Conformational Study of the Potent Peptide Hormone Antagonist [1-Penicillamine,2-leucine]oxytocin in Aqueous Solution†

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ABSTRACT: [1-Penicillamine,2-leucine]oxytocin is a conformationally restricted analogue of oxytoxin in which the half-cystine-1 and tyrosine-2 residues of the native hormone are replaced by half-penicillamine (β,β-dimethyl-half-cystine) and leucine, respectively. This analogue is a surprisingly potent oxytocin antagonist [Hruby, V. J., Deb, K. K., Yamamoto, D. M., Hadley, M. E., & Chan, W. Y. (1979) *J. Med. Chem. 22*, 7]. Extensive proton magnetic resonance experiments were performed to determine the conformational properties of this analogue in aqueous solution, and the results were compared with the previously published model for the conformation of [1-penicillamine]oxytocin. The results are consistent with a conformation similar to that of [1-penicillamine]oxytocin except that, while [1-penicillamine]oxytocin in aqueous solution possesses two 1←3 (C_7) type turns involving the isoleucine-3 peptide amide proton and the half-penicillamine-1 carbonyl and the asparagine-5 peptide amide proton and the isoleucine-3 carbonyl, [1-penicillamine,2-leucine]oxytocin has only the latter 1←3 turn. This difference between the antagonists is reflected in the different ϕ and ψ angles in the three N-terminal residues of the two inhibitor analogues and in differences in the preferred side-chain conformations for several residues. One particular result of these conformational differences is that, whereas for [1-penicillamine]oxytocin the tyrosine-2 side chain is unable to assume the rotamer for maximal binding to the uterine receptor, [1-penicillamine,2-leucine]oxytoxin retains conformational and dynamic properties at residues two and three which are more similar to those of oxytocin. It is postulated that these conformational and dynamic properties are consistent with the stronger binding and, hence, greater antagonist activity for this penicillamine analogue relative to [1-penicillamine]oxytocin.

A major goal in efforts to understand the chemical–physical basis for information transfer in biological messengers such as peptide hormones is to differentiate those conformational and dynamic features important to the interaction of the hormone with its receptor (the binding message) from those important to the biological response (the biological activity message). For this purpose, examination of the conformational and dynamic differences of peptide hormone agonists and antagonists should provide important insights (Meraldi et al., 1977; Hruby, 1980). This is because, while agonists such as the native hormone contain the necessary information to both bind to the receptor and transduce a biological effect (agonist activity), competitive inhibitors (antagonists) can bind to the receptor but lack some critical structural, conformational, and/or dynamic property necessary for biological activity.

In previous investigations, we have examined and compared the conformational and dynamic properties in aqueous solution of the peptide hormone, oxytocin, H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2, and related agonists, and the conformationally restricted oxytocin inhibitor, [1-penicillamine]oxytocin ([Pen^1]oxytocin), an analogue in which the half-cystine-1 residue is replaced by a half-penicillamine (β,β-dimethylcysteine) residue.[1] Using high-resolution proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy (Meraldi et al., 1975, 1977; Hruby et al., 1979a), carbon-13 spin–lattice relaxation (T_1) studies (Meraldi et al., 1977), and CD and laser Raman spectroscopy (Hruby et al., 1978) we showed that, while oxytocin had considerable conformational and dynamic flexibility, [Pen^1]oxytocin was conformationally and dynamically more restricted. The spectroscopic data were consistent with a conformation for the 20-membered ring moiety of [Pen^1]oxytocin that possessed two 1←3 intramolecular hydrogen bonds (C_7 structures) (Meraldi et al., 1975, 1977; Hruby, 1980), a disulfide bond with a dihedral angle of 110–115° and right-handed chirality (Hruby et al., 1978), and a restricted Tyr^2 side chain in which the rotamer that would place the aromatic side chain pointing toward the 20-membered disulfide ring was excluded. We suggested that the restricted dynamic and conformational properties were important for the antagonist properties of [Pen^1]oxytocin. As a further test of this model, we more recently prepared [Pen^1,Leu^2]oxytocin and found that it was a very potent oxytocin antagonist, much more potent than expected (Hruby et al., 1979b). Carbon-13 chemical shift and T_1 measurements (Hruby et al., 1979b) and CD and laser Raman studies (Hruby et al., 1978) indicated that it had very similar conformational and dynamic properties to those of [Pen^1]oxytocin. In this paper, we further examine the conformational properties of [Pen^1,Leu^2]oxytocin by using proton NMR (^{1}H NMR). The results are consistent with a conformation in which [Pen^1,Leu^2]oxytocin retains a restricted conformation in the 20-membered disulfide ring similar to that previously proposed for [Pen^1]oxytocin, but with considerably more flexibility of the side chain at the 2 position and greater conformational restrictions at the 5 and 6 positions. These

† From the Departments of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721. *Received November 4, 1980.* This research was supported by grants from the U.S. Public Health Service (AM-17420) and the National Science Foundation. Experiments performed on the Nicolet NT 360 NMR spectrometer were performed at the Purdue University Biochemical Magnetic Resonance Laboratory under the direction of Dr. J. Markley, which is supported by National Institutes of Health Grant RR-01077. We thank Dr. Markley for the use of this facility. Preliminary experiments on the Bruker HX-270 were performed at the Francis Bitter National Magnet Laboratory of the Massachusetts Institute of Technology which is supported by National Science Foundation Contract C-670 and National Institutes of Health Grant RR-00995.

