STABILIZATION OF $\beta$-TURN CONFORMATIONS IN PRO-X SEQUENCES BY DISULPHIDE BRIDGING.
SYNTHESIS AND SOLUTION CONFORMATIONS OF FIVE CYCLIC CYSTINE PEPTIDES

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(Received in the UK 25 July 1983)

Abstract—Five cyclic peptide disulphides of the type Boc-Cys-PRO-X-Cys-NHMe have been synthesized, where X = Gly (1), L-Ala (2), D-Ala (3), Aib (4) and L-Leu (5). $^1$H NMR studies at 270 MHz in CDCl$_3$ and (CD$_3$)$_2$SO provide evidence of a Pro-X $\beta$-turn conformation, stabilized by a transannular 4$\cdots$1 hydrogen bond involving the Cys(4) NH, in all the peptides. In addition peptides 2, 4 and 5 also possess a second intramolecular hydrogen bond involving the -NHMe group. The spectroscopic data are consistent with a consecutive type III $\beta$-turn conformation for peptides 2, 4 and 5, a type I(III) $\beta$-turn structure for 1 and a type II $\beta$-turn structure for 3.

$\beta$-Turn conformations$^1$-$^4$ and disulphide linkages$^5$ are important elements of peptide and protein structures.$^6$-$^7$ The structural diversity and wide spectrum of biological activity of natural and synthetic peptides has provided a powerful impetus for the development of the experimental$^8$-$^9$ and theoretical$^{10}$ methods of peptide conformational analysis. The refinement of spectroscopic methods of conformational analysis, necessarily requires the availability of well defined model peptides. Cyclic peptides have proved attractive systems in view of their relatively restricted range of conformations.$^{11}$-$^{12}$ Cyclic peptides incorporating stereochromically constrained residues like, $\alpha$-aminoisobutyric acid (Aib)$^*$ have been developed more recently as conformational models.$^{13}$-$^{16}$

In this report, we describe the synthesis and spectroscopic analysis of a series of cyclic peptide disulphides (Fig. 1), in which an L-Pro-X sequence separates two Cys residues. The choice of the Pro-X sequence was dictated by the high probability of $\beta$-turn formation.$^{17}$ The disulphide bridge provides a means of "covalently locking" the $\beta$-turn structure and additionally allows these peptides to be applied to the development of CD$^{*}$,$^{19}$ and Raman spectroscopy,$^{25}$-$^{22}$ in determining the stereochemistry of the $\cdots$S$\cdots$ linkage. The 14-membered disulphide loop is expected to possess only limited structural flexibility, thereby generating conformationally well defined peptides.$^{23}$ The results establish that these cyclic peptide disulphides provide models for type I, II and consecutive type III $\beta$-turns.$^1$

RESULTS

Synthesis and characterization

The peptide disulphides were synthesized using the classical oxidative cyclization of the peptide dithiols, carried out under conditions of high dilution.$^{24}$ Peptides 1-5 were obtained as homogeneous solids and thus far single crystals, suitable for X-ray diffraction have been obtained in the case of the Pro-Aib (4) and Pro-Leu (5) disulphides. The mononeric nature of the cyclic disulphides was established by mass spectrometric observation of the molecular ion, using field desorption or fast atom bombardment mass spectrometry.$^{25}$ Elemental analysis and 270 MHz $^1$H NMR spectra were fully consistent with the structures. Further confirmation of the presence of the 14-membered disulphide ring in 4 has been obtained by X-ray diffraction.$^{26}$-$^{27}$ The yields of the cyclic peptide disulphides were low, ranging from 12 to 25% (Table 1). Side reactions involving cleavage of Cys-Pro bonds during the reductive cleavage of S-benzyl groups by Na/lqiquid NH$_2^+$ and the formation of polymeric products during the subsequent oxidation, presumably account for the poor yields. An attempt to establish a correlation between possible Pro-X $\beta$-turn formation in the acyclic precursor and yields of cyclization did not result in a fruitful correlation.

