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A Raman Spectroscopic Investigation of the Disulfide Conformation in Oxytocin and Lysine Vasopressin†

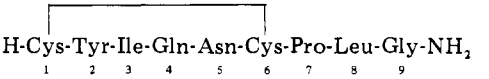
Frederick R. Maxfield‡ and Harold A. Scheraga*

ABSTRACT: The conformation of the CCSSCC moiety in oxytocin and lysine vasopressin is investigated using laser Raman spectroscopy. The Raman spectra of solutions of these hormones in water and in dimethyl sulfoxide show an intense band at 508 cm^{-1} which is assigned to the S-S stretching mode. The presence of shoulders on this band between 490 and 525 cm^{-1} shows that there is an equilibrium among several conformations for the disulfide unit of these hormones in solution. Most of the CS-SC dihedral angles are within 30° of ±90°, but some of the molecules have CS-SC dihedral angles strained away from this value by more than 30°. The previously published circular dichroism spectra of these hormones are reinterpreted, and it is shown that the circular dichroism spectra indicate the presence of more than one conformation for the disulfide unit, in agreement with the Raman results.

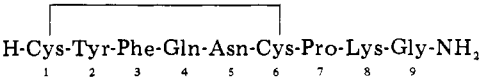
Recently, Van Wart and Scheraga (1976a, b, 1977) and Van Wart et al. (1976a, b) have shown that Raman spectroscopy can be useful for investigating the conformations of disulfides. In this paper, the results of a Raman spectroscopic investigation of the disulfide moiety in oxytocin and lysine vasopressin are reported, and the conformational implications of the Raman spectra, based on the analysis of Van Wart and Scheraga (1976a, b, 1977) and Van Wart et al. (1976a, b), are discussed. The conformations of oxytocin and lysine vasopressin have been investigated previously using NMR[1] (Von Dreele et al., 1971, 1972a; Brewster and Hruby, 1973; Brewster et al., 1973; Glickson et al., 1976; Meraldi et al., 1977), CD (Beychok and Breslow, 1968; Urry et al., 1968, Frič et al., 1975), and conformational energy calculations (Kotelchuck et al., 1972; Brewster et al., 1973). The NMR studies have yielded considerable information about the conformation of these molecules in water and in dimethyl sulfoxide, but this technique has not been useful for studying the conformation around the disulfide bond. CD has been used previously to study the disulfide conformation in these hormones (Beychok and Breslow, 1968; Urry et al., 1968; Frič et al., 1975), but the bands due to the disulfide chromophore are partially obscured by the CD of tyrosine (Frič et al., 1975), making band assignments difficult. In this paper, we present a new interpretation of the CD of these hormones which is consistent with our Raman results and with recent theoretical studies of the optical activity of disulfides (Linderberg and Michl, 1970).

Materials and Methods

Oxytocin



and lysine vasopressin



were generously synthesized, assayed, and supplied by Drs. Roderich Walter and Clark Smith, and were used as received. The oxytocin possessed 495 ± 25 units of avian vasodepressor activity per mg, and the lysine vasopressin had 275 ± 15 units of rat pressor activity per mg. These values may be compared with 507 ± 15 units/mg of avian vasodepressor activity for oxytocin (Chan and du Vigneaud, 1962) and 275–300 units/mg of rat pressor activity for lysine vasopressin (Meienhofer and du Vigneaud, 1960). Solutions were prepared by dissolving the hormones in dilute aqueous acetic acid or in dimethyl sulfoxide, with concentrations ranging between 7 and 10% (w/v). The pH of the aqueous solutions was adjusted to a value between 4 and 4.5 with acetic acid. After the Raman spectra

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; CD, circular dichroism.

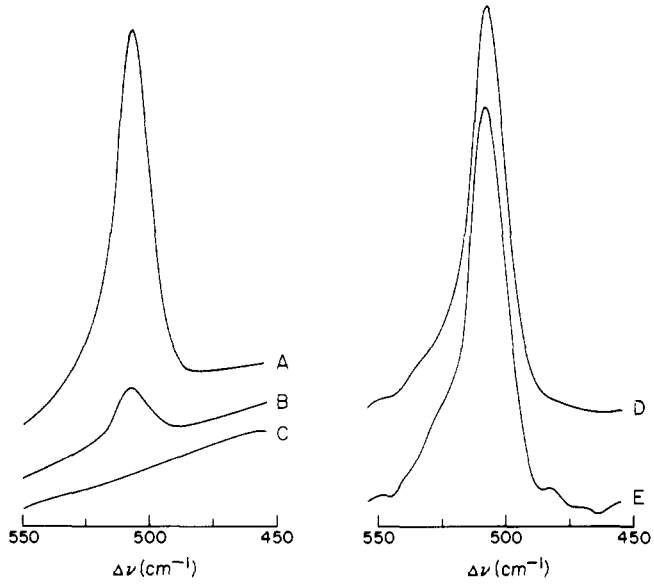

FIGURE 1: Raman spectra of cystine in solution: (A) 0.2 M cystine in 1 N HCl; (B) 0.02 M cystine in 1 N HCl; (C) 1 N HCl; (D) same as in A, but with the solvent subtracted out; (E) same as in B, but with the solvent subtracted out. The intensity scale is arbitrary.

were obtained, the solutions were diluted with water, lyophilized, and returned to Dr. Clark Smith for an assay of the avian vasodepressor activity of the oxytocin and the rat pressor activity of the lysine vasopressin, both of which were unchanged from the original material. In order to check which Raman bands were due to the disulfide moiety, a small sample of each hormone was treated with performic acid for 2.5 h at −5 °C to convert the cystine to cysteic acid (Hirs, 1956).

