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Proton n.m.r. spectroscopic evidence for sulfur-aromatic interactions in peptides

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The downfield shift of the tyrosyl proton resonances and an increased chemical shift difference between the resonances for the 2′,6′ and 3′,5′ hydrogens in a series of deamino-oxytocin analogs modified in the disulfide bridge provide evidence for aromatic-sulfur interactions in d_6-dimethylsulfoxide solutions.

Key words: carba analogs; deamino-oxytocin; sulfur-aromatic interactions

Sulfur-aromatic interactions have been observed in several crystal structures of small proteins (1–4) and are thought to be important for the stabilization of secondary and tertiary structure (5, 6). The enthalpy for sulfur-aromatic interactions has been calculated to be 3–5 times greater (0.7 to 1.0 kcal/mol) than the estimates for van der Waals' forces alone (5, 6). The interaction of the disulfide bridge of oxytocin (Cys-Tyr-Ile-Asn-Gln-Cys-Pro-Leu-Gly-NH_2, OT) with Tyr^2 was first suggested (7) based on circular dichroic studies of deamino-oxytocin derivatives containing single or double replacements of the sulfur atoms by a methylene group (carba-1-, carba-6- and dicarba-OT). Replacement of sulfur by a methylene group does not appear to influence the overall peptide backbone conformation because there is no observable change in the amide regions of the CD spectra. However, in the absence of sulfur at position 6 (deamino-carba-6-OT, deamino-dicarba-OT), the tyrosine n-π^* band is shifted to shorter wavelengths as compared to the CD spectra of OT analogs with sulfur at position 6 (7).

More recently, time-resolved fluorescence studies were used to compare the decay kinetics of tyrosine fluorescence in the presence (OT, deamino-OT) or absence (deamino-dicarba-OT) of the disulfide bridge (8). In addition to demonstrating that the tyrosyl fluorescence is quenched by the disulfide bridge, the authors analyzed the decay kinetics of this process and concluded that only the gauche(-) side chain rotamer of tyrosine was able to interact with the disulfide bridge.

Other evidence for the interaction of sulfur

Abbreviations: All optically active amino acids are of the L configuration unless otherwise noted. Symbols and abbreviations are in accord with recommendations of the IUPAC-IUB Joint Commission of Biochemical Nomenclature (*European J. Biochem.* (1984) 138, 9–37). Other abbreviations include: OT, oxytocin; deamino-oxytocin, [1-β-mercaptopropionic acid] oxytocin; HPLC, high performance liquid chromatography; n.m.r., nuclear magnetic resonance; CD, circular dichroism; TSP, sodium trimethylsilyltetradeuteriopropionate; TFA, trifluoroacetyl; Dmp, deaminopenicillamine, β, β-dimethyl-β-mercaptopropionic acid; Maa, mercaptoacetic acid; Cha, β-cyclohexylalanine; Apim, α-aminopimelic acid.

in position 6 with tyrosine may be found in the chromatographic behavior of these derivatives (9). Greatly reduced reversed-phase HPLC retention times are observed for derivatives of deamino- and deamino-carba-1-OT as compared with deamino-carba-6-OT and deamino-dicarba-OT derivatives. This suggests that the sulfur-aromatic interaction makes the aromatic residue less accessible for interaction with the stationary phase. Furthermore, the sulfur at position 1 in deamino-carbo-6-OT is more readily oxidized than the sulfur at position 6 of deamino-carba-1-OT (10).

Proton n.m.r. spectroscopy is a valuable technique for conformational studies of peptides (11). The introduction of modern instrumentation and new pulse sequences makes this method comparable to X-ray crystallography, especially when through-space atomic interactions are observed. Since the interaction of sulfur with the tyrosyl aromatic ring might result in changes of the chemical shifts of the aromatic proton resonances, we decided to examine the spectra of a series of modified deamino-OT derivatives. In addition to the carba analogs previously discussed, we include in this study analogs in which L-Tyr^2 is replaced by D-Tyr^2, and in which the 20-membered ring is reduced, or contains various substitutions in the peptide chain.

