Cyclic Peptide Disulfides. Solution and Solid-State Conformation of Boc-Cys-Pro-Aib-Cys-NHMe, a Disulfide-Bridged Peptide Helix

A. Ravi, B. V. Venkataram Prasad, and P. Balaram*

Contribution from the Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India. *Received March 12, 1982*

Abstract: The solution and solid-state conformations of the peptide disulfide Boc-Cys-Pro-Aib-Cys-NHMe have been determined by NMR spectroscopy and X-ray diffraction. The Cys(4) and methylamide NH groups are solvent shielded in $CDCl_3$ and $(CD_3)_2SO$, suggesting their involvement in intramolecular hydrogen bonding. On the basis of known stereochemical preferences of Pro and Aib residues, a consecutive β-turn structure is favored in solution. X-ray diffraction analysis reveals a highly folded 3_{10} helical conformation for the peptide, with the S–S bridge lying approximately parallel to the helix axis, linking residues 1 and 4. The backbone conformational angles are Cys(1) $\phi = -121.1°$, $\psi = 65.6°$; Pro(2) $\phi = -58.9°$, $\psi = -34.0°$; Aib(3) $\phi = -61.8°$, $\psi = -17.9°$; Cys(4) $\phi = -70.5°$, $\psi = -18.6°$. Two intramolecular hydrogen bonds are observed between Cys(1) CO--HN Cys(4) and Pro(2) CO--HNMe. The disulfide bond has a right-handed chirality, with a dihedral angle (χ_{SS}) of 82°.

Disulfide bridges are an important structural feature of proteins and biologically active polypeptides.[1] The covalent cross-linking of two nonadjacent cysteine residues in a peptide chain results in the formation of well-defined loops of the type -Cys-$(X)_n$-Cys-, where X is an α-amino acid residue. If the number of intervening amino acids is large ($n \geq 4$), there is considerable conformational flexibility possible within the loop. However, if the number of amino acids separating the two Cys residues is small ($n < 4$), the cyclic structures formed will have significantly less conformational freedom and well-defined geometries may be obtained for the cyclic disulfide segments. While considerable effort has been directed toward the conformational analysis of 20-membered cyclic peptide disulfides ($n = 4$),[2] which constitute a key structural element of the hormones oxytocin and vasopressin,[3] very little attention has been centered on smaller ring peptide disulfides.[4-9] A systematic program to examine the conformational characteristics of 14-membered cyclic peptide disulfides ($n = 2$) has been undertaken in this laboratory. The presence of the -Cys-Gly-Pro-Cys- segment at the active site of the protein thioredoxin[10] provided an impetus for the study of the 14-membered cyclic disulfides. In order to substantially restrict backbone flexibility and to provide a rigid model system as a starting point, the amino acids proline[11] and α-aminoisobutyric acid (Aib)[12,13] were used as the spacer residues. This report describes the conformational analysis of the peptide Boc-Cys-Pro-Aib-Cys-NHMe (**1**) in solution and in the solid state.[14] The peptide backbone folds into a 3_{10} helical structure with an S–S bridge lying approximately parallel to the helix axis.

Experimental Section

1 was synthesized by solution-phase procedures using benzyl groups for thiol protection. Removal of *S*-benzyl groups in Na/liquid NH_3 was followed by oxidation with $K_3Fe(CN)_6$ in dilute solution at pH 6–7. Evaporation of water and silica gel chromatography of the residue yielded **1** as a homogeneous solid. The monomeric nature of the disulfide was established by mass spectral measurements (electron ionization M^+ = 517; fast atom bombardment MH^+ = 518). ^1H NMR spectra, 270 MHz, were fully consistent with the structure of **1**. Detailed synthetic procedures will be reported separately, together with the synthesis of related peptide disulfides.

