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Relative conformational rigidity in oxytocin and [1-penicillamine]-oxytocin: A proposal for the relationship of conformational flexibility to peptide hormone agonism and antagonism

(nuclear magnetic resonance/hormone receptor/structure-function studies/hormone inhibitor)

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ABSTRACT **A comparative study of the proton and carbon-13 nuclear magnetic resonance spectral parameters of the peptide hormone oxytocin and of its competitive inhibitor [1-L-penicillamine]oxytocin has been made, and the results analyzed in terms of comparative conformational and dynamic properties. The results indicate that oxytocin has a flexible conformation, while [1-L-penicillamine]oxytocin has a more restricted conformation. The results provide a framework for understanding the mechanism of peptide hormone agonism and antagonism for these compounds, and an approach for understanding some features of the interaction of the hormone and related compounds with their receptor.**

Nuclear magnetic resonance (NMR) spectroscopy has been widely used to investigate the three-dimensional structure of small peptides in solution (1). The mean conformation obtained is generally an average of a manifold of conformations, and the question arises of the application of these findings to the biological activity for these compounds. Even if the hypothesis that the native conformation of a protein is the one of lowest free energy (2) is accepted, this cannot necessarily be extrapolated to the case of small peptides with biological activity (3, 4). From NMR and other conformational studies, information regarding the *biologically active* conformation(s) cannot generally be obtained in an absolute manner unless the following conditions can be met: (*a*) the peptide is flexible in solution and criteria can be established for a direct unequivocal relationship between the mean conformation in solution and that adapted by the hormone at the receptor, or (*b*) the conformation is so rigid that we can assume it will not be significantly modified by interaction with the receptor.

The problem encountered in (*a*) is extremely complicated because we have very little knowledge of hormone receptors, of peptide hormone interactions with receptors, or of the physical-chemical basis for the mechanism of hormone action. Nonetheless some approaches have been suggested (ref. 5 and refs. therein) to define the relationship mentioned in (*a*). To our knowledge, the situation under point (*b*) has never been demonstrated experimentally for small peptide hormones. It was suggested some time ago (6), and there is increasing evidence (1, 7–9) that small peptide hormones do not have a rigid solution conformation.

In this paper, we report on a comparative conformational study of the peptide hormone oxytocin, Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2, and [1-L-penicillamine]oxytocin, S-$C(CH_3)_2CH(NH_2)CO$-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 ([Pen^1]oxytocin), a competitive inhibitor of oxytocin (ref. 10 and refs. therein), in aqueous solution. From these studies we suggest that the conformational flexibility of oxytocin is an important property for its agonist activity at the receptor, and that the antagonist activity of [Pen^1]oxytocin is related to its *relative* coformational rigidity. We also briefly discuss the effect of relative rigidity on the binding kinetics of the antagonist and its consequence in an *in vitro* bioassay.

MATERIALS AND METHODS

Oxytocin and [Pen^1]oxytocin were synthesized and purified by standard procedures (10). The compounds have identical proton NMR spectra (7, 12) and biological properties as previously reported. Carbon-13 and T_1 measurements were made at Arizona on a Bruker WH-90 FT spectrometer. T_1 data were acquired using a $180°-\tau-90°-t_\infty$ pulse sequence, where t_∞ is at least five times the longest T_1. The experimental error is ±10–15%. The high-resolution proton NMR spectra were obtained on Varian Associates HR-300 or Bruker HX-270 NMR spectrometers. For the proton NMR experiments, concentrations were 5 mg/ml. A drop of t-butan[^{2}H]ol was used as internal reference.

RESULTS

The results and conclusions presented here were made using aqueous (H_2O, D_2O) solutions of oxytocin and [Pen^1]oxytocin. A similar study was also attempted using dimethylsulfoxide solutions, but could not be readily done or interpreted due to a molecular association or interaction of these compounds in this solvent (12, 13).