The Raman spectra were obtained on an instrument which has been described previously (Van Wart et al., 1976a), except that the number of photons counted was stored on a tape cartridge and transferred to a Prime 300 computer, and the spectra were plotted on a Versatec printer/plotter. The 488.0-nm line of the argon ion laser was used at a power of 100–300 mW. All measurements were made with an instrumental resolution of 4 cm^{-1}, and the positions of the bands were calibrated using the emission lines of the argon ion laser.

All of the hormone solutions exhibited a large background fluorescence which was partially reduced after 1–3 h in the laser beam. This fluorescence was nearly constant in the vicinity of the disulfide stretching frequency, and the Raman scattering was clearly visible above this constant background. Spectra were obtained by stepping the monochromator in 1 cm^{-1} increments and counting photons for 1 min at each wavelength. This was repeated approximately ten times for each solution, and the results of multiple scans were averaged on the computer. A shaft encoder was used to allow the computer to determine the frequency and keep multiple scans in exact register. Raman spectra of the solvents used were obtained under similar conditions, and, in some cases, the solvent spectrum was subtracted from the solution spectrum to help determine band positions.

For comparison with the hormone spectra, Raman spectra of cystine were also obtained. First, a spectrum of 0.2 M cystine in 1 N HCl was obtained using a single scan and a 1-min counting time. This solution was then diluted tenfold with 1 N HCl, and the results of ten scans of the dilute solution were averaged on the computer. Raman spectra of solutions of the other constituent amino acids of oxytocin and lysine vasopressin were also obtained to check for bands due to other side chains in the vicinity of the ν(S–S) band.

Unless otherwise indicated, Raman spectra were obtained at room temperature. For measurements at other temperatures, the samples were held in contact with a brass block which was maintained at the desired temperature by a Forma bath. Temperatures were measured with a thermocouple placed in the solution when the laser beam was not incident upon the solution.

Results and Discussion

As discussed below, the disulfide stretching bands in the Raman spectra of oxytocin and lysine vasopressin exhibited small shoulders which would indicate the presence of some conformational flexibility in the disulfide moiety. Since the hormone solutions exhibited a large background fluorescence and the shoulders were small, it was necessary to use very long counting times (1 min per wavenumber) and about ten scans to increase the signal-to-noise ratio in the Raman spectra so that the shoulders could be observed with confidence. In order to demonstrate that these long counting times and multiple scans did not introduce artifacts (e.g., through laser instability or frequency drift), we have compared the Raman spectrum obtained for a single scan of a 0.2 M cystine solution with the average of ten scans of a 0.02 M cystine solution (Figure 1). Individual scans of the 0.02 M cystine solution had a signal-to-noise ratio similar to that observed in the single scans of hormone solutions. When the solvent spectrum (Figure 1C) is subtracted from the two solution spectra (Figure 1D, E), the spectra taken at the two concentrations are nearly identical. Both spectra have a strong peak at 507 cm^{-1} and a shoulder near 525 cm^{-1}, as reported previously (Van Wart and Scheraga, 1976a).

The Raman spectra of oxytocin and lysine vasopressin in the S–S stretch region from 460 to 550 cm^{-1} are shown in Figures 2 and 3. The spectra obtained from aqueous solutions are on sloping backgrounds due to the acetate Raman band at about 460 cm^{-1}. The spectra of dimethyl sulfoxide solutions curve up at the ends due to solvent bands at 674 and 388 cm^{-1}. The S–S stretching band shown in these figures is one of the most intense bands (excluding solvent bands) found in the Raman spectrum from 300 to 1800 cm^{-1}.

Before discussing these spectra, it will be helpful to summarize the results obtained by Van Wart and Scheraga (1976a, b, 1977) and Van Wart et al. (1976a, b), as they pertain to the conformations of cystine linkages. It was found that the S–S stretching frequencies, ν(S–S), in primary disulfides containing the CCSSCC moiety (e.g., cystine) varied between about 485 and 540 cm^{-1} depending on the values of the SS–CC and CS–SC dihedral angles. From spectra of model compounds with CS–SC dihedral angles near ±90°, it was found that disulfides with the trans or either of two nonequivalent gauche conformations about their C–S bonds all have values of ν(S–S) near 510 cm^{-1}. It was found, however, that disulfides with A conformations (defined as conformations with SS–CC dihedral angles near 30°) about one or both of their C–S bonds were responsible for disulfide stretching bands near 525 or 540 cm^{-1}, respectively. On the other hand, values of ν(S–S) less than about 505 cm^{-1} were found to be due to absolute values of the CS–SC dihedral angle less than about 60°. For CS–SC dihedral angles of ±60°, ±30°, and ±10°, the values of ν(S–S) were roughly 509, 495, and 486 cm^{-1}, respectively. No suitable model compounds with CS–SC dihedral angles greater than 90° were available for study, but it was suggested that increasing the dihedral angle above 90° would also tend to decrease ν(S–S). Thus, values of ν(S–S) significantly greater