All NMR studies were carried out on a Bruker WH-270 FT-NMR spectrometer, as described earlier.[15]

X-ray Diffraction. Single crystals of **1** (M_r = 517) were grown from methanol–ethyl acetate by using a sample recovered after NMR analysis in a mixture of chloroform and dimethyl sulfoxide. Rotation and Weissenberg photographs indicate an orthorhombic lattice, with the space group $P2_12_12_1$. The cell constants are $a = 8.646$ (1) Å, $b = 18.462$ (2) Å, and $c = 19.678$ (3) Å. The measured density, obtained by using the flotation method in a CCl_4–benzene mixture, was 1.27, yielding a molecular weight of 601 ($Z = 4$), suggesting the incorporation of solvent. A molecule of Me_2SO was found after structure determination.

Intensity data were collected on a CAD-4 diffractometer with a ω–2θ scan, up to a maximum Bragg angle of 75°, by use of Cu Kα radiation. A total of 3464 reflections with $I_0 > 3\sigma(I)$, representing 94% of reflections collected, were used in the structure determination and refinement. The structure was solved by the direct methods program MULTAN80.[16] A 20-atom fragment could be identified in the *E* map, corresponding to the best set of phases generated by MULTAN. Karle recycling[17] yielded nine more atoms. The refinement of the positional and isotropic thermal parameters, using a block diagonal least-squares procedure, converged to an *R* value of 0.32. The remaining non-hydrogen atoms of the molecule and four solvent peaks, of which one was very intense, were disclosed by difference electron density maps. Assuming the four solvent atoms to be carbons, two cycles of refinement resulted in an *R* value of 0.18. The high intensity of one solvent peak together with the peak connectivities suggested a Me_2SO molecule. Further refinement led to

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(13) Abbreviations used: Aib, α-aminoisobutyric acid; Boc, *tert*-butyloxycarbonyl; Me_2SO, dimethyl sulfoxide. IUPAC–IUB conventions are followed for atom numbering.[26] However, in the discussion and Table III the residues are sequentially numbered (1–4) for convenience.
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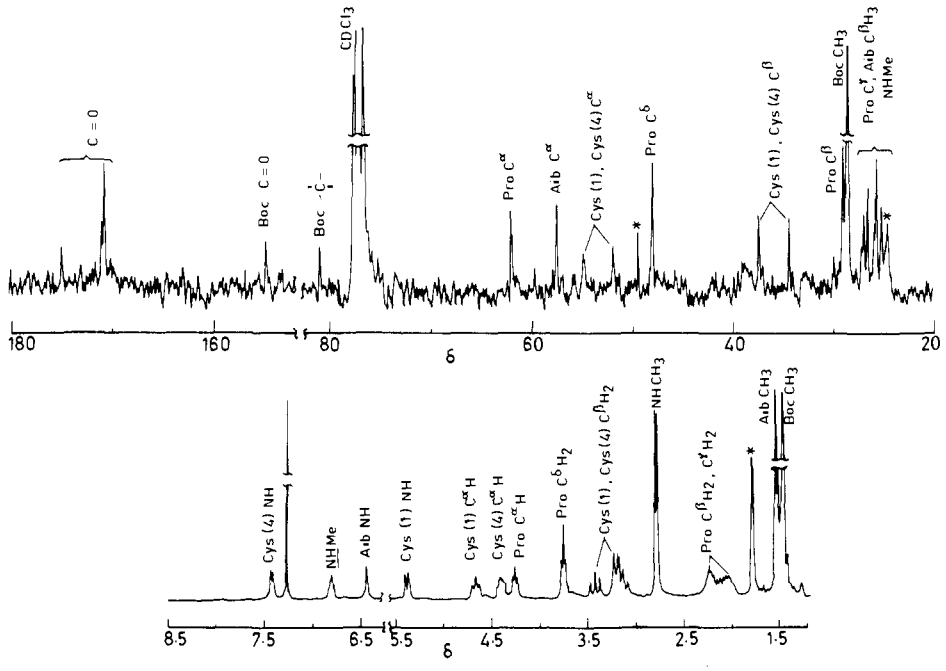

Figure 1. (Top) 67.89-MHz ^{13}C NMR spectrum of **1** in $CDCl_3$; (bottom) 270-MHz ^1H NMR spectrum of **1** in $CDCl_3$.