A 300 MHz proton NMR spectrum of [Pen^1]oxytocin in H_2O (D_2O) is shown in Fig. 1 along with the assignments as determined by double resonance and other experiments as previously described for oxytocin (7). Tables 1 and 2 summarize the chemical shift and coupling constant [$J_{NH\text{-}\alpha CH}$, $J_{\alpha CH\text{-}\beta CH}(J_{\alpha\beta})$, $J_{\alpha CH\text{-}\alpha' CH}(J_{\alpha\alpha'})$, and $J_{\beta CH\text{-}\beta' CH}(J_{\beta\beta'})$] parameters for oxytocin and [Pen^1]oxytocin used in a partial computer simulation (12) of the spectra. The temperature dependnce of the peptide NH protons in [Pen^1]oxytocin (12) and oxytocin (7) were determined, and are the same as previously reported. The fractional populations $p(a)$, $p(b)$, $p(c)$ of rotational isomers around the C_α–C_β bonds in oxytocin and [Pen^1]oxytocin were calculated from data in Tables 1 and 2 by standard procedures (14), and the results are reported in Table 3.

The ^{13}C NMR spectrum of [Pen^1]oxytocin in D_2O was obtained and assigned (Fig. 2); the aromatic carbons(not shown) were also assigned. The assignments were made by comparison of the spectrum of [Pen^1]oxytocin with oxytocin (15), [Pen^1, Leu^2]oxytocin, and several deuterated derivatives of oxytocin (V. J. Hruby, J.-P. Meraldi, D. Yamamoto, D. Upson, and K. K. Deb, unpublished data). The empirical rules for chemical

Abbreviations: NMR, nuclear magnetic resonance; Pen, penicillamine; β-Mpa, β-mercaptopropanoic acid.

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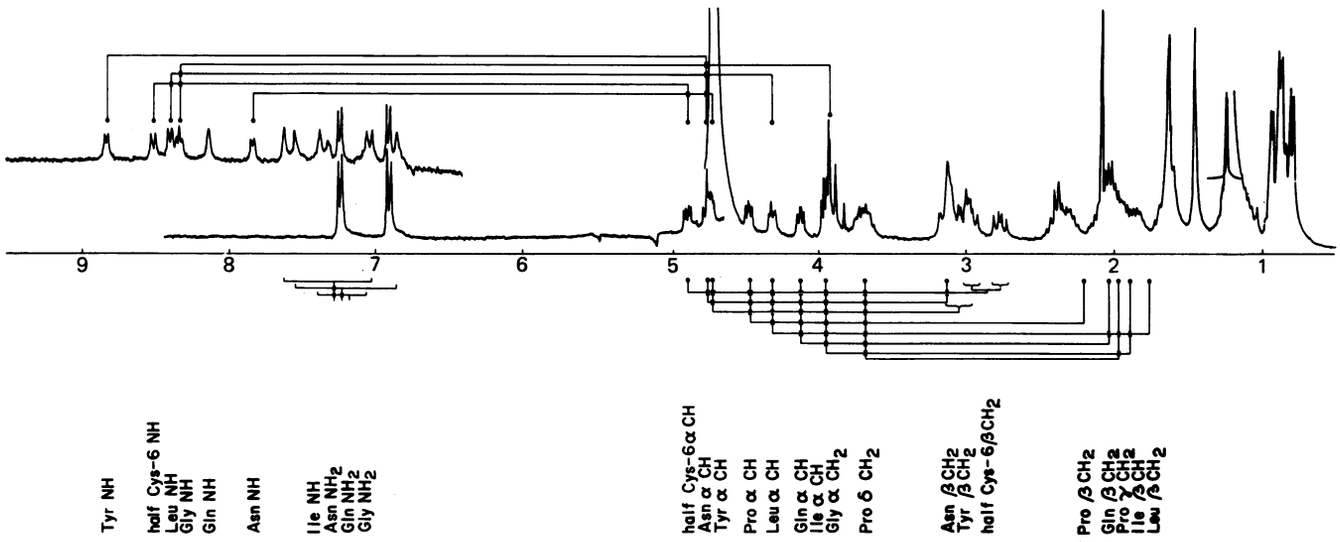

FIG. 1. Composite 300 MHz NMR spectrum of [Pen[1]]oxytocin in H_2O (D_2O) at pH 3.8, 28°C. The upper spectrum is in H_2O, the lower spectrum in D_2O.

shifts of alkanes (16) were used to assign the Pen-1 carbon resonances. The NT_1 values of individual carbon atoms in [Pen[1]]-oxytocin are given in Fig. 3 for a 7.8×10^{-2} M solution.