$^1$H NMR studies

Figure 3 shows a representative 270 MHz $^1$H NMR spectrum of the Pro-Gly disulphide 1 in CDCl$_3$. $^1$H NMR studies on disulphide 4 have been described earlier, in conjunction with X-ray diffraction studies.$^{27}$ Extremely well resolved spectra were obtained in all cases permitting unambiguous assignment of resonances. In all five peptides the Cys(1)NH could be unequivocally assigned to the high field NH ($\sim 5.5$) in CDCl$_3$, in view of the known tendency of urethane NH groups to appear at high field in this solvent.$^{29}$-$^{30}$ The X$\cdots$NH groups in 1 (X = Gly) and 4

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Footnotes:

$^*$Abbreviations used: Aib, $\alpha$-aminoisobutyric acid; Boc, t-butyloxy carbonyl; DCC, N,N'-dicyclohexyl carbodimide; Bz, benzyl; DMF, N,N-dimethylformamide; THF, tetrahydrofuran; NMM, N-methylmorpholine; NOE, Nuclear Overhauser Effect. Where configuration is not indicated, L-amino acids are implied.
Table 1. Characterisation of cyclic peptide disulphides Boc-Cys-Pro-X-Cys-NHMe

<table>
<thead>
<tr>
<th>X</th>
<th>Yield (%)</th>
<th>Mass Spectrum</th>
<th>MP (°C)</th>
<th>Elemental Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M⁺</td>
<td>M⁺-H⁺</td>
<td>A</td>
</tr>
<tr>
<td>Gly</td>
<td>25.4</td>
<td>-</td>
<td>490²</td>
<td>140-149</td>
</tr>
<tr>
<td>Ala</td>
<td>13.6</td>
<td>505²</td>
<td>504⁰</td>
<td>227-228</td>
</tr>
<tr>
<td>Ala</td>
<td>15.9</td>
<td>503²</td>
<td>504⁰</td>
<td>198-199</td>
</tr>
<tr>
<td>Aib</td>
<td>12.2</td>
<td>517²</td>
<td>516⁰</td>
<td>188-190</td>
</tr>
<tr>
<td>Leu</td>
<td>15.3</td>
<td>545²</td>
<td>-</td>
<td>155-157</td>
</tr>
</tbody>
</table>

a) A = 15% Methanol in chloroform; B = Ethylacetate-pyridine-acetic acid-water (5:5:1:1).
b) Analytical Data computed with a molecule of (CD₃)₂SO
c) Field Desorption
d) Fast Atom Bombardment
e) Electron impact
f) C = 0.2, CH₂OH

(X = Aib) are unambiguously recognized as triplet and singlet resonances, respectively. In peptides 2, 3 and 5 both the Cys(4) and X-NH groups are doublets. Unequivocal assignments were made by double resonance experiments, which establish the connectivities between C₆H₂, C₆H and NH resonances; the C₆H₂, Cys resonances being clearly distinct from the Ala C₆H₃ or Leu C₆H₂ peaks. The methylamide NH resonances are readily identified as broad quartets. Assignments in (CD₃)₂SO were made on the basis of spin decoupling and by monitoring NH chemical shifts in mixtures of CDCl₃ and (CD₃)₂SO. The chemical shifts and JH-NH values for the NH groups in peptides 1-5 are listed in Table 2.

Delineation of hydrogen bonded NH groups

The presence of solvent shielded or intramolecularly hydrogen bonded NH groups was established using three criteria: (i) temperature dependence of NH chemical shifts in (CD₃)₂SO, (ii) solvent dependence of NH chemical shifts CDCl₃-(CD₃)₂SO mixtures and (iii) rates hydrogen-deuterium (H-D) exchange in CDCl₃-D mixtures. A typical H-D exchange experiment is shown in Fig. 3 (inset) for peptide 1. It is clear that the exchange rates follow the order Gly(3)NH > Cys(1)NH > NHMe > Cys(4)NH. The Cys(4) and methylamide NH protons exchange significantly slower than the other two, indicative of their relative inaccessibility to solvent. A similar order of H-D exchange rates is observed in the other peptide disulphides (2-5) although the precise value of the exchange half lives (t₁/₂) vary. The use of heterogeneous solvent system precludes comparison between different sets of peptides. No attempt has been made to quantify the H-D exchange data since a qualitative ordering of NH groups is sufficient for delineating solvent shielded NH groups.