[1] Standard abbreviations and nomenclature for amino acids, peptides, and peptide derivatives are used throughout. All amino acids except glycine are of the L configuration. Other abbreviations used: NMR, nuclear magnetic resonance; CD, circular dichroism; ^{1}H NMR, proton magnetic resonance; T_1, spin–lattice (longitudinal) relaxation time; Pen, half-penicillamine.

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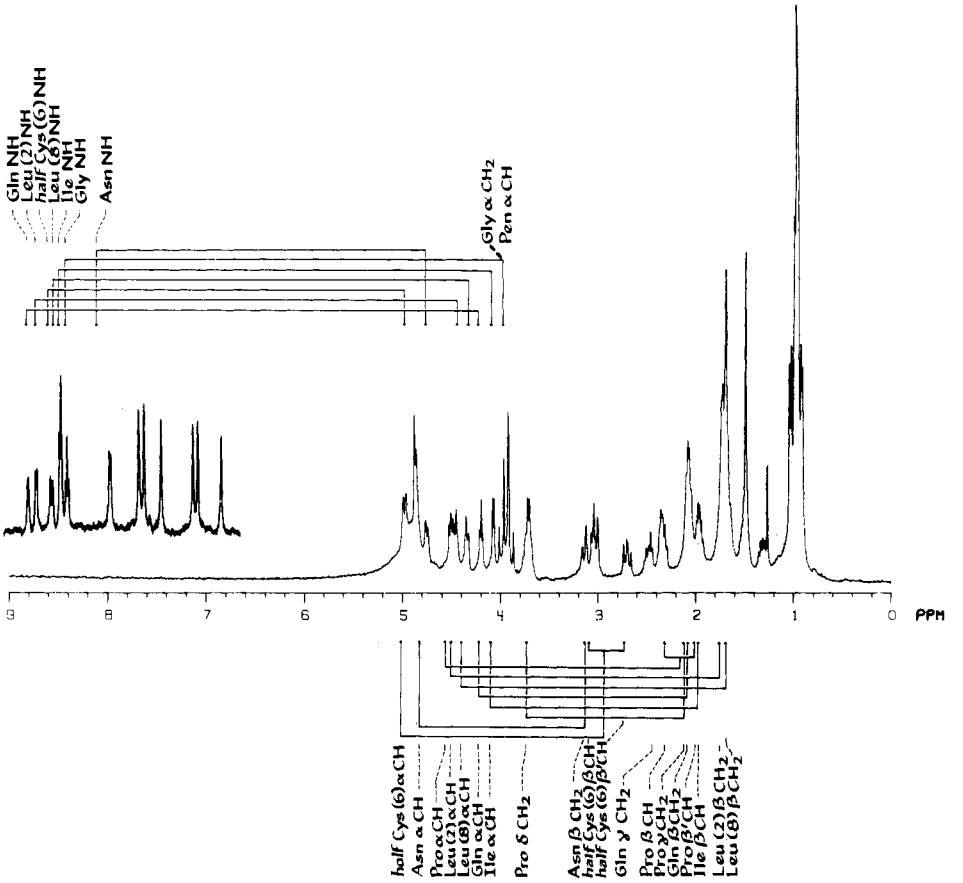

FIGURE 1: Composite 360-MHz proton NMR spectrum of [Pen^1,Leu^2]oxytocin in H_2O (D_2O) at pH 3.7, 28 °C. The upper spectrum is in H_2O, the lower spectrum in D_2O. Connecting lines indicate coupled spin systems and assignments as determined by decoupling experiments.

properties apparently allow for stronger binding to the receptor.

Experimental Procedures

Synthesis and Purification of Peptides. [Pen^1,Leu^2]oxytocin was prepared by the solid-phase method and purified as described previously (Hruby et al., 1979b). The purified peptide gave single uniform spots on thin-layer chromatography in three solvent systems as previously reported.

Sample Preparation for ^1H NMR Studies. [Pen^1,Leu^2]-oxytocin (~15 mg) was dissolved in approximately 5 mL of D_2O to exchange water-labile protons with deuterium and lyophilized. ^1H NMR samples were then prepared by dissolving the peptide in either 99.96% D_2O (Aldrich Chemical Co., Milwaukee, WI) or distilled, deionized H_2O containing aproximately 10% D_2O to serve as a lock signal in the ^1H NMR experiments. The pH was adjusted to 3.7 (direct meter reading) by using CD_3COOD. The prepared samples were ~0.5 mL and were 10–15 mM in [Pen^1,Leu^2]oxytocin. A small amount of *tert*-butyl alcohol was present in all samples as a chemical shift reference. Wilmad 528-PP 5-mm o.d. tubes were used for all ^1H NMR experiments.

^1H NMR Spectra. Unless otherwise noted, all ^1H NMR experiments were performed on a Nicolet NT 360, 360-MHz pulse and Fourier-transform spectrometer located at the Purdue University Biochemical Magnetic Resonance Laboratory in West Lafayette, IN. Decoupling experiments on D_2O solutions were performed by using either an accumulation of free induction decays following 90° pulses or a (180°–τ–90°–T) WEFT sequence (Patt & Sykes, 1972) in which τ is chosen such that the residual HDO peak is nulled and $T \sim 5T_1$ where T_1 is the longitudinal relaxation time of the HDO proton. All decoupled spectra were compared with a spectrum in which the decoupling frequency was chosen to correspond to a region of the spectrum containing no resonances.