DISCUSSION

Interpretation of the NMR Results. From previous NMR studies in aqueous solution (7, 12, 17–19), it is known that oxytocin has a flexible conformation, and potential energy calculations support this conclusion (20–22). Obviously the condition of ring closure requires certain restrictions in the geometrical conformations allowed for the oxytocin disulfide-containing 20-membered ring relative to a random coil peptide. However, if the molecule can occupy most of the remaining conformational space, the resulting coupling constants should be similar to those for a random coil (6.5 ± 1 Hz; refs. 23 and 24). Considering the $J_{NH\text{-}\alpha CH}$ values of the cyclic portion of oxytocin in Table 1 and keeping in mind the above considerations, the values are quite representative of a random coil. On the other hand, the same parameters for [Pen[1]]oxytocin (Table 2) are often characterized by more extreme values (<4 Hz, >8 Hz), suggesting a more well-defined conformation and thus a more rigid structure than for oxytocin. In addition, the coupling constants of [Pen[1]]oxytocin remain unchanged between 20°C and 80°C; in oxytocin changes are seen, particularly in the cystine residue. This is consistent with circular dichroism data, which indicate considerable internal motion for the cystine residue of oxytocin (ref. 25 and refs. therein).

Both the Ile-3 and the Asn-5 peptide amide protons of [Pen[1]]oxytocin have a near zero temperature dependence of their chemical shifts (12). This indicates that these two protons are shielded from solvent over a wide temperature range and could be involved in intramolecular hydrogen bonding (26). Whether these protons are hydrogen bonded or "buried" in the molecule, these results present further evidence for a degree of rigidity in the conformation since, in a small peptide like [Pen[1]]oxytocin, no proton is far from solvent and if the ring moiety is highly flexible, every amide proton would be in contact with solvent some of the time.

In Table 3 we summarize the results of a comparison of the side-chain rotamer populations about the C_α–C_β bond in various residues of [Pen[1]]oxytocin and oxytocin. We are aware that the choice of values of J_t and J_g in our calculations is somewhat arbitrary (see, for example, ref. 27). However, since we are primarily interested in a comparison of various residues in the two peptides and the same relative values would be obtained using other values, the procedure used here is justified. Examination of the data shows that the side chains of Leu-8, Gln-4, and especially Tyr-2 are more conformationally restricted in [Pen[1]]oxytocin than in oxytocin. These conformational features further support the suggestion that the 20-membered ring of [Pen[1]]oxytocin is less flexible than in oxytocin.

The ^{13}C spin-lattice relaxation times (T_1) of [Pen[1]]oxytocin (Fig. 3) are qualitatively similar to those reported for oxytocin (28), but a direct comparison is difficult owing to the difference in the concentration and viscosity of the solution (13). The T_1 values of the α-carbon atoms in the ring moiety of oxytocin and [Pen[1]]oxytocin are similar. However, due to the ring closure condition, which should be considered in the derivation of the correlation functions, we cannot describe this part of the molecules in terms of relative internal motions of the backbone as is done for a linear chain (29). A more useful comparison is that of some of the T_1 values of the α-carbon atoms relative to the side-chain carbon atoms. Such a comparison can provide us with a measure of the flexibility of the side chains relative to the backbone for the two molecules. In this regard, the T_1 measurements confirm a more restricted motion for the side chain of the Gln-4 residue in [Pen[1]]oxytocin than in oxytocin. Unfortunately, it was not possible to accurately determine the T_1 value of the β-carbon of the Tyr-2 residue in [Pen[1]]oxytocin. Our primary purpose in measuring the T_1 values of [Pen[1]]-oxytocin was to determine qualitatively if the COOH-terminal acyclic tripeptide (tail) moiety is experiencing more mobility than the ring moiety, as was shown for oxytocin (18, 28). As can be seen in Fig. 3, the T_1 values of the α-carbon atoms increase in going from the NH_2 to the COOH-terminus of the a cyclic tripeptide portion of [Pen[1]]oxytocin, as found in oxytocin (28), though much less dramatically. A comparison of the ratio of the T_1 values of Leu-8 and glycinamide-9 α-carbon atoms to those of the α-carbon atoms of the 20-membered rings suggest the tail is somewhat more restricted in its motion in [Pen[1]]oxytocin than in oxytocin. However, the time scale of this restriction appears to be too short to be noticed in the proton NMR spectra. A conformation for the ring portion of [Pen[1]]oxytocin has been suggested (12) and the results reported here are consistent with this.