Table I. NMR Parameters of NH Resonances in Boc-Cys-Pro-Aib-Cys-NHMe

NH group	δ, ppm		$J_{HNC^\alpha H}$, Hz		dδ/dT (ppm/°C)
	$CDCl_3$	$(CD_3)_2SO$	$CDCl_3$	$(CD_3)_2SO$	
Cys(1)	5.35	7.48	6.2	7.0	0.0074
Aib(3)	6.42	8.33			0.0045
Cys(4)	7.43	7.44	9.1	9.0	0.0015
NHMe	6.78	7.65			0.0034

[a] Solvent, $(CD_3)_2SO$.

an R value of 0.145. Further cycles of refinement, with anisotropic temperature factors for non-hydrogen atoms, positional and isotropic thermal parameters for 38 hydrogen atoms (stereochemically fixed), and a σ-weighting scheme, gave a final R value of 0.069. The temperature factors of the hydrogen atoms in the Me_2SO molecule show large variations. Hence, these atoms were not included in the refinement, but were retained in the structure factor calculations. It may be noted that the Me_2SO molecule has a high probability of being deuterated, since the sample was originally recovered from a $CDCl_3$–$(CD_3)_2SO$ (99.5 atom % D) mixture. However, for purposes of X-ray scattering no distinction was made between H and D. The scattering factors were those of Cromer and Waber[18] for non-hydrogen atoms and of Stewart et al.[19] for hydrogen atoms.

Results and Discussion

NMR Studies. The 270-MHz ^1H NMR spectrum of **1** is shown in Figure 1. All assignments are based on spin-decoupling experiments and chemical shifts determined with model peptide fragments. The assignment of the NH resonances is straightforward. The Aib(3) and methylamide NH groups appear as a singlet (δ 6.42, $CDCl_3$) and broad quartet (δ 6.78, $CDCl_3$), respectively. The two Cys NH doublets are distinguished on the basis of the well-established tendency of urethane NH groups to resonate at high field in $CDCl_3$.[15,20] Thus, the δ 5.35 resonance is assigned to Cys(1) NH and the δ 7.43 resonance to Cys(4) NH. The corresponding Cys $C^\alpha H$ and $C^\beta H_2$ resonances were identified by successive double resonance experiments. The specific assignments of the Cys NH groups in $(CD_3)_2SO$ were made by

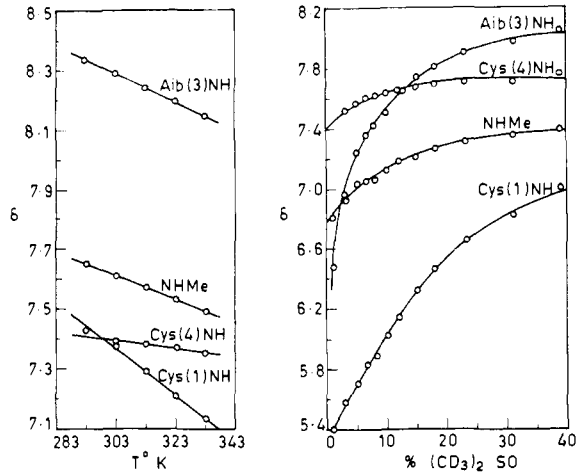

Figure 2. (Left) Temperature dependence of NH chemical shifts in $(CD_3)_2SO$ for **1**; (right) solvent dependence of NH chemical shifts in $CDCl_3$–$(CD_3)_2SO$ mixtures.

monitoring chemical shifts in $CDCl_3$–$(CD_3)_2SO$ mixtures. The various NH NMR parameters are summarized in Table I. The 67.89-MHz ^{13}C NMR spectrum of **1** in $CDCl_3$ is shown in Figure 1. The position of the C^β resonance of Pro(2) at δ 28.4 is characteristic of a trans Cys–Pro bond.[21a] There is no evidence for the presence of any cis conformer.