Decoupling experiments in H_2O solutions for which the decoupling frequency was in the region of the large H_2O resonance were performed at 200 MHz on Nicolet, Bruker, or Varian instruments. The problem of trying to observe a 10 mM signal (amide proton) in the presence of an ~100 M proton signal (H_2O) was solved by using either a saturating pulse at the H_2O resonance frequency or double-precision data collection and a floating point transform to increase the dynamic range. The former method often leads to complications in the form of broadened, less-intense amide signals due to exchange with the saturated water protons. For the case studied here, the pH was such that the exchange rate was slow, and as a result, no significant transfer of saturation was apparent.

The temperature dependence of the amide proton chemical shifts in H_2O solution was determined by using a Redfield 2–1–4 pulse sequence (Redfield et al., 1975) optimized such that the H_2O resonance was not excited.

The ^1H NMR spectra were collected by using quadrature detection, a 16K data base, and a spectral bandwidth of 5000 Hz. Approximately 100 pulses were accumulated for each experiment. The resulting FID was multiplied by an exponential corresponding to a 0.4-Hz line broadening prior to Fourier transformation. The chemical shifts reported are relative to DSS (not present in the sample) calculated by setting the *tert*-butyl alcohol (internal) methyl resonance equal to 1.247 ppm.

Results

Spectra. The 360-MHz ^1H NMR spectrum of [Pen^1,Leu^2]oxytocin is presented in Figure 1 as a composite of spectra taken in D_2O (bottom spectrum) and H_2O (down-

Table I: Chemical Shift and Coupling Constant Data from Computer Simulation of ^{1}H NMR Spectra of [Pen^1,Leu^2]oxytocin and [Pen^1]oxytocin[a]

residue	δ [b]	$J_{NH-\alpha CH}$ [d]	$J_{\alpha\beta}$	$J_{\alpha\beta'}$	$J_{\beta\beta'}$
Pen^1	δ_{NH} = —	—			
	δ_α = 3.89 (3.92)				
	δ_γ = 1.66 (1.62), 1.46 (1.44)				
Leu^2 (Tyr^2)[c]	δ_{NH} = 8.66 (8.82)[c]				
	δ_α = 4.42 (4.74)[c]	4.7 (6.2)[c]	7.4 (10.0)[c]	6.9 (3.1)[c]	
	δ_β = 1.7				
Ile^3	δ_{NH} = 8.39 (7.33)	<2.0 (3.0)	5.5 (5.8)		
	δ_α = 4.05 (3.94)				
	δ_γ = 1.9 (1.86)				
Gln^4	δ_{NH} = 8.71 (8.14)	3.0 (2.0)	6.0 (8.4)	5.6 (5.2)	
	δ_α = 4.17 (4.11)				
	δ_β = 2.04 (2.01)				
	δ_γ = 2.4 (2.4)				
Asn^5	δ_{NH} = 7.92 (7.84)	6.1 (6.0)	8.3 (7.5)	2.3 (7.3)	14.1 (14.5)
	δ_α = 4.73 (4.76)				
	δ_β = 3.12 (3.11), 3.02 (3.08)				
Cys^6	δ_{NH} = 8.50 (8.51)	9.4 (9.0)	11.9 (10.0)	3.5 (4.0)	14.8 (15.8)
	δ_α = 4.96 (4.88)				
	δ_β = 3.01 (3.0), 2.68 (2.75)				
Pro^7	δ_{NH} = —	—	8.2 (8.4)	5.4 (5.2)	
	δ_α = 4.48 (4.46)				
	δ_β = 2.31 (2.2), 1.9				
	δ_γ = 2.1 (2.0)				
	δ_δ = 3.68 (3.70)				
Leu^8	δ_{NH} = 8.40 (8.39)	7.0 (5.8)	10.0 (9.6)	4.0 (3.6)	
	δ_α = 4.31 (4.3)				
	δ_β = 1.7 (1.7)				
Gly^9	δ_{NH} = 8.34 (8.33)	5.8 (6.2)	$J_{\alpha\alpha'}$ = 17.3 (16.8)		
	δ_α = 3.96 (3.93), 3.87 (3.85)	5.8 (5.2)			

[a] Values for [Pen^1]oxytocin in parentheses (Meraldi et al., 1977). [b] All δ values are at 28 °C, pH 3.8, and are ±0.005. [c] Tyrosine is in position 2 of [Pen^1]oxytocin. [d] All J values are ±0.2 Hz.

Table II: Temperature Dependence of the Chemical Shifts of the Peptide Amide Protons in [Pen^1,Leu^2]oxytocin and [Pen^1]oxytocin

amino acid residue	-10^3 (dδ/dT)
Leu^2 (Tyr^2)	4.7 (6.5)[a,b]
Ile^3	9.4 (0.5)
Gln^4	8.7 (9)
Asn^5	0.8 (0.5)
Cys^6	4.8 (4.5)
Leu^8	9.0 (6.5)
Gly^9-NH$_2$	7.0 (8)

[a] Values in parentheses are for [Pen^1]oxytocin. [b] Tyr residue in [Pen^1]oxytocin.

field inset). The assignments designated were made via homonuclear decoupling experiments linking the resonances as shown and by analogy with those for [Pen^1]oxytocin (Meraldi et al., 1977) and oxytocin (Brewster & Hruby, 1973) in aqueous solution. The experimental spectrum was partially simulated by using the LAOCN3 program (Bothner-By & Castellano, 1968), and the best-fit chemical shift and coupling constant parameters of the simulation are given in Table I.