Table 1. Chemical shifts and coupling constants for protons of oxytocin used in a computer simulation of the NMR spectrum

Residue no.	Proton δ (ppm)	J_{NH}-αCH	Coupling constants $J_{\alpha\beta}$	$J_{\alpha\beta'}$	$J_{\beta\beta'}$
Half Cys-1	δ_{NH} = —		3.8	6.0	15.0
	δ_α = 4.30				
	δ_β = 3.31, 3.49				
Tyr-2	δ_{NH} = 8.99	6.4			
	δ_α = 4.79		8.1	6.2	14.0
	δ_β = 3.02, 3.17				
	δ_δ = 6.8				
	δ_ϵ = 7.2				
Ile-3	δ_{NH} = 7.96	6.0			
	δ_α = 4.04				
	δ_β = 1.9		6.0		
Gln-4	δ_{NH} = 8.22				
	δ_α = 4.12	4.0			
	δ_β = 2.05		6.6	6.8	
	δ_γ = 2.4				
Asn-5	δ_{NH} = 8.34	8.0			
	δ_α = 4.74				
	δ_β = 2.86, 2.86		7.5	7.5	
Half Cys-6	δ_{NH} = 8.21	6.5			
	δ_α = 4.89				
	δ_β = 2.98, 3.24		9.6	3.0	15.9
Pro-7	δ_{NH} = —				
	δ_α = 4.6				
	δ_β = 2.2		6.5	5.0	
	δ_γ = 2.02				
	δ_δ = 3.73				
Leu-8	δ_{NH} = 8.45	6.0			
	δ_α = 4.32		8.6	4.4	
	δ_β = 1.65				
Gly-9	δ_{NH} = 8.36	6.0			
	δ_α = 3.87, 3.94	4.5	$J_{\alpha\alpha'}$ = 17.0		

Table 2. Chemical shifts and coupling constants for protons of [Pen¹]oxytocin used in a computer simulation of the NMR spectrum

Residue no.	Proton δ (ppm)	J_{NH}-αCH	Coupling constants $J_{\alpha\beta}$	$J_{\alpha\beta'}$	$J_{\beta\beta'}$
Half Pen-1	δ_{NH} = —				
	δ_α = 3.92				
	δ_γ = 1.62, 1.44				
Tyr-2	δ_{NH} = 8.82	6.2			
	δ_α = 4.74		10.0	3.1	14.7
	δ_β = 2.95, 3.13				
	δ_δ = 6.9				
	δ_ϵ = 7.16				
Ile-3	δ_{NH} = 7.33	3.0			
	δ_α = 3.94		5.8		
	δ_β = 1.86				
Gln-4	δ_{NH} = 8.14	2.0			
	δ_α = 4.11		8.4	5.2	
	δ_β = 2.01				
	δ_γ = 2.4				
Asn-5	δ_{NH} = 7.84	6.0			
	δ_α = 4.76		7.5	7.3	14.5
	δ_β = 3.08, 3.11				
Half Cys-6	δ_{NH} = 8.51	9.0	10.0	4.0	15.8
	δ_α = 4.88				
	δ_β = 2.75, 3.0				
Pro-7	δ_{NH} = —				
	δ_α = 4.46				
	δ_β = 2.2		8.4	5.2	
	δ_γ = 2.0				
	δ_δ = 3.70				
Leu-8	δ_{NH} = 8.39	5.8			
	δ_α = 4.3		9.6	3.6	
	δ_β = 1.7				
Gly-9	δ_{NH} = 8.33	6.2			
	δ_α = 3.85, 3.93	5.2	$J_{\alpha\alpha'}$ = 16.8		