MATERIALS AND METHODS

Analogs used in this study have been previously described in the literature (for references see Table 1). They were synthesized *de novo* (analogs I–III), or were obtained from Dr. K. Jost (analogs IV–VI). Deamino-carba-6-tocinoic

TABLE 1

Chemical shifts for the aromatic protons of Tyr^2 in deamino-OT derivatives in DMSO

Compound[b]	Chemical shift, p.p.m.[a] 2′, 6′	3′, 5′	Δ[c]	Ref.[d]
TFA-Gly-Gly-Tyr-Ala-OMe	7.041	6.661	0.380	12
OT	7.150	6.710	0.440	13
Deamino-OT (I)	7.126	6.692	0.434	14
Deamino-carba-1-OT (II)	7.115	6.705	0.410	15
Deamino-carba-6-OT (III)	7.047	6.670	0.377	16
Deamino-dicarba-OT (IV)	7.046	6.672	0.374	16, 17
Deamino-carba-1-OT sulfoxide (V)	7.064	6.679	0.386	18
[D-Tyr^2]deamino-OT (VI)	7.033	6.639	0.394	19
[D-Tyr^2]deamino-carba-1-OT (VII)	7.029	6.644	0.385	20
[D-Tyr^2]deamino-carba-6-OT (VIII)	7.026	6.635	0.391	20
[Dmp^1]carba-6-OT (IX)	7.038	6.644	0.394	21
[Dmp^1]OT (X)	7.102	6.677	0.425	22
[Thr^4]deamino-carba-1-OT (XI)	7.117	6.708	0.409	23
[Thr^4]deamino-carba-6-OT (XII)	7.058	6.671	0.387	23
[Glu^4]deamino-carba-1-OT (XIII)	7.114	6.693	0.421	24
[Cha^3]deamino-OT (XIV)	7.110	6.689	0.421	Roy, J. (unpublished communication)
Deamino-carba-6-tocinoic acid (XV)	7.039	6.665	0.376	–
[Cys(C_2H_4CO)^{6,1}]OT (XVI)	7.060	6.692	0.368	25
[Apim^{6,1}]OT (XVII)	7.056	6.678	0.378	25
[Maa^1]OT (XVIII)	7.127	6.695	0.432	26

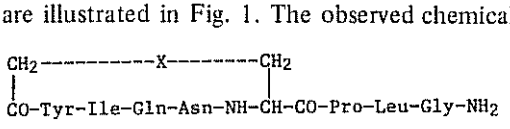
[a]Referenced to TSP.
[b]OT, oxytocin; Dmp, deaminopenicillamine; β, β-dimethyl-β-mercaptopropionic acid; Cha, beta-cyclohexyl-alanine; Apim, aminopimelic acid; Maa, mercaptoacetic acid.
[c]Chemical shift difference between the aromatic signals.
[d]Reference is to paper reporting synthesis of the peptide.

acid was synthesized by solid phase methodology using Fmoc protection for Nα-amino groups and cyclization on the resin (27) using diphenylphosphorylazide.

Proton n.m.r. spectra were recorded on Bruker WM-250 and AM-250 spectrometers in d_6-dimethylsulfoxide or in a $D_2O/H_2O/CD_3$-COOD (5:3:2) mixture using sodium 3-trimethylsilyl-tetradeuteriopropionate (TSP) as an internal standard.

RESULTS

The structures of the analogs used in this study are illustrated in Fig. 1. The observed chemical



I, X = CH_2-S-S

II, X = CH_2-CH_2-S

III, X = CH_2-S-CH_2

IV, X = CH_2-CH_2-CH_2

V, X = CH_2-CH_2-SO

IX, X = $C(CH_3)_2$-S-CH_2

X, X = $C(CH_3)_2$-S-S

XVI, X = CH_2-S

XVII, X = CH_2-CH_2

XVIII, X = S-S

FIGURE 1

Structure of some of the analogs examined in this study.

shifts for the tyrosine protons are listed in Table 1. A reference value for unperturbed tyrosine aromatic protons is provided by the tetrapeptide TFA-Gly-Gly-Tyr-Ala-OMe (12). Our analysis utilized four different series of compounds: (a) compounds maintaining the 20-membered ring of oxytocin, but differing in the position of sulfur (I–IV); (b) compounds in which the stereochemistry of Tyr^2 is changed (VI–VIII); (c) compounds modified at other positions in oxytocin (IX–XV); and (d) compounds in which the ring size is reduced (XVI–XVIII).