The results of experiments to determine the solvent and temperature dependences of NH chemical shift...
Table 2. $^1$H NMR parameters of NH group in the peptides Boc-Cys-Pro-X-Cys-NHMe

<table>
<thead>
<tr>
<th>X</th>
<th>$\delta_{CDCl_3}$ (ppm)</th>
<th>$\Delta \delta^a$ (ppm)</th>
<th>$\delta_{CDCl_3}$ (ppm)</th>
<th>$\Delta \delta^a$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>5.61</td>
<td>7.55</td>
<td>6.82</td>
<td>7.15</td>
</tr>
<tr>
<td>L-Ala</td>
<td>5.44</td>
<td>7.26</td>
<td>6.55</td>
<td>6.35</td>
</tr>
<tr>
<td>D-Ala</td>
<td>5.72</td>
<td>7.67</td>
<td>6.97</td>
<td>7.13</td>
</tr>
<tr>
<td>Aib</td>
<td>5.35</td>
<td>7.43</td>
<td>6.78</td>
<td>6.42</td>
</tr>
<tr>
<td>Leu</td>
<td>5.4</td>
<td>7.22</td>
<td>6.54</td>
<td>6.2</td>
</tr>
</tbody>
</table>

In peptides 2(-Ala) and 3(-Ala) are shown as illustrative examples in Fig. 4. The values of the temperature coefficients ($d\delta/dT$) and solvent shift values ($\Delta \delta$) for the NH groups in the various peptides are summarized in Table 2. In the Pro–Gly (1) and Pro–D–Ala (3) disulphides only the Cys(4)NH group appears to be shielded from the solvent ($d\delta/dT < 0.004$ ppm/$^\circ$C) presumably by involvement in an intramolecular hydrogen bond. In the Pro-L-Ala (2) and Pro-L-Leu (5) disulphides two NH groups, Cys(4) and NHMe, show evidence for being hydrogen bonded. In the Pro-Aib (4) disulphide, Cys(4)NH is strongly solvent shielded, while NHMe has an intermediate $d\delta/dT$ value in (CD$_3$)$_2$SO (0.0034 ppm/$^\circ$C). In all peptides the $d\delta$ values for the Cys(1) and X-NH groups are large (1.5–2.0 ppm), whereas the Cys(4)NH exhibits very little solvent dependence ($\Delta \delta < 0.3$ ppm). The NHMe group has an intermediate $\Delta \delta$ value for all five peptides (0.8–1.1 ppm), suggesting that the solvent titration experiments may be less reliable in differentiating free and hydrogen bonded terminal methylamide groups.