Relationship of Conformational Features to Biological Activities. From the biological point of view, there is a difference between oxytocin and [Pen¹]oxytocin: the former is an agonist, the latter an antagonist. From the conformational point of view, the results reported here suggest a change from a flexible conformation for oxytocin to a somewhat more rigid conformation for [Pen¹]oxytocin. As discussed above, it is very difficult to arrive at the biologically active conformation (the conformation at the receptor) for a flexible molecule. However, as discussed by Burgen *et al.* (ref. 30 and refs. therein), simple thermodynamic considerations lead us to deduce that the binding of oxytocin to the receptor occurs in a stepwise manner. The reaction coordinate for the binding of oxytocin to the receptor can be viewed as a series of binding steps, where the initial state corresponds to the free ligand and free receptor, all intermediate states correspond to various binding steps of the hormone to the receptor of a biologically inactive state, and the concluding step corresponds to the activation of the hormone-receptor complex to its *biologically active state*. On the other hand, though the antagonist [Pen¹]oxytocin is somewhat less flexible than oxytocin, it can interact with the receptor in a similar manner but with a very important difference: the final state (activated hormone-receptor complex) is not obtained. We propose that some element of intrinsic rigidity in [Pen¹]oxytocin presents an energy barrier that prevents the formation of the biologically active form of the receptor necessary for transduction of the hormone message and, hence, inhibitory activity is manifested. In summary, without knowing the absolute active conformations of oxytocin and [Pen¹]oxytocin, we can explain the mechanism of inhibition using a *comparison* of the conformational features of these peptides and can deduce that conformational flexibility probably is an important feature for biological activity in this hormone as in other small organic compounds (31–33). We further predict that for small peptide hormones, an analog designed to reduce the flexibility of the hormone has a high probability of either reduced agonist bio-

Table 3. Rotamer populations of some side-chain residues in [Pen¹]oxytocin and oxytocin

Residue	[Pen¹]oxytocin $p(a)$*	$p(b)$*	$p(c)$*	Oxytocin $p(a)$*	$p(b)$*	$p(c)$*
Tyr-2	0.28	0.67	0.05	0.17	0.50	0.32
Gln-4	0.22	0.53	0.23	0.26	0.36	0.38
Asn-5	0.14	0.44	0.42	0.12	0.44	0.44
Leu-8	0.26	0.63	0.11	0.30	0.54	0.16

* $\chi^1_a = +60°$, $\chi^1_b = -60°$, $\chi^1_c = \pm 180°$.