TABLE I: Raman and CD Data for Disulfides.[a]

χ(CS-SC) (deg)	ν(S-S)[b] (cm^{-1})	λ_1[c] (nm)	$[\theta]_1$ (deg-cm^2/dmol)	λ_2[d] (nm)	$[\theta]_2$ (deg-cm^2/dmol)	CD ref
≃0	485	370	~0[e]	280	~0[e]	*f*
≃+30	495	330	+8300	262	−5000	*f*
≃+60	509	280–294	+11 500 to +18 000	238–244	−6700 to −18 800	*f, g*
+90	509	250[h]	~0	*i*	*i*	*j, k*
≃+120	NA[m]	272	−7550	*i*	*i*	*l*

[a] This table covers positive values of χ(CS-SC). For negative values, the signs of the two CD bands will change, but all other CD and Raman parameters will remain the same. Some of the data listed in the table were obtained from compounds with negative values for χ(CS-SC). [b] The Raman data are from Van Wart and Scheraga (1976b) and apply to cystine-like molecules which are not in the A conformation for the C-S bond. [c] The longest wavelength disulfide band. [d] The second longest wavelength disulfide band. [e] The disulfide chromophore is inherently symmetric when χ(CS-SC) is 0°. Molar ellipticities of −1700 and −890 deg-cm^2/dmol for $[\theta]_1$ and $[\theta]_2$, respectively, have been observed,[f] and were attributed[f] to the influence of a dissymmetric environment. [f] Neubert and Carmack (1974). [g] Carmack and Neubert (1967). [h] From UV absorption. [i] Not observed. [j] Donzel et al. (1972). [k] Mez (1974). [l] Ludescher and Schwyzer (1971). [m] NA, not available.

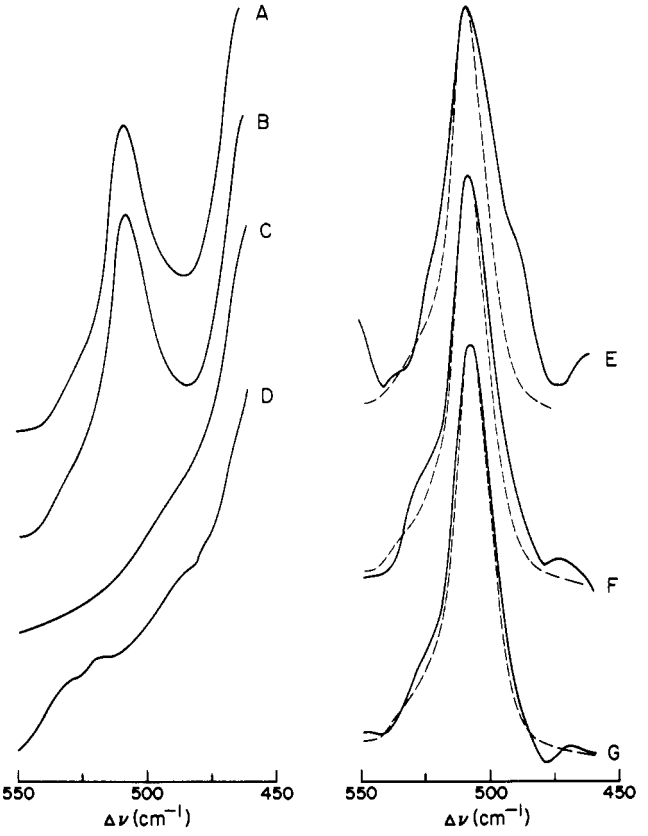

FIGURE 2: Raman spectra of oxytocin in solution: (A) oxytocin in water at 5 °C, adjusted to pH 4 with acetic acid; (B) oxytocin in water at room temperature, adjusted to pH 4 with acetic acid; (C) acetic acid in water, pH 4; (D) oxytocin (treated with performic acid) in water, adjusted to pH 4 with acetic acid; (E) oxytocin in dimethyl sulfoxide at room temperature; (F) same as in A, but with the solvent subtracted out; (G) same as in B, but with the solvent subtracted out. The dashed lines under curves E-G are the cystine Raman spectrum from Figure 1D. The intensity scale is arbitrary.