Hydrogen Bonding. The participation of NH groups in intramolecular hydrogen bonding was established by using the temperature dependence of NH chemical shifts in $(CD_3)_2SO$[21] and solvent dependence in $CDCl_3$–$(CD_3)_2SO$ mixtures.[22] The results are shown in Figure 2. The temperature coefficient (dδ/dT) values in Table I clearly show that Cys(4) NH is strongly shielded from solvent. The intermediate dδ/dT value for the methylamide NH does not permit definitive conclusions. However, the Aib NH and Cys(1) NH have dδ/dT values characteristic of solvent-ex-

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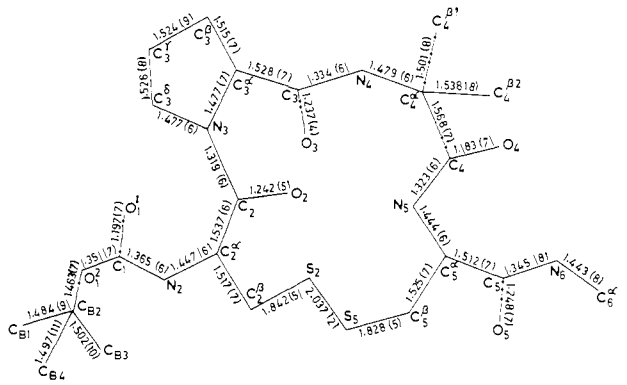

Figure 3. Bond lengths determined in **1**; estimated standard deviations are indicated in parentheses.

posed NH groups. In the solvent titration experiment (Figure 2) the Cys(4) and methylamide NH resonances show very small downfield shifts with increasing concentration of $(CD_3)_2SO$. On the contrary, the Cys(1) and Aib(3) NH groups move very rapidly downfield with addition of $(CD_3)_2SO$, a feature indicative of their exposure to the solvent. A hydrogen–deuterium (H–D) exchange experiment[23] in $CDCl_3$–D_2O mixtures yielded an exchange half-life ($t_{1/2}$) of >1 day for Cys(4) NH, while the Aib(3) and Cys(1) NH groups had $t_{1/2} \sim 15$ min. The methylamide NH had a $t_{1/2} \sim 25$ min.

These results suggest that Cys(4) NH is involved in a strong intramolecular hydrogen bond, while the methylamide NH may be participating in a weaker interaction. The possibility that these NH groups are sterically shielded from solvent must also be considered. A number of stereochemical constraints are incorporated in the structure of **1**. The 14-membered ring necessarily requires chain reversal. The presence of Pro and Aib residues further restricts backbone conformations.[11,12] Pro-Aib sequences have a strong tendency to occur in β-turn conformations.[24] The various 4→1 hydrogen-bond-stabilized β turns formed by all trans peptide backbones that need to be considered are as follows: type I $\phi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\phi_{i+2} = -90°$, $\psi_{i+2} = 0°$; type II $\phi_{i+1} = -60°$, $\psi_{i+1} = 120°$, $\phi_{i+2} = 80°$, $\psi_{i+2} = 0°$; type III $\phi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\psi_{i+2} = -60°$, $\psi_{i+2} = -30°$. In addition, the enantiomeric structures, in which all the signs of the dihedral angles are reversed,[25] must be considered. ϕ,ψ represent the backbone torsion angles[26] for the two corner residues ($i + 1$ and $i + 2$) in a β turn. The types I and III β turns differ slightly in the values of ϕ_{i+2} and ψ_{i+2}. For purposes of discussing solution conformations, we shall consider them as equivalent. For L-Pro, $\phi_{Pro} \sim -60°$ and the values of $\psi_{Pro} \sim -30°$ (type III) and $\psi_{Pro} = 120°$ (type II) are sterically accessible. For Aib[10] the allowed ϕ, ψ values are largely restricted to $\phi_{Aib} \pm 60°$ and $\psi_{Aib} \pm 30°$. This is borne out by X-ray diffraction studies of a large number of Aib-containing peptides,[27] with a single exception being noted in the cyclic tetrapeptide dihydrochlamydocin.[28] Considering the limited