The ^{1}H NMR experiments were performed at a slightly acid pH (3.7) (as have most other studies of peptide conformations in aqueous solution) in order to slow the exchange of NH protons with H_2O and thus obtain accurate coupling constants. The question of whether the conformation would be different at other pHs (e.g., 7) is not addressed here. However, previous CD investigations over a wide pH range (Hruby et al., 1978) suggest that only minor changes in conformation accompany changes in pH.

Temperature Dependence of Amide Chemical Shifts. The temperature dependence of the peptide amide proton chemical shifts of [Pen^1,Leu^2]oxytocin over the temperature range of 10–80 °C is shown in Figure 2, and the results are summarized and compared with [Pen^1]oxytocin in Table II. Particularly

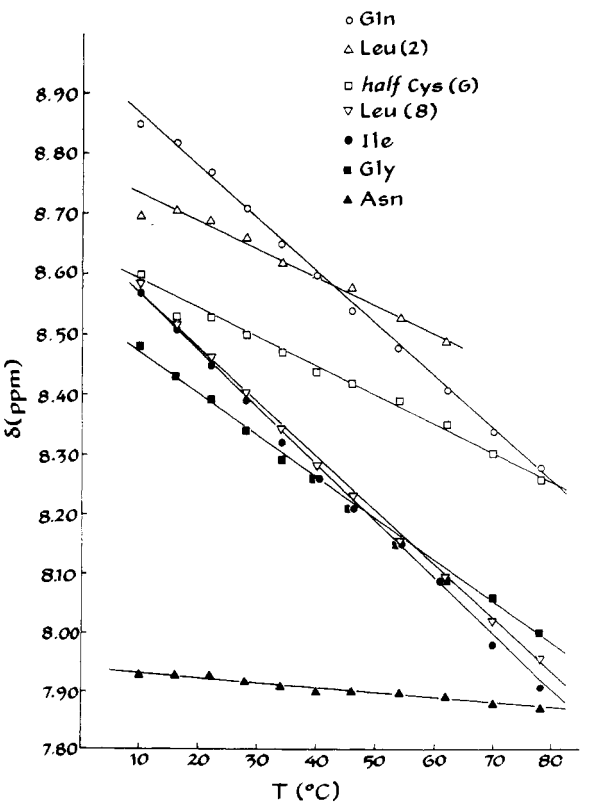

FIGURE 2: Temperature dependence of peptide amide protons of [Pen^1,Leu^2]oxytocin in H_2O at pH 3.7. The various peptide amide protons are Asn^5 (▲), Gly^9 (■), Ile^3 (●), Leu^8 (▽), Cys^6 (□), Leu^2 (△), and Gln^4 (○).

noteworthy is the very small temperature dependence of the Asn^5 peptide amide proton (see below).

Backbone and Side-Chain Conformations. The best-fit values of $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ given in Table I were used to calculate

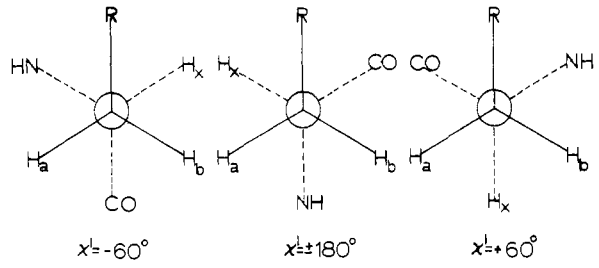

FIGURE 3: The classical, staggered rotamers about the C_α–C_β bond of an amino acid residue of the L configuration; $\chi^1 = -60°$ (a), $\chi^1 = \pm 180°$ (b), $\chi^1 = 60°$ (c). R represents a side-chain group at the β position of the peptide. For an amino acid residue at the amino terminal, the ND_3^+ group replaces the NH group, and for an amino acid residue at the carboxy terminus, the CO group is replaced by a CO_2^- group.

Table III: Classical Rotamer Populations Calculated[a] for [Pen^1,Leu^2]oxytocin and for [Pen^1]oxytocin

	[Pen^1,Leu^2]oxytocin			[Pen^1]oxytocin		
residue	$P(a)^b$	$P(b)^b$	$P(c)^b$	$P(a)^b$	$P(b)^b$	$P(c)^b$
Leu^2 (Tyr^2)	0.44	0.39	0.17	0.67	0.05	0.28
Gln^4	0.31	0.27	0.42	0.53	0.24	0.23
Asn^5	0.52	0	0.48	0.44	0.42	0.14
Cys^6	0.85	0.08	0.07	0.64	0.13	0.20
Pro^7	0.51	0.25	0.24	0.53	0.24	0.23
Leu^8	0.67	0.13	0.20	0.64	0.09	0.27

[a] Values calculated from Pachler (1964). Conformations a, b, and c are those shown in Figure 3. $P(a) = (J_{13} - J_g)/(J_t - J_g)$; $P(b) = (J_{12} - J_g)/(J_t - J_g)$; $P(c) = 1 - (P(a) + P(b))$; $J_g = 2.6$ Hz; $J_t = 13.56$ Hz. [b] Fractional rotamer populations are ±0.02.

rotamer populations about the χ^1 (C_α–C_β) bond (Figure 3) in the usual manner (Pachler, 1964). The results are presented in Table III and compared with the rotamer populations for [Pen^1]oxytocin (Meraldi et al., 1977). The assignments of $P(a)$ and $P(b)$ are arbitrary since the β and β' protons cannot be stereochemically assigned in these experiments. The convention used is to arbitrarily assign J_{13} to be the larger of the two coupling constants, $J_{\alpha\beta}$ and $J_{\alpha\beta'}$. The analysis is valid if only classical rotamers contribute to side-chain group conformations.