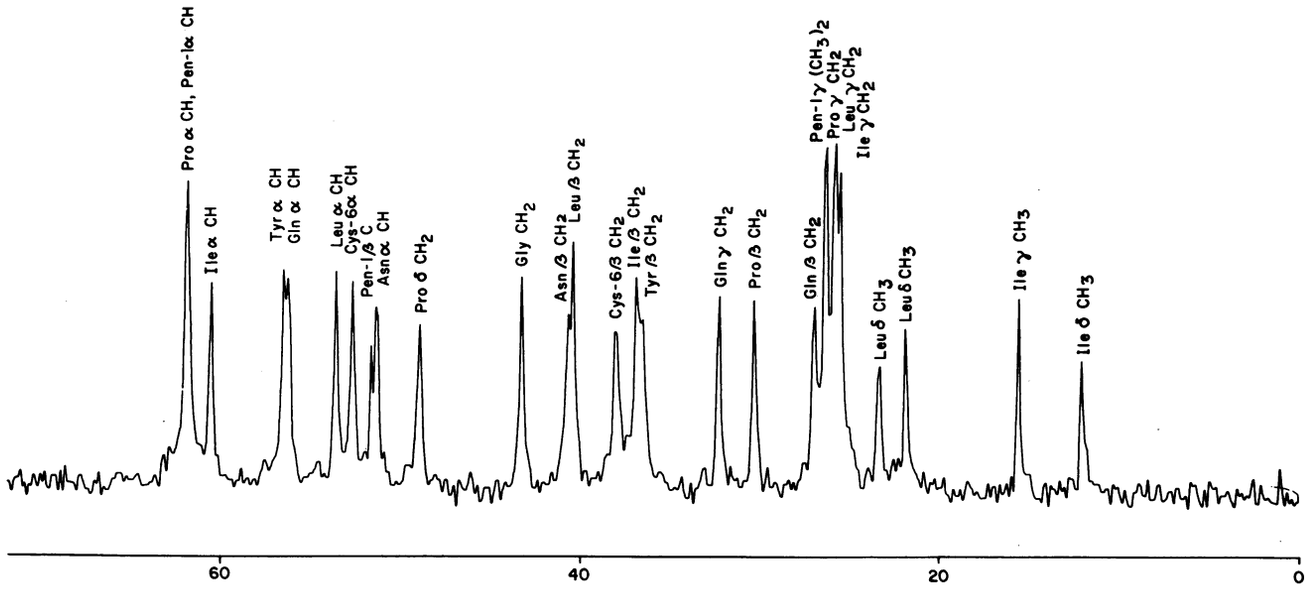

FIG. 2. ^{13}C NMR spectrum of the aliphatic portion of [Pen^1]oxytocin in D_2O at pH 3.8, 28°C.

logical activity or, under favorable circumstances, hormone antagonist activity.

An alternative explanation for the inhibitor activity of [Pen^1]oxytocin might be suggested, namely, that replacement of the half cystine-1 residue by penicillamine results in a nonspecific binding of the hormone analog which cannot lead to the active state. Several experimental facts argue against this, however, and suggest a selective binding of the antogonist to the receptor similar to that of oxytocin. (*i*) Deaminooxytocin ([1-β-mercaptopropanoic acid]oxytocin, [1-β-Mpa]oxytocin) is more active than oxytocin (oxytocic, avian vasodepressor, and milk-ejecting assays), and this is at least in part due to a better binding to the receptor (34). In a similar manner, [1-deaminopenicillamine]oxytocin ([1-β-Mpa(β-Me_2)]oxytocin) is a better inhibitor than [Pen^1]oxytocin (35) and thus parallels the behavior of the agonist. It also confirms a slight repulsive interaction of the N-terminal amino group with the receptor of the hormone. (*ii*) [1-β-Mercapto-ββ-diethylpropanoic acid]-oxytocin ([1-β-Mpa(β-Et_2)]oxytocin) is a more potent inhibitor than [Pen^1]oxytocin. Its derivative, [1-β-Mpa(β-Et_2), 4-Leu]-oxytocin, has an inhibitory activity (36) relative to [Pen^1]oxytocin which closely parallels the agonist activity of [1-β-Mpa, 4-Leu]oxytocin (37) relative to deamino-oxytocin.

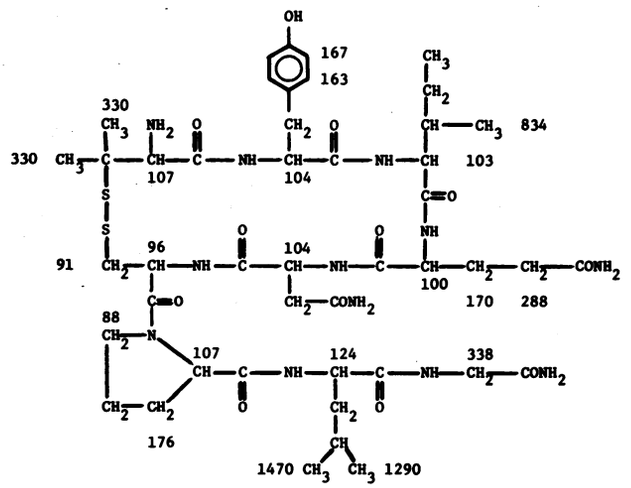

FIG. 3. NT_1 values of the resolved carbons of [Pen^1]oxytocin (7.8×10^{-2} M) in D_2O at pH 3.8, 28°C.