Table II. Fractional Coordinates (×10⁴) for Non-Hydrogen Atoms with Their Isotropic Equivalent Temperature Factors[a]

atom	x	y	z	B
C_{B1}	11459 (9)	−2171 (4)	5421 (4)	4.18
C_{B2}	12218 (7)	−1452 (3)	5370 (3)	5.16
C_{B3}	12978 (9)	−1370 (4)	4688 (4)	4.72
C_{B4}	13309 (10)	−1368 (5)	5955 (5)	4.82
O_1^2	11135 (4)	−852 (2)	5468 (2)	4.72
C_1	9993 (7)	−735 (3)	5013 (3)	4.02
O_1^1	9531 (5)	−1156 (2)	4597 (2)	5.61
N_2	9467 (5)	−43 (2)	5087 (2)	3.63
C_2^α	8317 (6)	255 (2)	4631 (2)	3.29
C_2	8941 (5)	934 (2)	4269 (2)	4.00
O_2	8333 (4)	1536 (2)	4360 (2)	3.30
C_2^β	6826 (6)	423 (3)	5005 (3)	3.13
S_2	5276 (2)	632 (1)	4394 (1)	4.18
N_3	10106 (5)	865 (2)	3842 (2)	3.44
C_3^α	10606 (6)	1510 (3)	3455 (3)	3.71
C_3^β	11966 (6)	1240 (3)	3042 (3)	4.80
C_3^γ	11734 (7)	423 (4)	2993 (3)	5.58
C_3^δ	11068 (6)	225 (3)	3687 (3)	4.18
C_3	9300 (6)	1821 (3)	3022 (2)	3.81
O_3	9254 (5)	2484 (2)	2930 (2)	5.10
N_4	8274 (4)	1365 (2)	2750 (2)	3.61
C_4^α	6965 (6)	1620 (3)	2329 (2)	3.97
$C_4^{\beta 1}$	7483 (8)	1980 (4)	1684 (3)	6.31
$C_4^{\beta 2}$	5953 (7)	958 (3)	2164 (3)	5.53
C_4	5866 (6)	2125 (3)	2750 (2)	3.60
O_4	5005 (5)	2507 (2)	2450 (2)	5.42
N_5	5976 (5)	2096 (2)	3420 (2)	3.41
C_5^α	5096 (6)	2570 (2)	3857 (2)	3.73
C_5^β	5165 (6)	2312 (3)	4593 (2)	3.95
S_5	4095 (2)	1486 (1)	4796 (1)	4.82
C_5	5613 (7)	3352 (3)	3840 (2)	4.10
O_5	4738 (5)	3831 (2)	4070 (2)	5.85
N_6	7034 (6)	3495 (2)	3596 (2)	4.93
C_6^α	7642 (11)	4223 (3)	3574 (4)	6.91
S_D	2920 (3)	4176 (1)	2261 (1)	7.66
O_D	1976 (5)	4803 (2)	2019 (2)	6.52
C_{D1}	1632 (13)	3430 (7)	2187 (13)	28.65
C_{D2}	4041 (23)	3875 (6)	1569 (7)	16.52

[a] Esd's are given in parentheses.