The $J_{NH-\alpha CH}$ values of Table I can be related to the dihedral angles about the N–C_α bonds via Karplus-like relations (Bystrov et al., 1973; Hruby, 1974). This will be elaborated upon below.

Discussion

^1H NMR Chemical Shifts. The ^1H NMR spectra of [Pen^1,Leu^2]oxytocin and [Pen^1]oxytocin are quite similar; this is reflected in the chemical shifts presented in Table I. In addition to the obvious differences for the second residue which differs in the two polypeptides, there are significant differences only at the Ile^3 and to a lesser extent in the Gln^4 amide proton chemical shifts and a moderate difference in the Ile^3 α-proton shift. The remaining corresponding chemical shifts are strikingly similar and are generally within about 0.05 ppm. These results suggest that the overall conformations of the two compounds are probably similar, as was previously indicated in carbon-13 NMR (Hruby et al., 1979b) and laser Raman and CD (Hruby et al., 1978) studies. The differences observed at Ile^3 and Gln^4 probably reflect significant local conformational differences between the two polypeptides, and indeed there is further evidence to support this interpretation (see below). However, it is possible that some of the observed differences may also be due to the presence of ring current effects from the aromatic side chain of Tyr^2 in [Pen^1]oxytocin and the lack of these effects in [Pen^1,Leu^2]oxytocin. Estimation of this ring current effect is very difficult since the number of relative orientations between the tyrosine ring and the nearby protons of other residues is large. This is a result of the multiple values of χ^1 for the tyrosine, the lack of information concerning the orientation of the tyrosine ring about the C_β–C_γ bond, and the lack of information concerning the Ile^3 χ angles. Nonetheless, the lack of any significant chemical shift differences for the Ile^3 and Gln^4 *side chains* in both the carbon-13 (Hruby et al., 1979a) and proton NMR spectra of [Pen^1]oxytocin and [Pen^1,Leu^2]oxytocin suggests that a ring current effect is not a significant contributor to the observed differences.

Temperature Dependence of Amide Proton Chemical Shifts. From Figure 2 and Table II, it can be seen that all but one of the amide protons of [Pen^1,Leu^2]oxytocin exhibit a temperature dependence in the range of $(4.7–9.4) \times 10^{-3}$ ppm/K. These temperature dependences can be explained by the accessibility of the amide protons to solvent, with greater temperature dependence implying hydrogen bonding to the solvent, H_2O (Urry, 1970). The lack of pronounced effect on the Asn^5 peptide amide proton is consistent with either the presence of an intramolecular hydrogen bond involving this proton or a manifestation of a highly populated conformation which does not allow access of water molecules to the Asn^5 peptide amide proton. In view of the rather small size of the polypeptide and the relative flexibility of polypeptides, the latter explanation seems less likely than the former.

The temperature dependence results for [Pen^1,Leu^2]oxytocin can be compared with those of [Pen^1]oxytocin which also are shown in Table II. It is apparent that the major difference is in the behavior of the Ile^3 peptide amide proton. In [Pen^1]oxytocin, this proton was found to have a very small temperature dependence (Meraldi et al., 1975, 1977), indicative of its participation in an intramolecular hydrogen bond. No such indication is found in [Pen^1,Leu^2]oxytocin; indeed, this proton is now exposed to the aqueous environment. Therefore, we conclude that while [Pen^1]oxytocin contains two peptide amide protons involved in intramolecular hydrogen bonds, [Pen^1,Leu^2]oxytocin contains only one.

Backbone Conformations and Side-Chain Rotamer Populations. Vicinal proton coupling constants can be related to conformation in peptides from two different aspects of structure. If a single, rigid conformation is assumed, then empirical relationships (Barfield & Karplus, 1969; Bystrov et al., 1973; Karplus, 1963; Kopple et al., 1973) can be used to arrive at possible dihedral (torsional) angles. In the case of [Pen^1,Leu^2]oxytocin, use of $J_{NH-\alpha CH}$ for determining the backbone conformation of the 20-membered disulfide ring (tocin ring) seems particularly promising because of the presence of an intramolecular hydrogen bond, the presence of two very small and one large NH–αCH coupling constants, and the known fixed dihedral angle and chirality of the disulfide bridge (Hruby et al., 1978). All of these features are indicative of a well-defined conformation in the 20-membered tocin ring of [Pen^1,Leu^2]oxytocin. It is anticipated that the constraints placed on possible conformations by these parameters as well as others to be discussed will permit a determination of a reasonable backbone conformation for this analogue.

A second approach, first discussed by Pachler (1964), allows calculation of rotamer populations for the three possible staggered (lowest potential energy) conformations (Figure 3) from the experimental vicinal coupling constants. This approach is appropriate for the less constrained situations that obtain for side chains in polypeptides. Though this method

has various limitations (Hruby, 1974; Feeney, 1976; see above), it can give relative side-chain conformational probabilities which in turn can provide considerable insight into conformations which are possible for a peptide.