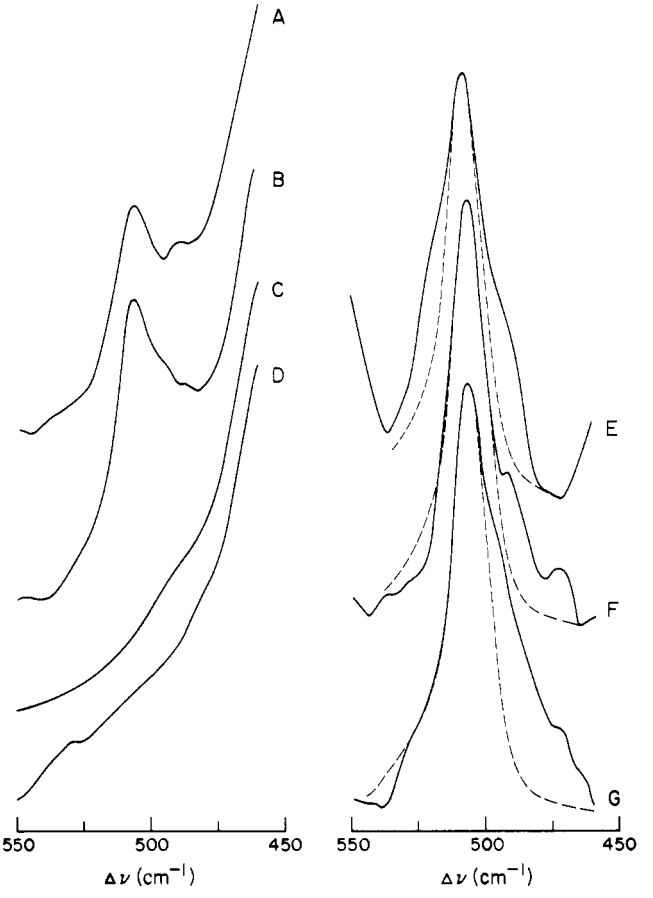

FIGURE 3: Raman spectra of lysine vasopressin in solution: (A) lysine vasopressin in water at 5 °C, adjusted to pH 4 with acetic acid; (B) lysine vasopressin in water at room temperature, adjusted to pH 4 with acetic acid; (C) acetic acid in water, pH 4; (D) lysine vasopressin (treated with performic acid) in water, adjusted to pH 4 with acetic acid; (E) lysine vasopressin in dimethyl sulfoxide at room temperature; (F) same as in A, but with the solvent spectrum subtracted out; (G) same as in B, but with the solvent spectrum subtracted out. The dashed lines under curves E-G are the cystine Raman spectrum from Figure 1D. The intensity scale is arbitrary.

than 510 cm^{-1} indicate the presence of A conformations about C-S bonds, and values less than about 505 cm^{-1} indicate that the CS-SC dihedral angle is strained away from its usual value of ±90° by at least 30°. Values of 510 ± 5 cm^{-1} for ν(S-S) arise for several conformations about the C-S bond and for CS-SC dihedral angles within about 30° of ±90°. The Raman data for strained disulfides are summarized in Table I.

The Raman spectra of oxytocin in water and in dimethyl sulfoxide, shown in Figure 2, all have an intense peak near 508 cm^{-1}. This peak disappears completely after performic acid treatment, supporting its assignment as ν(S-S). Since this peak is near 510 cm^{-1}, it is not possible to determine whether it is due to a single conformation or to an equilibrium of several conformations of the CCSSCC entity which all have ν(S-S) near 510 cm^{-1}.

The performic acid oxidized oxytocin exhibits weak Raman bands at ~475, 490, 525, and 535 cm^{-1} (Figure 2D). These bands also appear in the Raman spectra of solutions of Ile, Tyr, cysteic acid, and Ile, respectively (data not shown), and may

be due to vibrational modes of the side chains of those amino acids. A band at 525 cm^{-1} is present in cysteic acid, but it does not appear in the Raman spectrum of cysteine, indicating that the band is due to the sulfonic acid group. These weak bands are not evident in the Raman spectra of aqueous solutions of oxytocin (Figure 2A,B).

When the solvent spectrum is subtracted out from the spectra obtained from aqueous solutions (Figure 2F,G), a small shoulder near 525–530 cm^{-1} becomes evident. If this shoulder is due to the disulfide stretching band, then it would indicate the presence of a small percentage of A conformations. However, the 536 cm^{-1} band found in Ile and a weaker band at 533 cm^{-1} in Leu may be contributing significantly to this shoulder. The bandwidth and overall shape of the ν(S–S) band in oxytocin (Figure 2F,G) are very similar to those observed in cystine. The Raman spectra indicate that the CS–SC dihedral angle of oxytocin in aqueous solution is within 30° of ±90°, and that the SS–CC groups are predominantly in a gauche or trans but not in the A conformation.

