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Conformational Analysis of Small Disulfide Loops. Spectroscopic and Theoretical Studies on a Synthetic Cyclic Tetrapeptide Containing Cystine†

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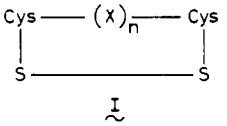
ABSTRACT: The conformational analysis of the synthetic peptide Boc-Cys-Pro-Val-Cys-NHMe has been carried out, (S–S) as a model for small disulfide loops, in biologically active polypeptides. ^{1}H NMR studies (270 MHz) establish that the Val(3) and Cys(4) NH groups are solvent shielded, while ^{13}C studies establish an all-trans peptide backbone. Circular dichroism and Raman spectroscopy provide evidence for a right-handed twist of the disulfide bond. Analysis of the vicinal ($J_{\alpha\beta}$) coupling constants for the two Cys residues establishes that $\chi^1 \sim \pm 60°$ for Cys(4), while some flexibility is suggested at Cys(1). Conformational energy calculations, imposing intramolecular hydrogen bonding constraints, favor a β-turn (type I) structure with Pro(2)-Val(3) as the corner residues. Theoretical and spectroscopic results are consistent with the presence of a transannular 4 → 1 hydrogen bond between Cys(1) CO and Cys(4) NH groups, with the Val NH being sterically shielded from the solvent environment.

The formation of disulfide bonds between cysteine residues located at different points in a polypeptide chain leads to the generation of compact structures. When the number of intervening amino acids separating the two Cys residues is large, the loops formed can have considerable conformational flexibility. However, if the number of spacer amino acids is small, then the reduction in ring size introduces stereochemical constraints. The formation of such conformationally well-defined structural units in proteins, hormones, and polypeptide toxins may have some importance in recognition processes like receptor interactions. Small ring peptide disulfides (I) ($n \leq$



I

4) are frequently observed in hormones, e.g., oxytocin and vasopressin (n = 4, 20-membered ring) (Hruby, 1981), insulin (n = 4) (Blundell et al., 1972), and snake toxins, e.g., long neurotoxin *Siamensis* (n = 4 and n = 3, 17-membered ring) (Karlson et al., 1971). An important example of an n = 2 loop (14-membered ring) is the -Cys-Gly-Pro-Cys- segment, which constitutes the active site of the redox protein thioredoxin (Holmgren, 1981). While considerable interest has been centered on the conformation of oxytocin, vasopressin, and their analogues (Urry & Walter, 1971; Fischman et al., 1980; Tu et al., 1979; Wyssbrod et al., 1977, 1979; Hruby et al., 1979a,b; Mosberg et al., 1981), relatively little information is available on smaller cyclic peptide disulfides.

The 8-membered ring (n = 0) -Cys-Cys- (S–S) fragment in malformin A (Ptak, 1973; Tonelli, 1978) and the 17-membered ring (n = 3) Cys-Ser-Gly-Gly-Cys (S–S), found in pepsin (Klis & Siemion, 1978), have been studied experimentally. Structural studies on the model peptides cyclo(L-cystine) (Donzel et al., 1972; Varughese et al., 1981) and cyclo(cysteinylcysteine) (Capasso et al., 1977) have also been reported. As part of a program to explore the conformational characteristics of cyclic peptide disulfides, we describe in this report the synthesis and

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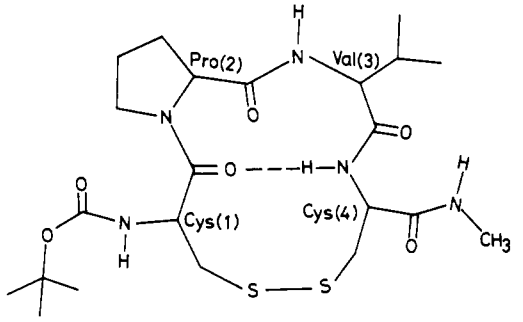

FIGURE 1: Structure of peptide disulfide **1**.

conformational analysis of Boc-Cys-Pro-Val-Cys-NHMe,[1] **1**.
S–S

In Pro-X sequences there is a high probability of peptide chain reversal or β-turn formation (Smith & Pease, 1980; Zimmerman & Scheraga, 1977). This structural feature should be promoted by hydrogen-bond formation between the Cys(1) CO and Cys(4) NH groups and by disulfide formation (Figure 1). Val was chosen as the X residue in order to enhance solubility in organic solvents and to facilitate ^{1}H NMR assignments. The detailed spectroscopic studies reported in this paper, in conjunction with theoretical calculations, support a β-turn conformation, within the disulfide ring.

Materials and Methods

Peptides were synthesized by solution phase procedures and checked for homogeneity by TLC on silica gel. All peptides were examined by 270- or 60-MHz ^{1}H NMR to establish structures.

Synthesis of Peptide Disulfide **1**. (*A*) *Boc-Cys(SBz)-Pro-OMe*. Boc-Cys(SBz) (3.1 g, 10 mmol) was dissolved in CH_2Cl_2 (15 mL) and cooled to 0 °C. Pro-OMe (1.3 g, 10 mmol) was added followed by dicyclohexylcarbodiimide (DCC) (2.16 g, 10 mmol), in 10 mL of CH_2Cl_2. The mixture was stirred in an ice bath for 4 h and at room temperature overnight. The dicyclohexylurea was filtered off, and the filtrate was washed with 1 N HCl, 1 N $NaHCO_3$, and H_2O and dried over Na_2SO_4. Evaporation of solvent yielded a residue, which was redissolved in a minimum volume of ethyl acetate. Insoluble material was filtered off, and the residue, after evaporation of solvent, was triturated with petroleum ether to yield the dipeptide as a solid: yield 3.5 g (77%); mp 91–93 °C.

(*B*) *Boc-Cys-(SBz)-Pro-OH*. The dipeptide ester (3.3 g, 7 mmol) was dissolved in CH_3OH (10 mL), and 2 N NaOH (7 mL) was added and left for 8 h at room temperature. H_2O (20 mL) was added, and the aqueous solution was washed with EtOAc (2 × 15 mL) to remove traces of peptide ester. The aqueous layer was acidified with 1 N HCl, saturated with NaCl, and extracted with $CHCl_3$ (4 × 20 mL). The $CHCl_3$ extracts were pooled, dried, and evaporated to yield the dipeptide acid as a chromatographically homogeneous oil: yield 2.9 g (75%).