Further evidence for the presence of intramolecular hydrogen bonded conformations was obtained from IR studies in CHCl$_3$ solution, at peptide concentrations of $\sim 3 \times 10^{-3}$ M (Fig. 5). The presence of the broad intense band at $< 3350$ cm$^{-1}$ is indicative of the presence of intramolecularly hydrogen bonded NH groups. In both $^1$H NMR and IR studies, effects due to peptide association have been disregarded for the following reasons: (i) non-specific aggregation effects tend to equalize the $d\delta/dT$ values for a set of NH groups in a peptide; (ii) in both NMR and IR studies, clear distinctions were obtained between the spectroscopic parameters ($d\delta/dT$, $\Delta \delta$ and area of the $\nu_{NH}$ (bonded) band) of the acyclic di–S-benzyl precursors and the cyclic peptide disulphides.
results suggest that in all five peptide disulphides, the Cys(4) NH is inaccessible to solvent. This observation is consistent with the involvement of the Cys(4)NH in a transannular 4+1 intramolecular hydrogen bond with the Cys(1)Co group, stabilizing a Pro-X β-turn (Fig. 6). The conformational angles in the ideal Pro-X-β-turns are type I \( \delta_{\rho_{\alpha}} = -60^\circ, \psi_{\rho_{\alpha}} = -30^\circ \), \( \phi_{\alpha} = -90^\circ, \psi_{\alpha} = 0^\circ \); type II \( \delta_{\rho_{\alpha}} = -60^\circ, \psi_{\rho_{\alpha}} = 120^\circ \), \( \phi_{\alpha} = 80^\circ, \psi_{\alpha} = 0^\circ \); type III \( \phi_{\rho_{\alpha}} = \phi_{\alpha} = -60^\circ, \psi_{\rho_{\alpha}} = \psi_{\alpha} = -30^\circ \). Note that type III turns are only a slight variant of the Type I conformation. This conformation is in accord with the established propensity of Pro-X sequences to adopt β-turn struc-

DISCUSSION

The number of intramolecular hydrogen bonds and the conformations consistent with the NMR data for peptides 1-5 are listed in Table 2. The NMR

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Fig. 4. Temperature and solvent dependence of NH chemical shifts in peptide disulphides (bottom) 2 and (top) 3.

The acyclic peptides, with the exception of Boc-Cys(SBz)-Pro-Aib-Cys(SBz)-NHMe, did not show evidence for the presence of intramolecularly hydrogen bonded NH groups. Aggregation effects, particularly via intermolecular β-sheet formation in organic solvents, would be expected to be more facile for the acyclic peptides. In the case of the Pro-Aib acyclic peptide, the presence of two stereochemically-constrained amino acids, would favour consecutive β-turn formation for the Pro-Aib-Cys-sequence in solution, a feature demonstrated in several cases in related peptides.

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Fig. 5. IR spectra of -Pro-X- disulphides ( \( \sim 3 \times 10^{-3} \)M) in CHCl₃. Only ν₅ stretching bands are shown.

Fig. 6. (top) Pro-X β-turn conformation proposed for peptides 1 and 3 (bottom consecutive β-turn conformation proposed for peptides 2, 4 and 5.)
sequence the bulky B-branched Val residue, lowers the probability of consecutive \( \beta \)-turn formation. In that sequence has often been used as a diagnostic for folded structures.\(^{27} \) Magnetic non-degeneracy of the Gly \( \text{CH} \) chemical shifts (at 230 MHz) precluded such an analysis. Magnetic non-degeneracy of the Gly \( \text{CH} \) and the X-NH resonances is expected for Pro-X structures by NOE and CD methods is described elsewhere.\(^{48} \)

The general scheme adopted is summarized in Fig. 2. All peptides were characterized by \( ^1 \text{H} \) NMR and checked for purity by TLC on silica gel. Elemental analyses were also carried out on the acyclic and cyclic tetrapeptides. The detailed protocol is described below for I.

**Synthesis of disulfide I**

**Boc-Cys(SBz)-NHMe.** Boc-Cys(SBz)-OH\(^{32} \) (3.1 g, 10 mmol) dissolved in anhyd THF (20 ml) was cooled in a freezing mixture (\(-15^\circ\text{C}\)). N-Methylmorpholine (1.1 ml) was added, and the mixture stirred for 2 hr. Insoluble salts were filtered off, the THF was evaporated, and the residue was dissolved in EtOAc and washed successively with \( 1\text{N} \) HCl, \( 1\text{N} \) NaHCO\(_3\), and sat aq NaCl. The organic layer was dried over Na\(_2\)SO\(_4\) and evaporated to yield a white solid (3.1 g, 95\%), m.p. 123°C.