The S–S stretching band of oxytocin in dimethyl sulfoxide (Figure 2E) is considerably broader than the bands observed for aqueous solutions of oxytocin, and there is also a small shoulder present at about 495 cm^{-1}. A comparison with the spectrum of cystine indicates that the broadness of this band may be due to a poorly resolved shoulder near 500 cm^{-1} in addition to the 495 cm^{-1} shoulder. These shoulders on the low-frequency side of the main peak indicate that some of the CS–SC dihedral angles of oxytocin in Me_2SO are strained by more than 30° from ±90°. There is also a shoulder on the high-frequency side of the main band, which indicates that some of the molecules adopt the A conformation. The Raman spectra indicate that there is some change in the disulfide moiety of oxytocin in going from aqueous solution to dimethyl sulfoxide. NMR measurements (Brewster and Hruby, 1973) have shown that the chemical shifts and relative positions of the α and β protons of half-cystine-1 in oxytocin are different in these two solvents, indicating that the conformations in the two solvents are different.

The Raman spectra of aqueous solutions of lysine vasopressin, shown in Figure 3, are somewhat more complex than the spectra obtained for oxytocin, indicating greater conformational flexibility in the CCSSCC moiety. The major peak is observed again at 508 cm^{-1} and disappears completely after performic acid treatment (Figure 3D). Two weak bands (near 485–490 cm^{-1} and 525 cm^{-1}) remain after performic acid treatment. The first of these is also present in the Raman spectrum of Phe and Tyr, and the latter is observed in the Raman spectrum of cysteic acid (but not cysteine). As with oxytocin, the peak at 508 cm^{-1} is identified as ν(S–S) and may be associated with one or more conformations of the CCSSCC group. In addition to the major peak, there are peaks or shoulders in the region between 490 and 505 cm^{-1}, which indicate that some of the lysine vasopressin molecules in solution have CS–SC dihedral angles that are strained away from ±90°. When the solvent spectrum is subtracted from the spectrum of an aqueous solution of lysine vasopressin at room temperature (Figure 3G), it can be seen that the ν(S–S) band is considerably broader than the corresponding band in oxytocin (Figure 2G) or cystine because of the presence of poorly resolved shoulders on the low-frequency side of the main peak. It is difficult to determine the frequencies of these shoulders, but it appears that one of the shoulders occurs near 498 cm^{-1} (Figure 3B,G), corresponding to a CS–SC dihedral angle of about ±40° (or to absolute values greater than 90°). Other shoulders may also be present. On the high-frequency side, a small shoulder near 525 cm^{-1} is evident.

When the temperature is reduced to 5 °C, some changes can be seen in the Raman spectrum (Figure 3A,F), indicating that the equilibrium populations of the various conformations are temperature dependent. The spectrum with the solvent subtracted out (Figure 3F) has a ν(S–S) band with a bandwidth nearly identical to that of cystine, except for a now fairly well-resolved shoulder near 492 cm^{-1}, corresponding to a CS–SC dihedral angle of ±30° (or to absolute values of the dihedral angle greater than 90°). This shoulder may also contain some contribution from the 485–490 cm^{-1} band observed in performic acid treated vasopressin, which was also observed in solutions of Phe and Tyr. However, the intensity of this band seems much greater when the disulfide is intact than when it is broken, and no similar band was observed in oxytocin (which has Tyr, but not Phe), indicating that the 492 cm^{-1} shoulder is due primarily to the presence of strained disulfides in lysine vasopressin. The shoulder near 498 cm^{-1}, which was present at room temperature, is greatly reduced in intensity at 5 °C, and it is probably the reduction in intensity of this intermediate shoulder which allows the 492 cm^{-1} shoulder to become well resolved at 5 °C. The shoulder which was observed near 525 cm^{-1} at room temperature is not present at 5 °C. The reduction in intensity of these shoulders as the temperature is lowered reflects a decreased population of these higher energy conformations at the lower temperature. A weak band is also observed in Figure 3F,G near 470 cm^{-1}, and this band is not observed in any of the amino acids which are found in vasopressin. This band occurs on the steeply sloping part of the Raman spectrum (Figure 3A,B), and is not easily observed before subtracting out the solvent spectrum. In any case, this band is outside of the expected range for ν(S–S).

The ν(S–S) band of lysine vasopressin in dimethyl sulfoxide is also observed at 508 cm^{-1}. This band is somewhat broader than the ν(S–S) of cystine in aqueous solution, with small shoulders occurring on both sides of the main peak. These shoulders indicate that some of the lysine vasopressin molecules in solution have CS–SC dihedral angles strained away from ±90°, and some of the molecules adopt the A conformation in one of the C–S bonds.

Since the correlation between ν(S–S) and the CS–SC dihedral angle is only approximate (and fourfold degenerate), it is difficult to draw any precise conclusions from the Raman spectra about the conformation of the strained disulfides. Since the main band occurs at 508 cm^{-1}, it is clear that, for all the cases studied, the predominant conformation has a CS–SC dihedral angle within 30° of ±90°. However, in the Raman spectra of oxytocin and lysine vasopressin in dimethyl sulfoxide and for aqueous solutions of lysine vasopressin, small shoulders have been observed on the low-frequency side of the main ν(S–S) band. The largest of these shoulders, occurring in the aqueous solution of lysine vasopressin at room temperature, accounts for about 15% of the total disulfide band intensity. Also, the small shoulders observed between 520 and 530 cm^{-1} for solutions of oxytocin and lysine vasopressin indicate that some of these molecules may adopt the A conformation about their C–S bonds in solution. These Raman spectra offer the first indication that these hormones may exist in an equilibrium among various conformations of the CCSSCC moiety.