(*C*) *Boc-Val-Cys(SBz)-OMe*. Boc-Val (2.2 g, 10 mmol) was coupled to Cys(SBz)-OMe (2.3 g, 10 mmol) as described above. The dipeptide ester was obtained as a solid: yield 3.0 g (72%); mp 115–116 °C.

(*D*) *Boc-Cys(SBz)-Pro-Val-Cys(SBz)-OMe*. Boc-Val-Cys(SBz)-OMe (2.3 g, 6 mmol) was dissolved in 10 mL of HCl/tetrahydrofuran (2 N). After 3 h the removal of the Boc group was essentially complete, as monitored by TLC. After evaporation of solvent, the residue was dissolved in H_2O, and the solution was carefully made alkaline with Na_2CO_3. Extraction with $CHCl_3$ (4 × 20 mL), followed by drying and removal of solvent, gave the free base Val-Cys(SBz)-OMe as an oil, which was directly used.

Boc-Cys(SBz)-Pro-OH (2.1 g, 5 mmol) in 10 mL of CH_2Cl_2 was cooled to 0 °C. Val-Cys(SBz)-OMe, obtained above, in 10 mL of CH_2Cl_2 and DCC (1.08 g, 5 mmol) in 5 mL of CH_2Cl_2 were added. Workup as described for the dipeptides gave the tetrapeptide ester as a white solid: yield 2.8 g (80%); mp 75–76 °C; NMR (^{1}H, 270 MHz, $CDCl_3$) δ 7.30, 7.28 (m, 10 H, phenyl), 7.00 (d, 1 H, Val NH), 6.86 [d, 1 H, Cys(4) NH], 5.26 [d, 1 H, Cys(1) NH], 4.72 [m, 1 H, Cys(4) C$^\alpha$H], 4.56 [m, 1 H, Cys(1) C$^\alpha$H], 4.53 (m, 1 H, Pro C$^\alpha$H), 4.28 (m, 1 H, Val C$^\alpha$H), 3.76 (s), 3.73 (s, 4 H, S-CH_2-phenyl), 3.71 (s, 3 H, ester CH_3), 3.68 (m, 2 H, C$^\delta$H$_2$, Pro), 2.85, 2.77, 2.59 [m, 4 H, C$^\beta$H$_2$ Cys(1) and Cys(4)], 2.23 (m, 1 H, Val C$^\beta$H), 1.97 (m, 2 H), 1.84 (m, 2 H, Pro C$^\beta$H$_2$, C$^\gamma$H$_2$), 1.45 (s, 9 H, Boc CH_3), and 0.88 (q, 4 H, Val C$^\gamma$H$_3$). Anal. Calcd for $C_{36}H_{40}O_7N_4S_2$: N, 7.95. Found: N, 8.49.

(*E*) *Boc-Cys(SBz)-Pro-Val-Cys(SBz)-NHMe*. The tetrapeptide ester was dissolved in absolute CH_3OH and saturated with dry methylamine gas at 10 °C. The flask was tightly stoppered and left overnight. The solvent was evaporated to give a white solid: yield 2.0 g (95%); mp 109–110 °C; NMR (^{1}H, 270 MHz, $CDCl_3$) δ 7.32, 7.31, 7.30 (m, 10 H, phenyl protons), 7.17 [d, 1 H, Cys(4) NH], 6.73 (m, 2 H, Val NH and $NHCH_3$), 5.13 [d, 1 H, Cys(1) NH], 4.61 [m, 1 H, Cys(1) C$^\alpha$H], 4.53 [m, 1 H, Cys(4) C$^\alpha$H], 4.41 (m, 1 H, Pro C$^\alpha$H), 4.10 (t, 1 H, Val C$^\alpha$H), 3.77 (s), 3.73 (s, 4 H, S-CH_2-phenyl), 3.75 (m, 2 H, Pro C$^\delta$H$_2$), 3.10 (q, 1 H), 2.89 (m, 2 H), 2.61 [q, 1 H, Cys(1) and Cys(4) C$^\beta$H$_2$], 2.28 (m, 2 H), 2.13 (m, 1 H), 2.01 (m, 2 H, Val C$^\beta$H, Pro C$^\beta$H$_2$ and C$^\gamma$H$_2$), 1.44 (s, 9 H, Boc CH_3), 0.95 (q, 6 H, Val C$^\beta$H$_3$). Anal. Calcd for $C_{36}H_{41}O_6N_5S_2$: N, 9.95. Found: N, 9.50.

(*F*) *Disulfide* **1**. The tetrapeptide methylamide (900 mg, 1.2 mmol) was dissolved in dry liquid NH_3 (150 mL). Small pieces of metallic sodium were added, while the solution was vigorously stirred. Sodium was added until the blue color persisted for 3 min. Then the excess of sodium was destroyed by careful addition of a few drops of glacial acetic acid. The ammonia was slowly evaporated under N_2 and the residue dissolved in 600 mL of H_2O (0.15% solution). The pH was brought to 6.8, and small amounts of 0.2 M $K_3Fe(CN)_6$ solution were added, while the pH was maintained at 6.8–7.0, until the yellow color persisted. A 5-mL excess of $K_3Fe(CN)_6$ was added to ensure oxidation. The solution was concentrated at 45–50 °C to 50 mL and extracted with ethyl acetate (4 × 25 mL), and the organic layer was washed with 1 N HCl, 1 N $NaHCO_3$, and H_2O and dried. Evaporation yielded a solid (300 mg) which had three closely moving components on TLC (silica gel, 5% CH_3OH–$CHCl_3$; R_f 0.7, 0.6, and 0.45). Chromatography on silica gel (250 mg loaded onto 30 g of silica gel in a 1.5 × 30 cm column) and elution with $CHCl_3$, 5% CH_3OH–$CHCl_3$, and CH_3OH afforded a clean separation (R_f 0.7, 60 mg; R_f 0.6, 25 mg; R_f 0.45, 100 mg). The compound with R_f 0.7 was established to be the desired disulfide: mp 210–212 °C; mass spectrum (field desorption) MH^+ 532 (M^+ Calcd 531). Anal. Calcd for $C_{22}H_{37}O_6N_5S_2$: N, 13.18. Found: N, 12.71. ^{1}H NMR (270 MHz) and ^{13}C NMR (67.89 MHz) spectra are shown in Figures 2 and 3.