Figure 3 depicts the three staggered side-chain rotamers whose populations are calculated and tabulated in Table III. As previously mentioned, an uncertainty exists in the calculated populations since the stereochemical assignments of the β and β' resonances are unknown. Thus, in Table III the values of $P(a)$ and $P(b)$ for any side chain could be reversed. Generally this makes direct comparison between different polypeptides tenuous. However, for structurally closely related peptides and for cases in which the same residue in two similar polypeptides yields very similar populations, i.e., nearly the same coupling constants, it seems reasonable to expect that similar side-chain conformations are reflected, and it is unlikely that $P(a)$ and $P(b)$ are reversed for one but not the other. On the other hand, for cases in which substantial differences occur in the calculated rotamer populations for the same side chain in two similar polypeptides, then whether or not $P(a)$ and $P(b)$ are reversed for one and not the other, there must be significant conformational differences between the two. Thus, there is considerable justification for comparing the calculated side-chain rotamer populations for [Pen^1,Leu^2]oxytocin to those for [Pen^1]oxytocin.

From Table III we see that the most similar rotamer populations for the two penicillamine analogues occur in the side chains of the "tail" portion of the peptides, Pro^7 and Leu^8. The corresponding rotamer populations for these residues are identical within experimental error. For [Pen^1]oxytocin, one rotamer of Cys^6 is essentially excluded. For [Pen^1,Leu^2]-oxytocin, this and a second rotamer are excluded, leading to an apparently fixed orientation about the C_α–C_β bond of this residue, and this, in turn, along with the geminal dimethyl groups of the Pen^1 residue, will be expected to limit possible conformations in the 20-membered ring of this peptide. There are significant differences in the rotamer populations of the Gln^4 residue and, of course, in the 2-position amino acid which differs for the two polypeptides. The most striking difference, however, occurs for the Asn^5 side chain. In [Pen^1,Leu^2]-oxytocin, one rotamer is excluded, and the remaining two are approximately equally populated. For [Pen^1]oxytocin, again two rotamers are equally populated, and the third is only sparsely populated. Note that, the absence of the absolute assignment of $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ notwithstanding, the excluded (or largely excluded) rotamers of the Asn-5 residue are different in the two oxytocin antagonists.

As discussed above, the $J_{NH-\alpha CH}$ experimental values can be used to determine the dihedral angles, ϕ_i, about the N–C_α bond. In general, one gets two possible values of the magnitude of the dihedral angle and, hence, four possible ϕ values for each peptide residue. The overwhelming number of possible conformations can be reduced by referring to contour maps of the interaction energy of a specific amino acid as a function of the dihedral angles ϕ and ψ, where ψ is the dihedral angle about the C_α–C′ bond (Ramachandran et al., 1963; Ramachandran & Sasisekharan, 1968; Bovey, 1974). In addition, the peptide bond dihedral angle, ω, can be taken (except in the case of proline) to be trans. In this manner, a tractable number of backbone conformations can be generated for [Pen^1,Leu^2]oxytocin, especially since its conformation is closely related to that of [Pen^1]oxytocin.

Previous workers have postulated on the basis of proton NMR transfer of saturation experiments (Krishna et al., 1979) and CD and laser Raman studies (Hruby et al., 1978; Tu et al., 1978) that in aqueous solution, oxytocin may possess, at least in part, β-turn structures. Examination of the NH–αCH coupling constants for Ile^3 and Gln^4 residues in [Pen^1,Leu^2]-oxytocin exclude the possibility of type I, II, or III β turns (Venkatachalam, 1968; Lewis et al., 1973). Since an intramolecular hydrogen bond is consistent with the temperature dependence of the Asn^5 peptide amide proton and its large unfield chemical shift relative to the other peptide amide protons (Urry, 1970), other ordered conformations were considered. From these considerations and a comparison of conformational parameters for [Pen^1]oxytocin, a plausible conformation for the 20-membered disulfide ring in [Pen^1,Leu^2]oxytocin is given in Table IV which is consistent with the ^{1}H NMR data given in Tables I and II, previous carbon-13 NMR studies (Hruby et al., 1979b), and CD and laser Raman investigations (Hruby et al., 1978). The conformation about the disulfide bond and the chirality of the disulfide bond were unambiguously determined previously (Hruby et al., 1978), and these results also are given in Table IV. Other major conformational features include a C_7 ring structure within the 20-membered ring. The C_7 structure contains a 7-membered ring formed by hydrogen bonding between the Asn^5 peptide NH proton and the Ile^3 CO oxygen. Extension of the C_7 structure to a γ-turn structure (Nemethy & Printz, 1972) was considered since the coupling constant data would be consistent with such a structure. However, this structure would involve a second intramolecular hydrogen bond between the Ile^3 NH proton and the Asn^5 CO oxygen, and no evidence for such an intramolecular hydrogen bond was found. Indeed, the very high temperature dependence of the Ile^3 NH proton strongly argues against a γ conformation for [Pen^1,Leu^2]oxytocin in aqueous solution. Yet another conformation considered was a C_5 ring structure (Maigret et al., 1970; Lewis & Scheraga, 1971) for the Asn^5 residue. This structure was possible, but was excluded since other models were better able to satisfy all the conformational and dynamic parameters obtained from the proton and carbon-13 NMR data and the CD and Raman results. Furthermore, the observation that, except for the Ile^3 residue, all of the nonvariable residues in [Pen^1,Leu^2]oxytocin and [Pen^1]oxytocin show chemical shifts and temperature dependences of peptide amide protons very similar to each other suggests that both antagonists have very similar conformations. The most noticeable difference, of course, is that [Pen^1,Leu^2]oxytocin has only one temperature-independent peptide amide proton, the Asn^5, while [Pen^1]oxytocin has two, the Asn^5 and the Ile^3 amide protons. In addition to the temperature-dependence differences, evidence that the Ile^3 amide proton is intramolecularly hydrogen bonded in [Pen^1]oxytocin but not in [Pen^1,Leu^2]oxytocin comes from the chemical shift of this proton, 7.33 and 8.39 ppm for