The principal method used in the past to study the disulfide conformation in oxytocin and vasopressin has been CD (Beychok and Breslow, 1968; Urry et al., 1968; Frič et al., 1975), and the results obtained in one of these CD studies (Urry et al., 1968) have been interpreted to show a single CS–SC dihedral angle of +90° (i.e., with a right-handed screw sense). Since the Raman results discussed above indicate that there may be more than one conformation about the disulfide bond, we have

examined the previously reported CD spectra of these hormones for further evidence of conformational flexibility. A reinterpretation of the CD spectra clearly indicates the presence of more than one CS–SC dihedral angle in these hormones. Furthermore, as discussed below, it appears that it is not possible at the present time to assign a screw sense to the predominant disulfide conformation with a CS–SC dihedral angle of ±90°.

Theoretical investigations of the disulfide chromophore (Bergson, 1962; Linderberg and Michl, 1970; Boyd, 1972) have shown that the two lowest energy transitions are nearly degenerate when the CS–SC dihedral angle is close to ±90°. In the absence of external perturbations, these transitions are predicted to have rotatory strengths of the same magnitude and opposite sign (Linderberg and Michl, 1970). Thus, because of the cancellation of rotatory strengths of opposite sign, the CD associated with the longest wavelength absorption is predicted to be nearly zero, even for molecules with a single screw sense, when the CS–SC dihedral angle is near ±90°. As the CS–SC dihedral angle is varied away from ±90°, the degeneracy is split, and a "quadrant rule" is predicted for the sign of the rotatory strength in the longest wavelength transition (Linderberg and Michl, 1970; Woody, 1973). This "quadrant rule" predicts that the CD band associated with the longest wavelength transition will change sign each time a new quadrant is entered on varying the CS–SC dihedral angle from −180° to +180°. Furthermore, the rotatory strength at the longest wavelength absorption is predicted to be zero when the CS–SC dihedral angle is −180°, −90°, 0°, or +90°. The longest wavelength transition is predicted to occur at 247 nm when the CS–SC dihedral angle is ±90°, and the wavelength at which it is observed is predicted to increase monotonically as the CS–SC dihedral angle is varied to 0° or 180° (Boyd, 1972).

The main features of these theoretical predictions have been verified in all quadrants, as summarized in Table I. In particular, the sign of the longest wavelength CD band is observed to change on varying the CS–SC dihedral angle from +60° to +120°. For disulfides with a single screw sense and a dihedral angle of 90°, the CD band associated with the longest wavelength transition is small (Donzel et al., 1972), and any CD observed for these dihedral angles is due largely to the influence of a dissymmetric environment (Casey and Martin, 1972).

The proposal that the CS–SC dihedral angle in oxytocin is approximately +90° was based on the assignment of a positive band near 250 nm and a negative band near 240 nm to the disulfide chromophore (Urry et al., 1968). By comparing the signs of these two longest wavelength CD bands with those of the CD bands observed for a molecule with a CS–SC dihedral angle of +60° (see Table I), Urry et al. (1968) concluded that the disulfide in oxytocin also had a right-handed helical sense (i.e., a positive value for the dihedral angle). It is clear, however, from the recent theoretical and experimental work on disulfides discussed above that the comparison between molecules with CS–SC dihedral angles of +60° and +90° is not valid. The assignment of the dihedral angle to ±90° (but *not* to +90°) on the basis of the wavelength of the transition is correct. Glickson (1975) has interpreted the small molar ellipticity of the CD band at 250 nm in oxytocin and vasopressin to indicate no significant preference for either screw sense of the disulfide. However, disulfides with a *single* screw sense have small CD bands if the CS–SC dihedral angle is near ±90° (Donzel et al., 1972). Since the CD of this band is strongly influenced by environmental influences (e.g., by the presence of dissymmetric α carbons), it is not possible to obtain any information about the screw sense of the disulfide from the sign or magnitude of this band (Frič et al., 1975).

Later CD studies on analogues of oxytocin and vasopressin have shown that the 240-nm transition is not due primarily to the disulfide chromophore, since this band is also present in analogues of oxytocin without a disulfide group (Frič et al., 1974a,b, 1975). On the basis of these later studies, it was concluded that oxytocin and vasopressin have a long-wavelength negative CD band (due to the disulfide) between 280 and 300 nm in addition to the positive band near 250 nm (Frič et al., 1975). The transition observed between 280 and 300 nm is partially obscured by the tyrosine CD, and is most clearly observed in analogues where tyrosine has been replaced by other amino acids (Frič et al., 1975). Since none of the model compounds with a single conformation in the disulfide unit have exhibited the *two* lowest energy transitions near 290 and 250 nm (see Table I), it is likely that these two bands arise from two different conformations of the disulfide unit.