Spectroscopic Measurements. ^{1}H NMR (270 MHz) and ^{13}C NMR (67.89 MHz) spectra were recorded on a Bruker WH-270 FT-NMR spectrometer at the Bangalore NMR

[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; NHMe, *N*-methylamide; TLC, thin-layer chromatography.

Table I: NMR Parameters of NH Groups in the Cyclic Peptide Disulfide **1** and Acyclic Precursor **2**

	Cys (1) in solvent[a]			Val (3) in solvent[a]			Cys (4) in solvent[a]			NHMe in solvent[a]		
	A	B	C	A	B	C	A	B	C	A	B	C
					Cyclic Peptide 1							
δ_{NH}	5.5	6.17	7.21	6.41	6.34	7.39	7.12	7.46	7.40	6.60	6.75	7.50
$d\delta/dT$ ($\times 10^3$ ppm/°C)		11.4	3.8		1.4	2.0		3.6	1.9		7.3	5.8
$t_{1/2}$ (h)	~24			>96			>96			~72		
$J_{HNC^{\alpha}H}$ (Hz)[b]	7.3	7.7	7.7	10.3	7.3	9.2	7.7	8.9	7.3			
					Acyclic Peptide 2							
δ_{NH}	5.13	5.89	7.16	6.73	7.49	7.96	7.17	7.90	7.83	7.07	7.47	7.96
$d\delta/dT$ ($\times 10^3$ ppm/°C)		11.7	7.8		15.5	5.3		15.0	5.0		17.1	5.8
$J_{HNC^{\alpha}H}$ (Hz)[b]	8.9	8.5		7.7	8.1		7.8	7.7				

[a] A, $CDCl_3$; B, C_6D_6; C, $(CD_3)_2SO$. [b] Errors in J values are ±0.4 Hz.

Facility as described (Nagaraj & Balaram, 1981). ABX spectra were simulated by using the ITRCAL program, with Nicolet computer attached to the NMR spectrometer. Spectra were simulated separately for the two Cys ABX systems, by using a line width of 1.7 Hz, and then added on the computer. Peptide concentrations in all NMR studies were fixed at 10 mg/mL (0.019 M).

CD spectra were recorded on a Jasco J-20 spectropolarimeter as described (Nagaraj & Balaram, 1981). Laser Raman spectra of the disulfide were obtained on a Spex Ramalog-5 spectrometer at Colorado State University (Ishizaki et al., 1981).

Theoretical Conformational Analysis. The dihedral angles defining the conformation of the molecule are defined according to IUPAC–IUB Commission on Biochemical Nomenclature (1970). Unless mentioned otherwise peptide bonds are assumed to be perfectly planar with trans geometry, having the standard dimensions (Corey & Pauling, 1953). The cyclization procedures used in this study are as described by Chandrasekaran (1968) and Renneboog-Squilbin (1980). The parameters and method employed for conformational energy calculations are as given by Ramachandran & Sasisekharan (1968) and Chandrasekaran & Balasubramanian (1969). The monopole charges on C, O, and N of the peptide unit preceding the pyrrolidine ring are taken from Holzwarth & Chandrasekaran (1969).

For examining model I (vide infra) the segment from N-[Cys(1)] and C[Cys(4)] defined by $\psi_{Cys(1)}$, ϕ_{Pro}, ψ_{Pro}, ϕ_{Val}, ψ_{Val}, and $\phi_{Cys(4)}$ was found to be sufficient, whereas for model II (vide infra) another peptide unit attached to C$^{\alpha}$[Cys(1)] whose orientation is defined by $\phi_{Cys(1)}$ was included. In regard to side-chain orientations, we have considered various puckering states of proline ring whereby a flexibility in ϕ_{Pro} is implicated and all the three staggered conformations of the valine side chain. The minimum energy puckering states of proline corresponding to ϕ_{Pro} = −50°, −60°, −70°, and −80° are taken from Table II of Ramachandran et al. (1970).

A variety of conformations were generated by varying the abovementioned dihedral angles in their allowed regions at an interval of 10°. Among these conformations only those that satisfied hydrogen-bonding criteria, i.e., N---O distance to be between 2.75 and 3.2 Å and HNO angle less than 35°, were chosen for cyclization tests (Chandrasekaran, 1968; Renneboog-Squilbin, 1980). These hydrogen-bonded and cyclized conformations were then subjected to conformational energy calculations.

Results and Discussion

NMR Assignments. The 270-MHz ^{1}H NMR spectrum of the disulfide **1** in $CDCl_3$ is shown in Figure 2. The Cys(1) NH is unambiguously assigned to the high-field doublet at δ 5.50, since urethane NH groups appear consistently at high field in a number of peptides. The assignment of the broad quartet at δ 6.60 to the methylamide NH is unequivocal. The doublets at δ 7.12 and 6.41 were assigned to Cys(4) and Val(1) NH groups by using double-resonance techniques. Irradiation of the Cys C$^{\beta}$H$_2$ and Val C$^{\beta}$H resonances, which can be unambiguously identified, shows that the Cys(1), Cys(4), and Val C$^{\alpha}$H protons form an overlapping multiplet between δ 4.6 and 4.8. The results of low-power spin tickling experiments, together with the unambiguous assignment of Cys(1) NH, led to the assignment of Cys(1), Cys(4), and Val C$^{\alpha}$H groups to the resonances centered at δ 4.75, 4.65, and 4.70, respectively. The differentiation of the Cys(1) and Cys(4) NH groups permits unambiguous assignment of the C$^{\alpha}$H and C$^{\beta}$H$_2$ resonances of the two Cys residues by establishing resonance connectivities by using double resonance. The inset to Figure 2 shows the partial 270-MHz ^{1}H NMR spectrum of **1** in C_6D_6. In this solvent the C$^{\alpha}$H resonances are very clearly resolved, with the Val C$^{\alpha}$H appearing as a doublet of doublets at δ 5.0. The enhancement of spectral dispersion in aromatic solvents has been widely used in studies of steroids and organic systems (Laszlo, 1967) but has so far not found great use in the spectroscopy of peptides (Venkatachalapathi & Balaram, 1981). In $(CD_3)_2SO$, satisfactory spectra could be obtained soon after dissolution. However, prolonged standing in this solvent results in the appearance of additional resonances, presumably as a consequence of disulfide instability in this solvent. Resonance assignments in C_6D_6 and $(CD_3)_2SO$ were established by using extensive decoupling studies. The Cys(1) NH was unambiguously assigned by its high-field position in C_6D_6. In $(CD_3)_2SO$ the position of this proton was established by using ^{1}H NMR spectra obtained in $CDCl_3$–$(CD_3)_2SO$ mixtures of varying composition. The ^{1}H NMR parameters