Table IV: Backbone Conformation for [Pen^1]oxytocin and [Pen^1,Leu^2]oxytocin

residue	[Pen^1,Leu^2]oxytocin[b] ϕ	ψ	χ^1	[Pen^1]oxytocin[a,b] ϕ	ψ	χ^1
Pen^1		−60	+60		−150	−60
Tyr^2 (Leu^2)	−175	180		95	−60	
Ile^3	−50	−60		−180	150	
Gln^4	−60	60		−50	70	
Asn^5	−160	−120		−165	−120	
Cys^6	60		−60	75		−60

disulfide: C–S–S–C, ~115°, chirality right handed

H bonds: [Pen^1]oxytocin Ile^3 NH to Pen^1 CO; Asn^5 NH to Ile^3 CO } 1←3 (C7) turns

[Pen^1,Leu^2]oxytocin Asn^5 NH to Ile^3 CO

[a] From Hruby (1980). [b] All torsional angles are ±30°.

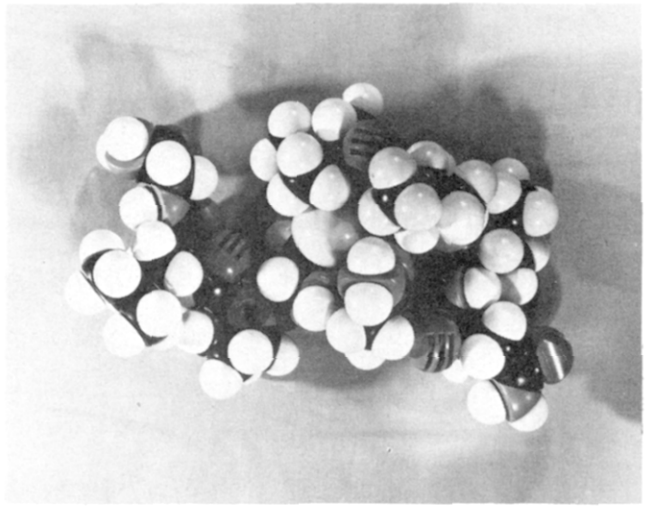

FIGURE 4: CPK model of [Pen^1,Leu^2]oxytocin in the conformation given in Table IV and the highest population side-chain conformations for each amino acid residue given in Table III. The placement of the C-terminal tripeptide side chain is arbitrary.

[Pen^1]oxytocin and [Pen^1,Leu^2]oxytocin, respectively. The former value is indicative of a proton inaccessible to solvent, while the latter value is typical of a solvent-exposed proton (Urry, 1970).

Since otherwise the chemical shift and coupling constant data are very similar in [Pen^1,Leu^2]oxytocin and [Pen^1]-oxytocin, a reasonable approach is to consider what local conformational perturbation would break the second C_7 hydrogen-bonded structure found in [Pen^1]oxytocin (between the Ile^3 NH proton and the Cys^1 CO oxygen) but still retain most of the other conformational features of the 20-membered ring proposed for [Pen^1]oxytocin. An obvious place to look is the 2 position since that is the variable position in the two antagonists. Examination of space-filling models of [Pen^1,Leu^2]oxytocin in the same conformation as [Pen^1]-oxytocin reveals a steric repulsion between the *sec*-butyl group of Ile^3 and the isobutyl group of Leu^2 in [Pen^1,Leu^2]oxytocin, which could be relieved by a conformational change in which the Ile^3 *sec*-butyl group assumed a slightly more equatorial position and the Leu^2 group a more axial-like position, with concomitant adjustments in the backbone of the Pen^1 conformation. Little else in the ring was affected. This change in conformation is consistent with the breaking of the hydrogen bond involving the amide proton of Ile^3, and, in fact, as indicated by its temperature dependence, this proton is now exposed to solvent. The conformation is also consistent with the further restriction of the Cys^6 rotamer to a single conformer, as shown in Table III. Finally, the conformation is consistent with the greater antagonist potency of [Pen^1,Leu^2]oxytocin vs. [Pen^1]oxytocin. As previously found (Meraldi et al., 1977; Hruby, 1980), the conformation of [Pen^1]oxytocin places the Tyr^2 aromatic side chain pointing away from the 20-membered ring and the Ile^3 side chain in an axial-like position. This placement of the Tyr^2 side chain in the weak antagonist [Pen^1]oxytocin is consistent with that aspect of the cooperative model for oxytocin (Walter, 1977) which proposed that for an *agonist*, the Tyr^2 aromatic side chain should be located over the 20-membered disulfide-containing ring in the biologically active conformation. Since this side chain is excluded from that conformation in [Pen^1]-oxytocin, this analogue is an inhibitor. However, the net effect of the placement of the Tyr^2 and Ile^3 side chains in these conformations is to diminish the binding of [Pen^1]oxytocin relative to oxytocin at the uterine receptor, and thus it is a weak inhibitor (Vavrek et al., 1972; Chan et al., 1967). In [Pen^1,Leu^2]oxytocin, on the other hand, both the Leu^2 and the Ile^3 side-chain groups have conformational properties similar to those found for oxytocin at the 2 and 3 positions (Meraldi et al., 1977; Wyssbrod et al., 1977). Consistent with the dynamic model of oxytocin activity (Meraldi, et al., 1977; Hruby, 1980), this permits greater attractive interaction of the antagonist with the uterine receptor, and, hence, a more potent antagonist is obtained. The elimination of the Asn^5 side-chain rotamer, mentioned previously, is also consistent with the suggested conformation but appears to require, in addition to the conformational properties indicated for the 20-membered disulfide ring moiety, that the proline residue on the tripeptide side chain be in a conformation that places its aliphatic ring near the asparagine residue. A suitable conformation would be one where the Cys^6 ψ torsional angle is between 0 and $-60°$. In this case, the other two classical rotamers should have equal weight in Asn^5. From the above, it is interesting to note that though there apparently is more conformational space available to the Leu^2 side chain in [Pen^1,Leu^2]oxytocin than to the Tyr^2 side chain in [Pen^1]-oxytocin, this is compensated for by an apparent decrease in the conformational space for the Cys^6 and Asn^5 side-chain moieties in the former analogue. It may be that since [Pen^1,Leu^2]oxytocin is a surprisingly potent inhibitor (Hruby et al., 1979b), its conformation in solution more closely resembles that required of the hormone for maximal binding than does that of [Pen^1]oxytocin.