From published CD spectra of oxytocin and vasopressin and their analogues (Beychok and Breslow, 1968; Urry et al., 1968; Frič et al., 1974a,b, 1975), it can be seen that the absolute value of the molar ellipticity for the 280–300-nm transition is always less than 400 deg-cm^2/dmol. The low molar ellipticity for the 280–300-nm transition indicates that only a small fraction of the molecules in solution contribute to this band, since other chiral disulfides with transitions between 270 and 300 nm have molar ellipticities between about 7000 and 18 000 deg-cm^2/dmol (Table I). The low molar ellipticity of this band may be due partially to the flexibility of oxytocin and vasopressin as compared to the fairly rigid model compounds used to obtain the data in Table I. The wavelength and sign of the CD band would indicate a CS–SC dihedral angle of about +130° or −60°, based on the CD of model compounds (Table I). Some of the published CD spectra of oxytocin and vasopressin and their analogues show weak, poorly resolved, negative bands at wavelengths greater than 300 nm, which would indicate that some CS–SC dihedral angles are strained more than 30° from ±90°. This interpretation of the CD spectra, showing that there is more than one conformation for the disulfide unit, is consistent with the results from our Raman study which show that, depending on conditions such as solvent and temperature, a small fraction of the oxytocin and lysine vasopressin molecules in solution have CS–SC dihedral angles more than 30° away from ±90°.

The combined Raman and CD evidence indicates that most of the oxytocin or vasopressin molecules in solution have CS–SC dihedral angles near ±90°, but a small fraction of the hormones have dihedral angles which are strained away from this value. A rough estimate would be that up to 20% of the hormone molecules in solution have a strained disulfide, with the fraction of strained disulfides being dependent on such conditions as solvent and temperature. It is not possible, at the present time, to assign a screw sense to the major conformation with a CS–SC dihedral angle of ±90°. It is also possible that both screw senses coexist in solution or that the screw sense changes on changing solvents.

Models have been proposed for the conformation of oxytocin, all of which include a disulfide dihedral angle of +90° (Urry and Walter, 1971; Kotelchuck et al., 1972; Brewster et al., 1973). An examination of a space-filling model indicates that the main features of these models can be maintained with a CS–SC dihedral angle of −90° if compensatory changes are made in the dihedral angles ψ, χ^1, and χ^2 of the N-terminal cystine residue. It is possible to open the CS–SC dihedral angle from +90° to +130° without encountering any steric overlaps. The other possible strained disulfide conformation suggested by the CD results, with a CS–SC dihedral angle near −60°,

can also be formed, but this conformation may require some rearrangement in the rest of the molecule due to crowding in the interior of the ring. Since NMR investigations indicate that there is considerable flexibility in these hormones (Brewster and Hruby, 1973; Brewster et al., 1973; Glickson, 1975; Meraldi et al., 1977), it is possible that CS–SC dihedral angles of −60° can also occur.

The evidence for the possibility of A conformations about the C–S bonds comes from the small high-frequency shoulders on the ν(S–S) band. Using space-filling models, it appears that it is possible to have an SS–CC dihedral angle near +30° on the side chain of cystine-6. It is difficult to estimate the relative stability of these various conformations from an examination of the models.

Conclusion

The Raman spectra of oxytocin and lysine vasopressin solutions in water and dimethyl sulfoxide show that most of the disulfide moieties in these molecules have CS–SC dihedral angles within 30° of ±90°. However, depending on the conditions, a small fraction of these molecules can have CS–SC dihedral angles which are strained away from ±90° by at least 30°, or they may adopt the A conformation about one of their C–S bonds (i.e., the SS–CC dihedral angle is near 30°).

Since the Raman data indicated that there is some conformational flexibility in the CCSSCC moiety, we examined previously reported CD spectra for further evidence of this flexibility. The CD bands at 250 nm and above 280 nm show that there are at least two conformations about the S–S bond in oxytocin and vasopressin. The negative sign of the CD above 280 nm indicates that the strained disulfides observed by this technique and by Raman spectroscopy have CS–SC dihedral angles in the quadrant between +90° and 180° or between 0° and −90°. The low molar ellipticity of this long-wavelength band and the small size of the shoulders on the Raman band indicate that only a few percent of the molecules in solution have strained disulfides. Unfortunately, it does not seem possible to determine the screw sense of the predominant (unstrained) conformation, which has a CS–SC dihedral angle near ±90°, from the sign of the 250 nm CD band.

Conformational energy calculations (Kotelchuck et al., 1972; Brewster et al., 1973) have indicated that there are several possible low-energy conformations for the peptide backbone in oxytocin. NMR experiments (Von Dreele et al., 1972a,b; Brewster et al., 1973; Brewster and Hruby, 1973; Glickson, 1975; Meraldi et al., 1977) have also shown that there is considerable flexibility in the backbone conformation of oxytocin and vasopressin and that the average conformation can be affected by changing solvents or by amino acid substitutions. However, the conformational energy calculations have been performed with the CS–SC dihedral angle held fixed at +90°, and NMR has not been useful for studying the CSSC group. Our results extend these previous studies by showing that conformational flexibility in the disulfide moiety must also be considered in trying to understand the properties of oxytocin and vasopressin and their analogues.