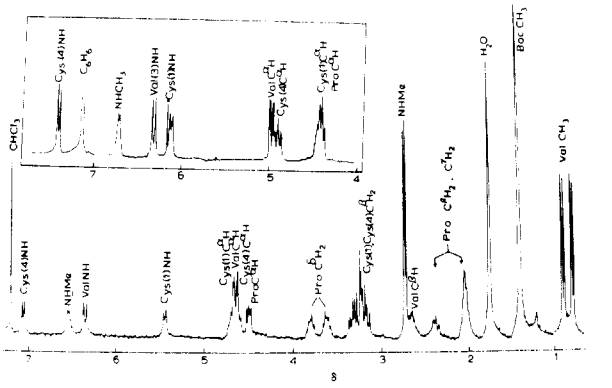

FIGURE 2: 270-MHz ^{1}H NMR spectrum of **1** in $CDCl_3$. (Inset) C$^{\alpha}$H and NH resonances of **1** in C_6D_6.

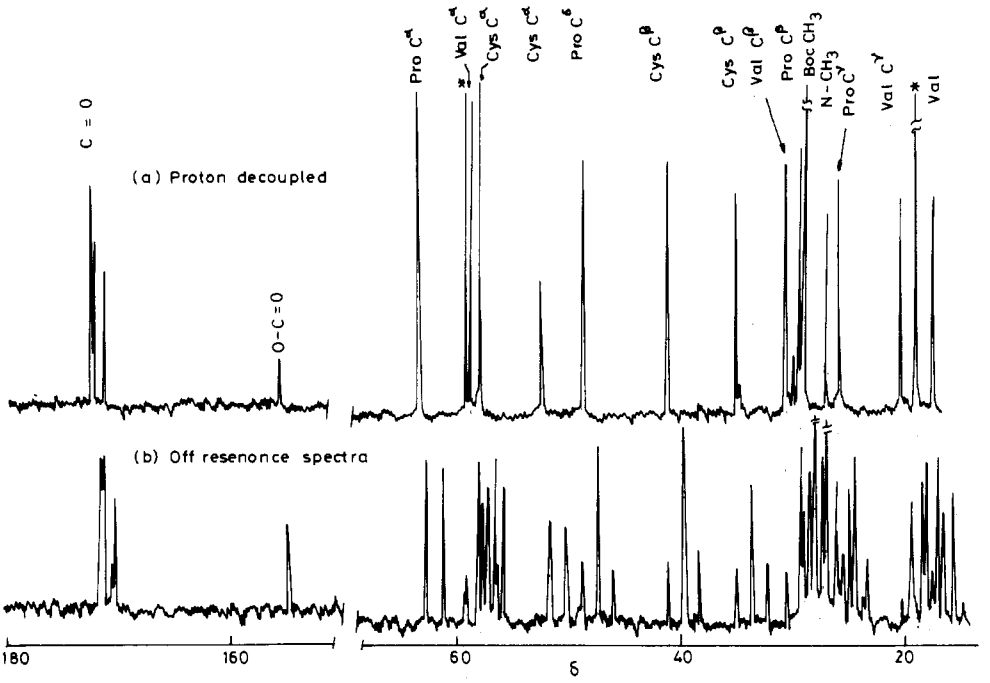

FIGURE 3: 67.89-MHz ^{13}C NMR spectrum of **1** in $CDCl_3$. (a) Proton decoupled and (b) off-resonance proton decoupled ^{13}C spectrum. Starred resonances correspond to ethanol.

for the NH groups in **1** are summarized in Table I. The corresponding values for the acyclic precursor Boc-Cys-(SBzl)-Pro-Val-Cys(SBzl)-NHMe (**2**) are also summarized for comparison. In **2**, additional resonances were observed which arise due to population of cis conformers about the Cys(1)–Pro(2) bond.

The 67.89-MHz ^{13}C NMR spectrum of **1** in $CDCl_3$ is shown in Figure 3a. Assignments of resonances were made by using known chemical shift values in model peptides. The Pro C^β and Val C^β resonances were distinguished by using off-resonance decoupled spectra (Figure 3b), where Pro C^β appears as a triplet while Val C^β yields a doublet. The positions of the C^β (δ 28.7) and C^γ (δ 25.3) resonances are characteristic of trans Cys(1)-Pro(2) conformers. The absence of additional minor resonances in the ^1H and ^{13}C spectra of **1** strongly suggests that only the trans Cys-Pro conformers are populated. In acyclic precursor **2**, the major C^β and C^γ resonances appear at δ 28.9 and 25.2, respectively, in $CDCl_3$, while minor resonances are detected at δ 31.0 and 23.3. These results suggest that while cis X-Pro conformers are populated in **2**, cyclization results in an almost exclusive preference for the trans geometry in **1**.

Delineation of Hydrogen-Bonded NH Groups. Hydrogen-bonded or solvent-shielded NH groups in peptides are frequently identified by establishing the solvent and temperature dependences of chemical shifts and by measuring the rates of hydrogen–deuterium (H–D) exchange (Hruby, 1974; Pitner & Urry, 1972; Stern et al., 1968). The results of solvent titration experiments in $CDCl_3$–$(CD_3)_2SO$ are represented in Figure 4b. While Cys(1) NH moves rapidly downfield on increasing the $(CD_3)_2SO$ concentration, the other NH groups are less affected. Figure 4a establishes the temperature dependences of NH chemical shifts in C_6D_6 and $(CD_3)_2SO$. The values of the temperature coefficients ($d\delta/dT$) are summarized in Table I. The $d\delta/dT$ values in $(CD_3)_2SO$ should be viewed with caution, since the disulfide slowly undergoes cleavage in this solvent. C_6D_6 has recently been shown to be a good solvent for delineation of solvent-shielded and -exposed NH groups (Venkatachalapathi & Balaram, 1981). Cys(4) NH and Val