The chemical shifts for the tyrosyl aromatic protons in the presence (I) of a disulfide bond are about + 0.08 p.p.m. downfield compared with the tyrosyl resonances in the dicarba derivative (IV), in which the disulfide group (S-S) is replaced by an ethylene (CH_2CH_2) group. The sulfur-tyrosyl interaction can be specifically assigned to the Cys^6 sulfur, since the resonances of the tyrosyl protons in the carba-1 analog (II) are + 0.06 p.p.m. downfield, while these resonances are normal in the carba-6 analog (III). The downfield shift is greatest for disulfide derivatives and the 2′,6′-protons of tyrosine are more strongly influenced than the 3′,5′-protons, resulting in an increased chemical shift difference between the resonances for these two sets of protons (Table 1). As might be expected, oxidation of the Cys^6 sulfur (deamino-carba-1-OT sulfoxide, V) eliminates this interaction and the observed Tyr^2 proton resonances are normal in V.

In three analogs containing D-Tyr^2 (VI–VIII), no change is observed for the chemical shifts of the 2′,6′-proton resonances regardless of the presence (VI, VII) or absence (VIII) of sulfur at position 6. In these compounds, the tyrosine residue cannot interact with the bridge without a dramatic change in the peptide backbone. Interestingly, the 3′,5′-protons are shifted slightly upfield in all of these analogs. This same upfield shift of the resonances for the 3′,5′-hydrogens is also observed in [Dmp^1]-carba-6-OT (IX). The substitution of penicillamine at position 1 is reported (28, 29) to decrease the conformational freedom of the tyrosyl side chain and steric constraints preclude the conformation that would place the aromatic group over the 20-membered ring

(28–30). However, the n.m.r. spectrum of the [Dmp^1]OT([1-β,β-dimethyl-β-mercaptopropionic acid]oxytocin (X)) shows that there might be an interaction of sulfur with tyrosine based on the increased difference between the aromatic proton signals. The upfield shift of both aromatic proton resonances of [Dmp^1]-OT (X) in comparison to deamino-oxytocin (I) might be caused by the sterically demanding methyl groups. A similar effect was observed in the pair of analogs III and IX, even though there is no sulfur-aromatic interaction.

Modification of other residues in the peptide sequence do not change the observed pattern of a downfield shift of the tyrosine 2′,6′-proton resonances whenever sulfur is present in position 6. The *N*-terminal amino group in oxytocin (OT) does not attenuate the downfield shift of the tyrosyl 2′,6′-protons, but rather increases it. Substitution of a bulky amino acid derivative at position 3 (XIV) or replacement of Gln^4 by Thr^4 (XI, XII) or Glu^4 (XIII) does not influence the observed pattern, nor does deletion of the *C*-terminal tripeptide (XV).

In analogs with a diminished ring size (XVI – XVIII), we observed the downfield shift of the tyrosine 2′,6′-protons only for [Maa^1]OT (XVIII). A sulfur-aromatic interaction was suggested (7) for analog XVI in which one of the sulfur atoms is deleted. We did not observe any significant change in the chemical shifts of the tyrosyl protons of either XVI or XVII (Table 1). Careful examination of the CD data (7) shows that the strong positive band above 240 nm observed at pH 12 cannot be attributed to a sulfur-aromatic interaction since it also is observed in the CD spectra of compound XVII, which has the same ring size but contains no sulfur. Therefore, the case for a sulfur-aromatic interaction in compound XVI is not so strongly supported by CD data as for analogs I and II.

TABLE 2

Chemical shift values for the tyrosyl protons of deamino-OT derivatives in D_2O

Compound	Chemical shift, p.p.m.[a] 2′,6′	3′,5′	Δ[b]
Deamino-OT (I)	7.155	6.832	0.323
Deamino-carba-1-OT (II)	7.173	6.848	0.325
Deamino-carba-6-OT (III)	7.156	6.834	0.322
Deamino-dicarba-OT (IV)	7.155	6.835	0.320

[a]Referenced to TSP.
[b]Chemical shift difference between the aromatic signals.