**Boc-Pro-Gly-Cys(SBz)-NHMe.** Boc-Cys(SBz)-NHMe (1.8 g, 5.5 mmol) was treated with dry THF saturated with HCl gas (10 ml). After Boc group removal was complete, as evidenced by TLC, the mixture was carefully neutralized with aqueous Na\(_2\)CO\(_3\) and extracted with CHCl\(_3\). The organic layer was dried over Na\(_2\)SO\(_4\), and the mixture stirred overnight in diethyl ether containing 3% diethylamine. The precipitate was filtered off and the filtrate was concentrated, and the residue was purified by flash chromatography (Silica gel) using hexanes:ether (95:5) to give a white solid (2.0 g, 85%).

**EXPERIMENTAL**

The **Synthesis of peptides**

The general scheme adopted is summarized in Fig. 2. All peptides were characterized by \( ^1 \text{H} \) NMR and checked for purity by TLC on silica gel. Elemental analyses were also carried out on the acyclic and cyclic tetrapeptides. The detailed protocol is described below for 1.
iated dicyclohexylurea was filtered off and the filtrate was washed with 1N HCl, 1N NaHCO
and H₂O. Drying and evaporation yielded the tripeptide as a white solid (2.1 g; 87%), m.p. 137°. The 270 MHz 'H NMR spectrum was fully consistent.

Boc-Cys(SBz)-Pro-Gly-Cys(SBz)-NHMe. Boc-Pro- Gly-Cys(SBz)-NHMe (2.0 g, 4.1 mmol) was deprotected with HCl/TIFH₂O as described above to give Pro-Gly-Cys(SBz)-NHMe as a colorless oil (yield 1.5 g, 97%). This was dissolved in 10 ml DMF, cooled to 0° and 1.2 g (4 mmol) Boc-Cys(SBz)-OH was added followed by 500 mg 1-hydroxybenzotriazole and 800 mg (4 mmol) DCC. The mixture was stirred overnight, the precipitated dicyclohexylurea filtered off, 30 ml EtOAc added and the product obtained after workup, as described above, as a white, hygroscopic solid, which slowly turns yellow on standing. Yield 2.5 g (97%). Column chromatography on silica gel with (3% CH₃OH-HCl) as the eluant needed the dicyclohexylurea was filtered off and the filtrate was

distilled liquid NH₃. Small pieces of metallic sodium (25.4%) of disulfide i. The physical characteristics are consistent.

Cyclic disulfide 1

2.0 g of acyclic tetrapeptide was dissolved in 400 ml of dry, distilled liquid NH₃. Small pieces of metallic sodium were added, while stirring vigorously until the blue color persisted. The excess of sodium was carefully destroyed by addition of a few drops of glacial acetic acid. The ammonia residue dissolved in 1800 ml H₂O 10.073% solution). The 270 MHz 'H NMR spectrum was fully consistent.

Synthesis of disulfides 2, 3, 4, 5

The disulfides 2-5 were synthesized using a protocol similar to that described for 1. The dipeptide acids Boc-Pro-X-OH were synthesized by the succinimide ester procedure, except for X = Alb which was prepared by saponification of Boc-Pro-Alb-OMe synthesized by the DCC/CH₂Cl₂ procedure. Except for X = Gly and Alb-1-hydroxybenzotriazole was used in the racemization suppressing step in the coupling of Boc-Pro-X-OH to Cys(SBz)-NHMe. In all cases the acyclic tetrapeptides were purified by silica gel column chromatography and fully characterized by elemental analysis and 270 MHz 'H NMR spectrum.

The yields of cyclization and various parameters characterizing the cyclic peptide disulfides are summarized in Table 1. All five disulfides gave extremely well resolved 270 MHz 'H NMR spectra. A representative spectrum is illustrated in Fig. 3.

Acknowledgements—We thank Dr S. Prasanna, Oregon Graduate Centre, for the mass spectral measurements. This research was supported by a grant from the Department of Science and Technology, Government of India. NMR studies were carried out at the Sophisticated Instruments Facility, Indian Institute of Science, Bangalore.

REFERENCES

1. C. M. Venkatachalam, Biopolymers 6, 1425 (1968).
Stabilization of \( \beta \)-turn conformations