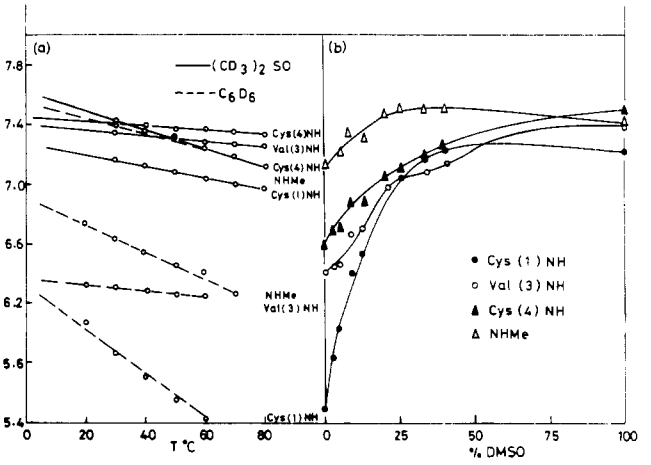

FIGURE 4: (a) Temperature dependence of NH chemical shifts in **1** in $(CD_3)_2SO$ and C_6D_6. (b) Solvent dependence of NH chemical shifts in $CDCl_3$–$(CD_3)_2SO$ mixtures.

NH have low $d\delta/dT$ values of 3.6×10^{-3} and 1.4×10^{-3} ppm/°C in C_6D_6, while Cys(1) and methylamide NH resonances have large $d\delta/dT$ values of 11.4×10^{-3} and 7.3×10^{-3} ppm/°C, respectively. In $(CD_3)_2SO$ also Cys(4) and Val NH resonances show $d\delta/dT$ values of 1.9×10^{-3} and 2.0×10^{-3} ppm/°C, which are characteristic of solvent-shielded NH groups. Cys(1) and methylamide NH groups show values of 3.8×10^{-3} and 5.8×10^{-3} ppm/°C, suggesting that they are solvent exposed. H–D exchange studies in $CDCl_3$–D_2O yielded exchange half-lives ($t_{1/2}$) of >96 h for the Val and Cys(4) NH group, ~72 h for NHMe, and a relatively low value of 24 h for Cys(1) NH. While H–D exchange rates in mixed solvent systems may be difficult to interpret quantitatively, the results suggest that Cys(1) NH is relatively more exposed to the solvent than the remaining NH groups. The temperature coefficient data in C_6D_6 and $(CD_3)_2SO$ lead to the conclusion that the Cys(4) and Val NH groups are strongly solvent shielded, in general agreement with the H–D exchange and solvent titration experiments.

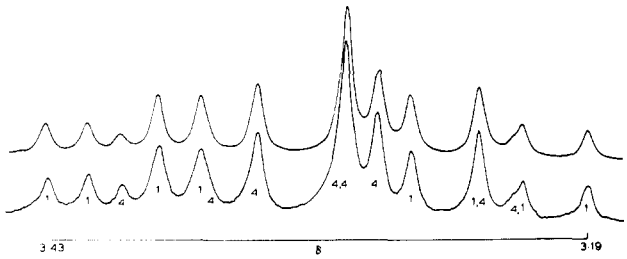

FIGURE 5: $C^{\beta}H_2$ resonances of Cys(1) and Cys(4) at 270 MHz. (Top) Experimental spectrum; (bottom) computer-simulated spectrum.

In the acyclic precursor **2** the $d\delta/dT$ values of all NH resonances are considerably higher than those for **1**, in both C_6D_6 and $(CD_3)_2SO$ (>5 × 10^{-3} ppm/°C in $(CD_3)_2SO$ and >7 × 10^{-3} ppm/°C in C_6D_6), suggesting that intramolecularly hydrogen-bonded structures are not significantly populated for **2** in these solvents.

Spin–Spin Coupling Constants. In conformationally rigid peptides information about the backbone ϕ angle may be readily derived from the vicinal $J_{HNC^{\alpha}H}$ coupling constant. Inferences about χ angles (side-chain conformations) may be drawn from $J_{HC^{\alpha}C^{\beta}H}$ values (Bystrov, 1976). The $J_{HNC^{\alpha}H}$ values for the Cys and Val residues of **1** and **2** are listed in Table I. In **1** the $C^{\alpha}H$–$C^{\beta}H_2$ protons of the two Cys residues form overlapping ABX spin systems, if the NH protons are exchanged for deuterium. The AB subspectral region for the $C^{\beta}H_2$ protons of Cys(1) and Cys(4) in $CDCl_3$ is shown in Figure 5. Unambiguous assignment of the transitions due to the individual Cys residues is possible as described earlier. Chemical shifts and coupling constants were derived by using the iterative program ITRCAL. The excellent agreement between the calculated and experimental spectra is shown in Figure 5. The coupling constants obtained are the following: Cys(1), J_{AX} = 4.9, J_{BX} = 7.9, and J_{AB} = 13.0 Hz; Cys(2), J_{AX} = 3.8, J_{BX} = 10.2, and J_{AB} = 15.4 Hz.

In view of the low barriers to rotation about the C^{α}–C^{β} bond, dynamic averaging of J values is possible. In such a case conformer populations may be estimated, using the treatment of Pachler (1964). Alternatively, if a fixed conformation is assumed, the χ values may be estimated from Karplus relationships (Karplus, 1959). Frequently "intuition rather than physical measurement is often used to decide whether rotational isomerism or a fixed angle pertains, and the appropriate form of analysis is then used" (Wyssbrod et al., 1977). The J values of 10.2 and 3.8 Hz for Cys(4) are close enough to extreme values to be suggestive of a fixed conformation, with one trans and one gauche $H_{\alpha}H_{\beta}$ relationship ($\chi \sim -60°$ or $\chi \sim +60°$). For Cys(1), the $J_{\alpha\beta}$ values (7.9 and 4.8 Hz) are more indicative of a conformation distorted from a perfectly staggered geometry or of the possibility of dynamic averaging. If a fixed conformation is assumed, χ^1_1 values close to the unfavorably eclipsed conformation of −120° are obtained. By use of the treatment of Pachler (1964), with J_{trans} = 13.56 and J_{gauche} = 2.6 Hz, the conformer populations estimated are the following: $\chi^1_1 = -60°$, 20%; $\chi^1_1 = 180°$, 48%; $\chi^1_1 = +60°$, 32%. Thus, it appears that flexibility may exist at the Cys(1) residue.