The observed differences in the chemical shifts for the 2′,6′-proton resonances were independent of concentration (3–30 mM/L). However, the downfield shift of these protons was not observed in aqueous solution (Table 2). This solvent dependency can be attributed to the increased conformational mobility of oxytocin in aqueous solution (31) compared to DMSO solution, where intramolecular hydrogen bond interactions are preserved (32, 33). This assumption is supported by the temperature dependencies of the aromatic proton resonances in dimethylsulfoxide solution (Table 3). In

TABLE 3

Temperature dependencies of the aromatic proton chemical shifts in DMSO

Compound	Temperature dependence (p.p.m. · deg^{-1} · 10^{-3})[a] 2′,6′	3′,5′
OT	−1.4[b]	0[b]
Deamino-OT (I)	−1.55	−0.35
Deamino-carba-1-OT (II)	−1.0	−0.48
Deamino-carba-6-OT (III)	−0.1	+0.1
Deamino-dicarba-OT (IV)	−0.2	−0.15
Deamino-carba-1-OT sulfoxide (V)	+0.04	−0.04

[a]All values are determined with the precision ± 0.07 × 10^{-3} p.p.m. · deg^{-1}.
[b]Taken from ref. 34.

those analogs for which a sulfur-aromatic interaction is proposed, the aromatic signals are significantly influenced by increased temperature. In analogs lacking this interaction, the aromatic signals are almost temperature-independent. These findings suggest that the interaction is weakened with increased molecular motion and around 80° sulfur-aromatic interactions no longer occur. At this temperature, the resonance signals of the aromatic protons are basically the same for all analogs.

DISCUSSION

We have observed a downfield shift of the 2′,6′-aromatic protons of the tyrosine-2 residue in analogs of oxytocin containing sulfur at position 6, suggesting a through-space interaction between sulfur and tyrosine. The greatest shift is for the protons *meta* to the phenol, suggesting that this interaction probably is not through hydrogen-bonding interactions of the tyrosine phenol with sulfur. Quantum mechanical calculations for the interaction of dimethyldisulfide with benzene have predicted that the lowest energy arrangement is one with the dimethyldisulfide molecule located above the plane of the benzene ring, with only one sulfur interacting with the π-electron shell (6). However, a recent X-ray examination of the geometry of aromatic-sulfur interactions in globular proteins suggests that the sulfur atoms preferentially orient toward the edge of the aromatic ring (4) and avoid the vicinity of the π-electrons. One could envision an attractive interaction between the negatively charged sulfur and the positively charged hydrogens of the aromatic ring. In the recently determined crystal structure of deamino-OT (35), the conformer with left-handed chirality of the disulfide fits our n.m.r. data, since the Tyr^2 side chain is available for interaction with the Cys^6 sulfur. Therefore, the conformation in dimethylsulfoxide solution might be similar to that observed in the crystal structure, especially for the analogs in which sulfur-aromatic interactions are suggested by the data in Table 1.

The preferential orientation of sulfur with tyrosine, and in these cases the biological activity of the "bioactive confirmation" of OT and this could be the reason for the higher *in vitro* biological activity of analogs containing sulfur in position 6 (deamino-carba-1-OT) compared to analogs with sulfur in position 1 (deamino-carba-6-OT)(36, 37). The *in vivo* biological activity of oxytocin carba-analogs is strongly influenced by oxidation to their respective sulfoxides, since the sulfoxide of deamino-carba-1-OT is 100 times less active than the sulfoxide of deamino-carba-6-OT (10). The oxidation of sulfur in position 6 not only eliminates the sulfur-aromatic interaction (*vide supra*), but also disturbs the spatial orientation of the tyrosine side chain. Consistent with this interpretation, oxidation of the sulfur in position 1 does not influence the side chain of tyrosine, and in these cases the biological activity of the deamino-carba-6-OT sulfoxide is only slightly decreased (36). The conservation of the biological activity (10) of the sulfoxide of XVI also supports our conclusion that there is no interaction between the sulfur and tyrosine in this analog.

In conclusion, we have found that sulfur-aromatic interactions in small peptides may be manifested by a downfield shift of the aromatic protons. Furthermore, examination of the biological activity of analogs of oxytocin suggests that this interaction may be important for high potency in agonist analogs.

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