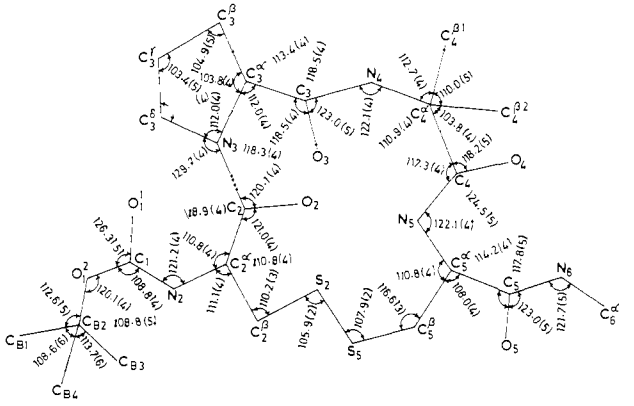

Figure 4. Bond angles determined in **1**; estimated standard deviations are indicated in parentheses.

conformational possibilities, only types III and II β turns need be considered for the Pro-Aib sequence. Both the structures would accommodate a 4→1 hydrogen bond between the Cys(1) CO and Cys(4) NH groups.

The NMR data provide some evidence, albeit less convincing, for the involvement of the methylamide NH in an intramolecular hydrogen bond. Aib-X sequences have a high propensity for formation of type III β turns when X is an L-amino acid residue.[12] The solution conformation of **1** could thus favor a consecutive β-turn structure having Aib(3) as a common corner residue, with the Cys(4) and methylamide NH groups being hydrogen bonded

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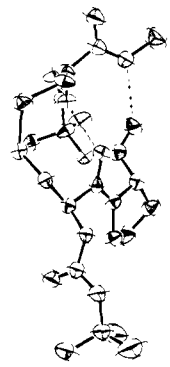

Figure 5. Molecular conformation of **1** in the solid state. Intramolecular hydrogen bonds are indicated by dotted lines. Non-hydrogen atoms are represented by thermal ellipsoids defined by the principal axes of thermal vibration and scaled to include 50% probability.

Table III. Torsional Angles[a] in the Solid State for Peptide 1

backbone residue	ϕ	ψ	ω
Cys(1)	−121.1 (5)	65.6 (5)	174.9 (4)
Pro(2)	−58.9 (6)	−34.0 (6)	179.4 (4)
Aib(3)	−61.8 (6)	−17.9 (6)	175.8 (4)
Cys(4)	−70.5 (6)	−18.6 (6)	−178.5 (5)

side chain	χ^1	χ^2	χ_{SS}
Cys(1)	−169.2 (3)	−138.7 (3)	81.6 (2)
Cys(4)	−71.3 (5)	73.3 (4)	

	χ^1	χ^2	χ^3	χ^4	θ
Pro(2)	22.8 (5)	−36.7 (6)	35.2 (5)	−22.1 (5)	−0.2 (5)

[a] In degrees. The angle about the urethane bond $O_1{}^2$–C_1–N_2–$C_2{}^\alpha$ is 175.2 (4)°. All torsional angles are defined as suggested in ref 26.

to the Cys(1) and Pro(2) CO groups, respectively. Two possible repetitive β-turn structures may be generated. These are type III–type III (3_{10} helical) and type II–type III′ conformations. The type III′ designation refers to a β turn enantiomeric with respect to type III. For the Pro-Aib-X sequence, when X is an L-amino acid, the type II–III′ structure is unlikely, since it would require positive ϕ, ψ values for the L residue. The $J_{HNC^\alpha H}$ value of 9 Hz for Cys(4) is also consistent with a ϕ value of ~−60° for this residue.[29]

The NMR results, together with the known stereochemical preferences of Pro-Aib and Aib-X sequences, thus favor a consecutive β turn or 3_{10} helical conformation of the peptide backbone in **1**, stabilized by two intramolecular 4→1 hydrogen bonds. Similar 3_{10} helical conformations have been observed in several acyclic Aib containing tetra[27] and pentapeptides (unpublished results). Preliminary computer model building studies establish the stereochemical feasibility of bridging residues 1 and 4 in a 3_{10} helix with an S–S linkage. In order to substantiate the conclusions of these NMR studies and to further define the conformational characteristics of the cyclic disulfide, a single-crystals X-ray diffraction analysis was carried out.