Circular Dichroism and Raman Studies. The CD spectrum of disulfide **1** in trifluoroethanol is shown in Figure 6. A weak +ve band around 270 nm, a −ve band of moderate intensity around 228 nm, and a strong +ve band below 210 nm are observed. The +ve and −ve bands at 270 and 228 nm are due to the n_a–σ^* and n_b–σ^* transitions of the S–S chromophore, while the band below 210 nm is probably due to the amide groups. Bands due to β-turn peptide conformations are expected ≤225 nm (Woody, 1974; Kawai & Fasman, 1978). Carmarck & Neubert (1967) have studied CD spectra of well-defined cyclic disulfides (in which dissymmetry is imposed solely by the disulfide twist) and observed that disulfides give two n–σ^* bands of opposite sign, above and below 250 nm. A −ve band above 250 nm and a +ve band below 250 nm have been observed for a left-handed twist of the disulfide, while a +ve band above 250 nm and a −ve band below 250 nm are seen for a right-handed twist. Boyd (1972), using molecular orbital calculations, has proposed a correlation between χ_{SS} and CD band position. The theoretical predictions have received some experimental support in studies of (2,7-cystine)-gramicidin S (Ludescher & Schwyzer, 1971). The CD data for disulfide **1** indicate a right-handed twist and are compatible with $\chi_{SS} \sim +60°$ or +120°.

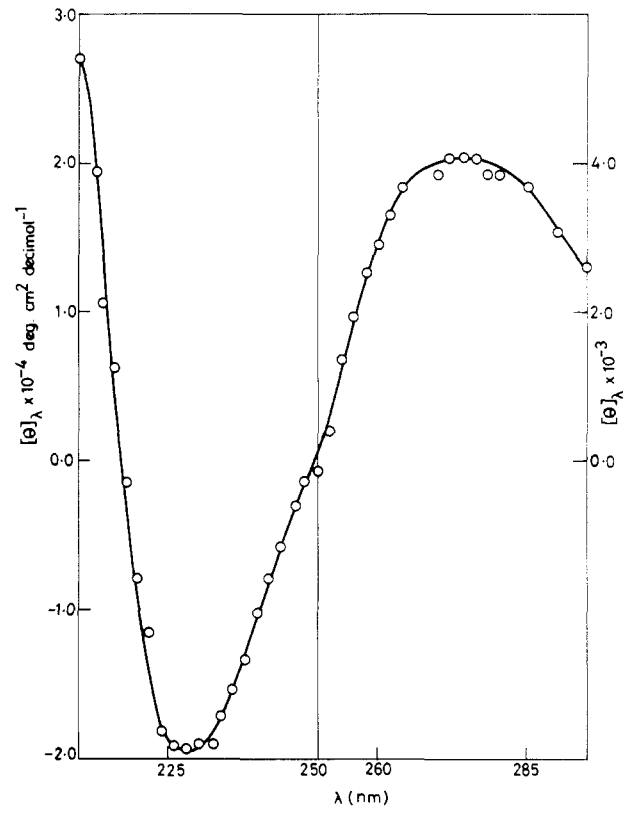

FIGURE 6: CD spectrum of **1** in trifluoroethanol. Concentration 1.9 mM. Note differences in ellipticity scales above and below 250 nm.

The laser Raman spectrum of disulfide **1** has been reported (Ishizaki et al., 1981). The major S–S band is observed at 522 cm^{-1}, with shoulders at 549 and 504 cm^{-1}. This is consistent with a gauche–gauche–trans (*ggt*) or trans–gauche–gauche (*tgg*) arrangement for the C^{α}–C^{β}–S–S–C^{β}–C^{α} segment. Minor conformations *tgt* and *ggg* may give rise to the observed shoulders at 549 and 504 cm^{-1}, respectively (Van Wart & Scheraga, 1976a,b). An examination of molecular models shows that the right-handed twist of the disulfide ($\chi_{SS} \sim +60°$ or +120°), as suggested by CD, can easily accommodate both *ggt* and *tgg* conformations. By use of the correlation suggested by Van Wart & Scheraga (1976a,b), the Raman band position of 522 cm^{-1} suggests a χ_{SS} value of ~90 ± 20°, in broad agreement with CD studies. The positions of the amide I band at 1668 cm^{-1} and the amide III band at 1267 cm^{-1} are suggestive of the presence of β-turn conformations (Ishizaki et al., 1981; Fox et al., 1981).

Conformation of **1**. The spectroscopic studies described above lead to the following conclusions: (1) The Cys(4) and Val NH groups are strongly solvent shielded. (2) For Cys(4)

Table II: Structural Parameters and Energies for Lowest Energy Conformations of Disulfide **1**