Figure 4 is a space-filling model of [Pen^1,Leu^2]oxytocin with the conformation for the 20-membered ring given in Table IV, the most populated side-chain rotamer given in Table III, and the Cys^6 ψ angle mentioned above. The model is consistent with all of the NMR, CD, and laser Raman spectral data which are given in this paper and in previous publications. It provides further evidence for the conformation-dynamic model of oxytocin antagonist activity which we previously presented (Meraldi et al., 1975, 1977; Hruby, 1980) and also is consistent with that aspect of the Walter cooperative model (Walter, 1977) which proposed that the placement of the tyrosine side-chain aromatic group toward the 20-membered ring is important to agonist activity. Further conformation and dynamic studies of conformationally restricted analogues substituted at the 2, 4, 7, and 8 positions should provde further insight into how the conformational property correlates to biological activity at these positions.

Acknowledgments

We thank Jackie Lelito and Robert Young for excellent technical assistance and Dr. Mac E. Hadley, Department of General Biology, University of Arizona, for the milk-ejecting assay. We especially thank Dr. Ray Salemme, Department of Biochemistry, University of Arizona, for use of his facilities for evaluation of peptide and protein conformations.

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Conformations of Synthetic Alamethicin Fragments. Evidence for 3_{10} Helical Folding from 270-MHz Hydrogen-1 Nuclear Magnetic Resonance and Circular Dichroism Studies†

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ABSTRACT: ^{1}H NMR studies at 270 MHz on the synthetic alamethicin fragments Z-Aib-Pro-Aib-Ala-Aib-Ala-OMe (1–6), Boc-Gln-Aib-Val-Aib-Gly-Leu-Aib-OMe (7–13), Boc-Leu-Aib-Pro-Val-Aib-OMe (12–16), and Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (11–16) have been carried out in $CDCl_3$ and $(CD_3)_2SO$. The intramolecularly hydrogen bonded amide hydrogens in these peptides have been delineated by using solvent titration experiments and temperature coefficients of NH chemical shifts in $(CD_3)_2SO$. All the peptides adopt highly folded structures, characterized by intramolecular 4 → 1 hydrogen bonds. The 1–6 fragment adopts a 3_{10} helical conformation with four hydrogen bonds, in agreement with earlier studies (Rao, Ch. P., Nagaraj, R., Rao, C. N. R., & Balaram, P. (1980) *Biochemistry 19*, 425–431]. The 7–13 fragment also appears to be folded in 3_{10} helical fashion, although some intramolecular hydrogen bonds are loosened in $(CD_3)_2SO$. The 11–16 fragment favors a structure with three intramolecular hydrogen bonds of the 4 → 1 and 5 → 1 types. CD studies in trifluoroethanol suggest a helical structure for the 1–13 and 1–17 fragments and alamethicin, while IR studies support a helical structure for the 1–13 peptide, stabilized by intramolecular hydrogen bonding. On the basis of fragment conformations and earlier studies of the stereochemistry of α-aminoisobutyric acid (Aib) containing peptides, a structure is suggested for the alamethicin backbone. A largely 3_{10} helical folding pattern is postulated for the hydrophobic 1–17 segment, with a polar flexible C-terminal tripeptide.

Cation transport across membranes may be mediated by a carrier mechanism involving lipophilic organic ligands capable of chelating metal ions or by formation of transmembrane channels or pores (Ovchinnikov et al., 1974; Pressman, 1976). While considerable effort has been expended on structural studies of carrier ionophores and their metal complexes, relatively little is known about the structure of membrane channels. The polypeptides gramicidin A (Sarges & Witkop, 1964) and alamethicin (Pandey et al., 1977a) are substances that affect the ionic permeabilities of membranes by formation of transmembrane structures (Urry, 1977). Of these, alamethicin also has the ability to induce excitability phenomena in artifical membranes (Mueller & Rudin, 1968). Structural models like the novel π_{LD} (Urry, 1971; Urry et al., 1971) and

† From the Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India. *Received September 24, 1980.* This work has been supported by grants from the University Grants Commission and the Department of Science and Technology. R.N. was a recipient of a fellowship from the Council of Scientific and Industrial Research, India.

0006-2960/81/0420-2828$01.25/0