The biological activity of the neurohypophyseal hormones is strongly affected by amino acid substitutions or chemical modification (see, for example, Walter et al., 1976), and there is considerable interest in understanding the relationship between the biological activities of these hormone analogues and their conformations in solution. Meraldi et al. (1977) have suggested that the flexibility observed in oxytocin may be necessary for biological activity. According to the presently available evidence, the disulfide in these hormones may be in either screw sense or in an equilibrium between the two screw senses. Whatever the screw sense, most of the CS–SC dihedral angles are near ±90°, but an observable fraction of the disulfides are strained away from this value by 30° or more. It is possible that the conformation of the disulfide group is related to biological function.

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Role of Coenzyme Q in the Mitochondrial Respiratory Chain. Reconstitution of Activity in Coenzyme Q Deficient Mutants of Yeast†

Gregory G. Brown‡ and Diana S. Beattie*

ABSTRACT: The reduction of cytochrome *c* by the reduced form of the 6-decyl analogue of coenzyme Q follows first-order kinetics with respect to cytochrome *c* and increases in a linear manner with added mitochondrial protein. The activity is completely sensitive to antimycin A in whole cell extracts of yeast as well as in isolated mitochondria and fractionates with markers for the mitochondrial electron-transport chain. The presence of both cytochrome *b* and c_1 in an approximately 2:1 ratio appears essential for enzymatic activity. Reduced coenzyme Q:cytochrome *c* reductase obeys Michaelis–Menten kinetics when assayed in mitochondria obtained from a yeast strain lacking coenzyme Q. Both reduced nicotinamide adenine dinucleotide and succinate:cytochrome *c* reductase activities were not detectable in six coenzyme Q deficient strains tested, but were restored after addition of the oxidized form of the coenzyme Q analogue. No marked difference in the concentration of the analogue required to restore the two activities was observed.

The mitochondrial electron-transfer chain is considered to be composed of discrete lipid–protein enzyme complexes, each of which catalyzes a distinct part of the overall oxidation reaction (Hatefi et al., 1962, 1975). This concept has been useful in the study of mitochondrial energetics, as it has allowed the identification and purification of many proteins directly involved in electron transfer. The role of coenzyme Q in this scheme is to act as a mobile electron carrier between the primary flavoprotein dehydrogenase complexes (complexes I and II) and the cytochrome *b*–cytochrome c_1 complex (complex III).

While it has long been accepted that coenzyme Q acts as a mediator of electron transfer between the NADH[1] dehydrogenase complex and complex III (Rieske, 1976), the role of coenzyme Q in the transfer of electrons from the succinate dehydrogenase complex has been the subject of some disagreement (Albracht et al., 1971). Furthermore, a functional heterogeneity and compartmentation of coenzyme Q within the mitochondria has been suggested to explain the results obtained in reconstitution experiments with coenzyme Q depleted mitochondria and submitochondrial particles (Lenaz et al., 1968; Gutman et al., 1971a). For example, maximum succinate oxidase activity can be achieved at lower concentrations of coenzyme Q than can NADH oxidase activity. In addition, the two oxidase activities differ with respect to the stimulatory and inhibitory effects of analogues and homologues of coenzyme Q (Lenaz et al., 1968, 1975; Jeng et al., 1968; Castelli et al., 1971). Kröger and Klingenberg (1973), however, have maintained that no such compartmentation exists because the coenzyme Q pool appears kinetically homogeneous.

The recent development by Folker's group of stable, low-molecular-weight analogues of coenzyme Q has provided a new tool to probe coenzyme Q function (Wan et al., 1975). We have previously used one of these analogues, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (DB) in which a straight chain aliphatic group of ten carbons is substituted for the isoprenoid side chain, to assay complex III formation in yeast undergoing respiratory adaptation (Brown and Beattie, submitted for publication). In the present study, a detailed investigation of the catalytic activity of complex III using DB as substrate was undertaken. The analogue was also used to reconstitute NADH and succinate:cytochrome *c* reductase activities in a series of coenzyme Q deficient mutants of yeast. The results obtained indicate that reduced DB can be used to monitor accurately the catalytic activity of the cytochrome *b*–cytochrome c_1 complex. The antimycin A sensitive reduction of cytochrome *c* by both NADH and succinate requires coenzyme Q with a similar concentration dependence. No evidence for the "functional heterogeneity" of coenzyme Q has been obtained.

Materials and Methods

Strains of Yeast: Saccharomyces cerevisiae. D273-10B, prototrophic (α) and coenzyme Q deficient mutants derived

† From the Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029. *Received March 21, 1977.* This work was supported, in part, by Grants HD-04007 from the National Institutes of Health and PCM 76-21599 from the National Science Foundation.

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[1] Abbreviations used are: DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; NADH, reduced nicotinamide adenine dinucleotide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.