conformational parameters	type A			type B						type C
$\psi_{Cys\ (1)}$ (deg)	70	80	90	70	80	90	80	100	90	30
ϕ_{Pro} (deg)	−70	−70	−70	−70	−70	−70	−70	−70	−60	−70
ψ_{Pro} (deg)	−20	−20	−30	−20	−20	−30	−10	−30	−50	−30
ϕ_{Val} (deg)	−70	−70	−70	−70	−80	−70	−90	−80	−50	−70
ψ_{Val} (deg)	−30	−20	−10	−30	−10	−10	−30	−10	−30	−20
$\phi_{Cys\ (4)}$ (deg)	−60	−80	−90	−130	−160	−160	−140	−170	−150	−70
$\chi^1_{Cys\ (1)}$ (deg)	200	181	190	200	190	190	210	190	180	217
$\chi^2_{Cys\ (1)}$ (deg)	230.5	222.6	218.1	230.7	214.8	218.3	210.4	200.3	113.1	−27.4
$\chi^1_{Cys\ (4)}$ (deg)	−80	−76	−80	80	90	80	80	80	80	−53
$\chi^2_{Cys\ (4)}$ (deg)	82.2	82.3	84.9	−82.3	−72.7	−69.8	−64.6	−48.1	108.5	164.3
χ_{SS} (deg)	65.9	77.5	69.2	128.5	129.7	127.8	109.1	110.8	119.9	−88.5
C^α–C^β–S–S–C^β–C^α	*tgg*$^+$	*tgg*$^+$	*tgg*$^+$	*tgg*$^-$	*tgg*$^-$	*tgg*$^-$	*tgg*$^-$	*tgg*$^-$	*tgg*$^-$	*g*$^-$*g*$^-$*t*
S–S (Å)	2.04	2.03	2.04	2.04	2.04	2.03	2.02	2.02	2.06	2.03
$C^\beta_{Cys\ (1)}$–S–S (deg)	108.3	114.0	99.7	107.1	108.1	98.5	103.3	103.6	113.1	108.0
S–S–$C^\beta_{Cys\ (4)}$ (deg)	113.4	102.1	101.6	113.3	100.6	109.8	113.9	106.0	108.5	112.0
$N_{Cys\ (4)}$···$O_{Cys\ (1)}$ (Å)	3.11	3.05	3.07	3.11	2.98	3.07	3.04	3.07	3.01	3.11
H–$N_{Cys\ (4)}$–$O_{Cys\ (1)}$ (deg)	26.9	17.7	19.7	26.9	17.2	19.8	33.3	23.7	34.1	25.8
total energy (kcal mol^{-1})	−33.0	−33.5	−31.9	−32.4	−31.9	−31.8	−31.3	−31.0	−31.0	−30.8

χ^1_4 is fixed at −60° or +60°, while there appears to be some conformational freedom at χ^1_1 [Cys(1)]. (3) A right-handed twist is favored for the –S–S bond, with a *tgg* or *ggt* arrangement about the C^α–C^β–S–S–C^β–C^α segment.

The NMR parameters suggest that Cys(4) NH is involved in an intramolecular hydrogen bond. A Pro(2)-Val(3) β turn, stabilized by a 4 → 1 hydrogen bond between Cys(4) NH and Cys(1) CO, would be consistent with the tendency of Pro residues to occupy the $i + 1$ position in β turns (Zimmerman & Scheraga, 1977; Chou & Fasman, 1977). While the Val residue has a relatively low probability of occurring in a β turn, in the disulfide **1**, cyclization imposes an additional constraint. In this context it is worth noting that where alternative reverse turn structures are feasible, the Pro-Val sequence does not adopt a β-turn conformation, even in a cyclic peptide like gramicidin S (Schwyzer, 1959; Stern et al., 1968; Hull et al., 1978). The Pro-Val β turn in **1** probably falls into the type I category ($\phi_{Pro} \sim -60°$, $\psi_{Pro} \sim -30°$, $\phi_{Val} \sim -90°$, and $\psi_{Val} \sim 0°$) (Venkatachalam, 1968). The $J_{HNC^\alpha H}$ values observed for the Val NH (Table I) are consistent with this ϕ value. Interestingly, the Val $J_{HNC^\alpha H}$ value shows a significant solvent dependence (7.3 Hz in C_6D_6 and 10.3 Hz in $CDCl_3$). However, the Karplus curve is rather steep in the vicinity of $\phi \sim -90°$. Therefore, even small changes in ϕ would lead to significant changes in J values (Bystrov, 1976).

The significant shielding of Val NH from the solvent may be due to its participation in a hydrogen bond or due to steric factors, which render it inaccessible to solvent. Intramolecular hydrogen-bonded structures that need to be considered are a Cys(1)-Pro(2) β turn or a C_7 turn at Pro(2). The former is unlikely, since X-Pro β turns are energetically highly unfavorable when X is an L-amino acid (Zimmerman & Scheraga, 1977). This is further borne out by the subsequent theoretical study. The C_7 turn is unlikely if Pro is already part of a type I β turn as suggested above. For further evaluation of the conformational preferences of **1**, a theoretical analysis was undertaken.

Theoretical Conformational Analysis. The energetics of different hydrogen-bonded structures were considered for **1** (see Materials and Methods). In model I, a single 4 → 1 hydrogen bond was imposed as a necessary criterion between Cys(4) NH and Cys(1) CO. In model II 4 → 1 hydrogen bonding was imposed as a condition between Cys(1) CO and Cys(4) NH and Val(3) NH and the Boc CO group. The ten lowest energy conformations along with selected structural parameters are listed in Table II. It is found that cyclization along with formation of Cys(4) NH···CO Cys(1) hydrogen bond demands $\psi_{Cys(1)} > 0°$. For all the minimum energy structures $\psi_{Cys(1)} > 30°$. This observation allows us to discriminate against model II, in which a further constraint of a Boc CO···Val NH hydrogen bond is introduced. For a good hydrogen bond in this case $\psi_{Cys(1)} \sim -30°$, which is a poor situation for cyclization. Introduction of nonplanar distortions of the peptide units ($\Delta\omega \pm 10°$) relieves the unfavorable steric contacts to some extent. However, all stereochemically ac-

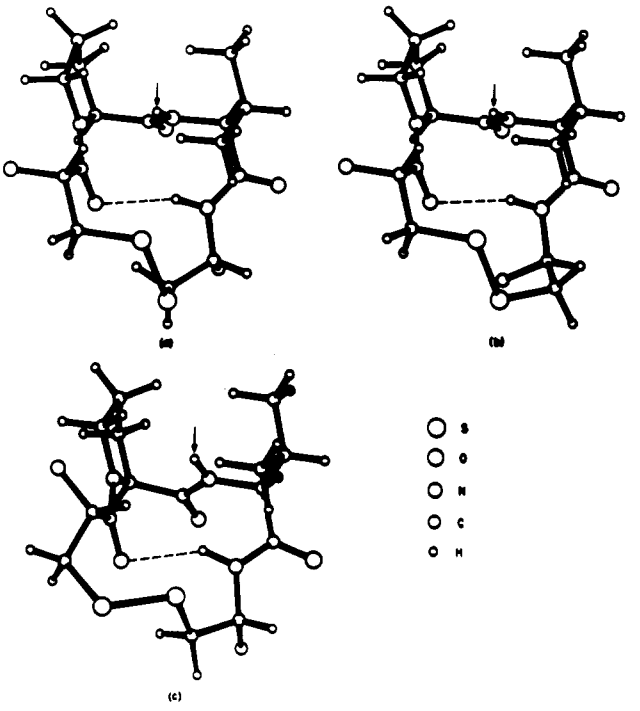

FIGURE 7: Perspective diagrams of three classes of theoretically determined low-energy conformations of **1** (see Table II). (a) Type A (−33.5 kcal mol^{-1}); (b) type B (−32.4 kcal mol^{-1}); (c) type C (−30.8 kcal mol^{-1}). The Val NH group is indicated by an arrow in each case.

ceptable model II conformations had energies very much higher than model I structures.