Disulfide Bridge and Biological Activity. By increasing the size of the substituents on the β-carbon of the 1 position in oxytocin, derivatives showed an increased inhibitory potency (36, 38), with [1-β-Mpa(β-$(CH_2)_5$)]oxytocin (I) more potent than [1-β-Mpa(β-Et_2)]oxytocin (II), which was more potent than [1-β-Mpa(β-Me_2)]oxytocin (III). It is likely that compounds I, II, and III have similar conformational and dynamical features as [Pen^1]oxytocin (IV), and hence also act as inhibitors via the same mechanism. Based on the bioassay results, it is clear that the increase in inhibitory activity is a result of a better binding of the antagonist to the receptor. A common feature of the antagonists I–IV is that they all have an aliphatic steric perturbation in the vicinity of the disulfide bridge. We conclude that these substituted groups act as a screening factor between the disulfide bridge and the receptor. In the first three cases, the size of these groups increase in the order I > II > III, corresponding to a better screening effect, increased binding, and increased inhibitory activity. The decreased inhibitory activity of IV relative to III has already been explained by the repulsive contribution of the α-amino group. We thus suggest that the disulfide bridge, or at least the sulfur of the half cystine-1 in oxytocin and derivatives, may have a slightly repulsive contribution on interaction of the hormone with the receptor much as the α-amino group does. This suggestion is further supported by the generally higher activities of the carba-analogues of oxytocin (ref. 39 and refs. therein) and can help explain the results of the bioassay of these compounds.

Some Aspects of Kinetics of Binding of Hormone to Receptor. We offer now a brief explanation for the correlation between kinetics of binding and the relative rigidity of the hormone and its derivatives. We first assume, as already discussed, that inhibitors such as [Pen^1]oxytocin, despite their increased rigidity relative to the hormone, still are sufficiently flexible to bind to the receptor in a stepwise manner. After the initial step in the binding, each or many of the additional steps in the binding process will be more difficult because the intrinsic rigidity of the molecule increases the energy barrier between each step. Under the same assay conditions, therefore, the overall rate of binding will be decreased. In other words,

the restriction in conformations easily available to the more rigid molecule will reduce the probability of selecting the proper conformation for the next step in the binding. The origin of this restriction could be steric, electronic, or dynamic, but the net effect would be the same, to decrease the rate of binding and thus increase the time to equilibrium. This possible difference in the kinetics of binding of hormone and inhibitor should be considered in the design of the bioassay to insure the determination of the true equilibrium inhibitory potency of the inhibitor.

CONCLUSIONS

In aqueous solution, the peptide hormone, oxytocin, has a relative flexible conformation, while the hormone antagonist [Pen^1]oxytocin appears to be somewhat more rigid in both its backbone and in certain side-chain residue conformations. The inhibitor activity of [Pen^1]oxytocin can be related to the relative rigidity of the molecule, and the results indirectly emphasize the biological importance of flexibility in a small peptide hormone such as oxytocin. The results are exciting in that they provide further understanding of the mechanism of hormone action and also suggest a useful approach to the design of hormone antagonists.

However, we still can say little regarding the biologically active conformation of the hormone or hormone antagonist because we know little regarding the nature of the interaction of the hormone with the receptor and the effect of this interaction on the hormone's conformation. A complete understanding of the conformational energy map of [Pen^1]oxytocin relative to oxytocin might provide valuable clues, but the present difficulty of such an approach is great. Alternative approaches are needed, such as studies of the dynamics and conformational effects of hormone agonist and antagonist binding to biologically significant macromolecules, including purified receptors. In the meantime, comparative studies of the type reported here can provide useful clues to the design of better hormone agonists and antagonists.

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