.502	0.374	0.124	1.13	2.23
		0.433	0.567	0.000		
		0.435	0.365	0.200		
$^1H^\alpha$, $^{15}N'$, $^{13}C'$	Phe-3	0.575	0.245	0.161	1.19	5.55
		0.567	0.367	0.067		
		0.659	0.235	0.106		
$^1H^\alpha$	Gln-4	0.547	0.256	0.197		
$^1H^\alpha$, $^{15}N'$	Asn-5	0.465	0.384	0.161		4.00[g]
				0.200[e]		
$^1H^\alpha$	Arg-8	0.584	0.174	0.243		
		L-Prolyl-L-leucylglycinamide				
$^1H^\alpha$		0.646	0.284	0.070		
$^{15}N'$	Leu-3	0.737	0.173	0.100	0.81	5.93
$^{13}C'$		0.571	0.309	0.120		

[a] For conditions, see Experimental Section. [b] The identities of populations are derived from the stereochemical assignments of Table III. Populations are calculated by standard methods (ref 2–4, 49, and 50), assuming equivalence of gauche states and the following standard (Hz) values for gauche and trans states: 2.6 and 13.56 (H^α–H^β), −1.8 and −4.8 (H^α–$^{15}N'$); 1.3 and 9.8 (H–$^{13}C'$). [c] Σp is the sum of those populations identified unequivocally by the sum of the two couplings from β protons to the α substituents, i.e., $p_{III}(H^\beta\text{–}H^\alpha) + p_{II}(H^\beta\text{–}^{15}N') + p_I(H^\beta\text{–}^{13}C')$. See ref 2. [d] R is the ratio of fitted R factors for the given assignments of β2 and β3 protons and their reverse case (see ref 2). For cases with six observed couplings, probabilities of correct assignment are 99% for $R > 3.8$, and 90% for $R > 1.8$. [e] The single coupling yields only a single population value. [f] The sum of couplings from the shift-equivalent β protons yields p_{III}. [g] Assignment shown agrees with that of ref 18b. [h] Small negative value, set to zero.

this type in the conformation of AVP in aqueous solution.

It had previously been suggested that biological function of the hormones can be related to differing populations in solution of half-cystyl staggered conformers.[44] Our data, in contrast, indicate that the χ^1's of half-cystyls-1 and -6 are very similar in the two peptides, that there is no interconversion between staggered rotamers, and that the $\chi_1{}^1$'s are most likely at fixed angles.

Table V. Independently Calculated Values for Gauche and Trans Standard States from Leucyl Residues in Oxytocin and PLG

calculated ^3J for standard state	from data for oxytocin	from data for PLG
gauche, ^{15}N–^1H	−1.58	−1.86
trans, ^{15}N–^1H	−5.38	−5.33
gauche, ^{13}C–^1H	0.47	1.95
trans, ^{13}C–^1H	13.18	9.43

Since the sequences of oxytocin and AVP were determined, there have been extensive investigation and speculation concerning the mechanisms of their different biological activities. The possible role of different conformations has been widely considered,[46] and many NMR investigations of increasing sophistication have been undertaken to determine these conformations (for brief reviews, see ref 46 and 48).

The techniques used in this present investigation have provided unequivocal assignments and conformational analyses of side chain not available from previous work. We have shown that side chain conformations in the two peptides are markedly similar; differences in the sequences are only slightly reflected in side chain conformation(s). The further investigation of the backbone conformation(s) is now being actively pursued in this laboratory with use of isotopic isomers[3] and other NMR techniques.

Acknowledgment. This work was supported by National Institutes of Health Grant AM-20357. NMR facilities at The Rockefeller University were purchased with funds from the National Science Foundation (PCM79 12083) and the Camille and Henry Dreyfus Foundation. NMR facilities at Carnegie-Mellon University (600 MHz) are supported by National Institutes of Health Grant No. RR-00292.

Supplementary Material Available: Additional spectra of isotopic isomers and synthetic detail and spectra of stereospecifically β-deuterated asparagines (6 pages). Ordering information is given on any current masthead page.

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