From Table II, it may be seen that three classes of conformations may be considered for **1**: type A, $\phi_{Cys(4)} \sim -70°$, $\chi^1{}_4 \sim -80°$; type B, $\phi_{Cys(4)} \sim -150°$, $\chi^1{}_4 \sim +80°$; type C, $\phi_{Cys(4)} \sim -70°$, $\chi^1{}_4 \sim -50°$. While types A and B have χ_{SS} as positive, type C has negative χ_{SS}. The type A conformations are slightly more favorable energetically, but both type A and type B structures are compatible with the spectroscopic results. The perspective diagrams of the three classes of conformations are shown in Figure 7. In all the cases the Val NH is sterically shielded by the Pro and Val side chains, a feature that accounts for the inaccessibility of this group to the solvent.

The stabilization of β-turn structures within cyclic peptides is a very well-established feature (Smith & Pease, 1980). Recent reports have described the use of ϵ-aminocaproyl residues to generate cyclized β-turn models (Deslauriers et al., 1979). The present study establishes that such chain reversals can be facilitated by disulfide formation, a feature important in several biologically active peptides. Conformationally constrained, small ring, cyclic peptide disulfides could serve as important model systems for the simultaneous spectroscopic characterization of β turns and disulfide linkages. Novel structural (Prasad et al., 1981) and spectroscopic properties (Mathew et al., 1981) have already been described for the constrained peptide disulfide Boc-Cys-Pro-Aib-Cys-NHMe.
S–S

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Swine Pepsinogen Folding Intermediates Are Highly Structured, Motile Molecules†

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ABSTRACT: Refolding of urea- or alkali-unfolded swine pepsinogen occurs by rapid formation of partially folded intermediates (I_s) which are slowly converted into the native protein (N). This slow reaction involves isomerization of proline residues in the protein to the configurations occurring in N. Kinetic studies on changes in absorbance or circular dichroism indicate I_s to be close to the native structure, while fluorescence and hydrogen exchange measurements show I_s to be much more open to solvent than N. Fluorescent probe binding suggests that I_s has a more hydrophobic surface than N. These contrasting results are interpreted to show that the presence of wrong proline residues does not greatly inhibit the overall folding of pepsinogen but prevents close packing of structural elements into the highly cooperative, stable, native form. I_s may be very similar to N in average structure, but is a much more fluctuating species.

Unfolding and refolding of low molecular weight proteins have recently been the subject of extensive studies, mainly by Baldwin and his group [reviewed in Kim & Baldwin (1982)]. These investigations have revealed a general mechanism (eq 1), in which the native protein (N)[1] rapidly equilibrates with

$$N \underset{K_u}{\leftrightarrow} U_f \rightleftharpoons U_s \quad (1)$$

a fast-folding unfolded form (U_f), which is in slow equilibrium with a mixture of slowing folding forms (U_s). It was originally proposed that these arose by cis–trans isomerization of proline residues in U_f (Brandts et al., 1975), but in some proteins, U_s could arise by configurational changes at peptide bonds not involving proline. Three such bonds have recently been detected in the high energy cis form in carboxypeptidase A (Rees et al., 1981). The kinetics of isomerization of all peptide bonds are very alike (Stewart & Siddall, 1970). With increasing time under unfolding conditions, the rate of refolding to N becomes limited by reversal of these slow reactions at "essential" residues, which must have the correct configurations for formation of N. Under strongly denaturing conditions, N is converted to U_f in a rapid reaction, but formation of U_s can be demonstrated by the double-jump procedure, in which protein is jumped to unfolding conditions, held for a variable delay time, and then returned to native conditions. The kinetics of refolding are found to vary with the delay time in a manner consistent with eq 1. When the native protein is only marginally unstable, the kinetics of unfolding are biphasic, corresponding to the two coupled steps in this mechanism (Brandts et al., 1975). Computer simulations by Creighton (1978) have shown that for high molecular weight proteins containing many prolines, cis–trans isomerization may drive unfolding to completion even when N is considerably more stable than U_f, because of the large number of U_s forms. Calculated unfolding curves showed slow, single phase kinetics with a rate constant which was essentially proportional to K_u, the equilibrium constant of the unfolding reaction (eq 1). These simulations also showed the dramatic effect of large numbers of proline residues in slowing down the rates of folding of such proteins. Since slow folding may be a disadvantage to a protein under physiological conditions, Creighton speculated on ways that the rate of folding of large proteins could be accelerated. He suggested intramolecular catalysis of proline isomerization during folding, division of large proteins into smaller independently folding domains, each containing fewer prolines, or rapid formation of partially folded intermediates containing incorrect proline isomers.

Swine pepsinogen is a large protein (M_r 40 000) containing a large number (i.e., 18) of prolines (Tang, 1976) which can be reversibly unfolded. Thus, it forms a good subject for the investigation of the mechanism of folding of such species. Previous studies which followed the ionization of tyrosine residues, normally buried in the native protein, and also the variation of potential pepsin activity indicated that pepsinogen unfolded very rapidly at pH 11.5 but was then slowly transformed into slowly refolding forms (U_s). The rate of this slow transformation was independent of pH and showed a temperature dependence characteristic of proline isomerization (McPhie, 1982). In contrast, unfolding by urea, at neutral pH, followed by absorbance and activity, was a slow reaction whose rate was dependent on pH, temperature, and urea concentration. The product of this reaction always refolded slowly (McPhie, 1980). The enthalpies of activation of the rates of unfolding and refolding were again consistent with the involvement of proline isomerization in the rate-limiting

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[1] Abbreviations: U_s and U_f, slow- and fast-folding forms of pepsinogen, respectively; I_s and I_n, folding intermediates; N, native form of pepsinogen; ANS, 8-anilino-1-naphthalenesulfonate; Tris, tris(hydroxymethyl)aminomethane.