Crystal Structure of 1. The final atomic coordinates in the cyclic disulfide are given in Table II, along with isotropic equivalents of the anisotropic thermal parameters. Hydrogen atom coordinates and anisotropic temperature factors are provided as supplementary material. Bond lengths and bond angles are summarized in Figures 3 and 4. These values are largely unexceptional. A perspective view of the molecular conformation is shown in Figure 5. Two intramolecular 4→1 hydrogen bonds are observed between the Cys(1) CO and Cys(4) NH (N--O = 2.94 Å, H–N–O = 28.1°) and Pro(2) CO and methylamide NH (N--O = 2.98 Å, H–N–O = 13.3°). These 4→1 hydrogen bonds stabilize Pro(2)-Aib(3) and Aib(3)-Cys(4) β turns. The conformational angles

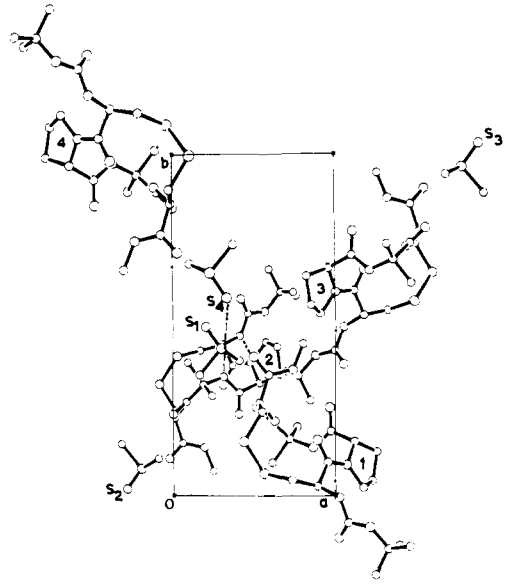

Figure 6. Crystal packing as viewed down the *c* axis. Only four peptides (numbered 1–4) and four Me_2SO molecules (numbered S_1–S_4) are shown. Molecule **1** is at x, y, z; 2 at $x - {}^1/_2, {}^1/_2 - y, \bar{z}$; 3 at $2 - x, {}^1/_2 + y, {}^1/_2 - z$; 4 at $-{}^1/_2 + x, 1 - y, {}^1/_2 + z$. Intermolecular hydrogen bonds are indicated by dotted lines.

for the peptide backbone are listed in Table III. The ϕ, ψ values for Pro(2), Aib(3), and Cys(4) clearly lie in the right-handed 3_{10} helical region. Figure 5 clearly illustrates the helical fold of the peptide backbone.

The S–S bridge lies approximately parallel to the helix axis. The observed C–S–S–C dihedral angle (χ_{SS}) is +82°. This is very close to the value of 90° expected in unstrained disulfides and similar, though opposite in chirality, to the value observed in *cyclo*(cystine).[9] The proline ring occurs in the C^γ endo conformation[30] and the endocyclic torsion angles are listed in Table III.

Molecular Packing. A view of the packing of peptide molecules in the crystal is illustrated in Figure 6. Only a limited number of molecules are shown to reduce the complexity of the presentation. The asymmetric unit consists of a single peptide molecule and a Me_2SO molecule. All four NH groups are hydrogen bonded. While the Cys(4) and methylamide NH groups are intramolecularly bonded, the Aib(3) NH is associated with the oxygen atom of Me_2SO (N--O = 2.93 Å, H–N–O = 14.8°). A single interpeptide hydrogen bond between Cys(1) NH of one molecule and Cys(4) CO of a neighbor is observed (N--O = 2.79 Å, H–N–O = 19.2°). The parameters obtained for the Me_2SO molecule are as follows: S_D–O_D 1.493 (5) Å, S_D–C_{1D} 1.778 (12) Å, S_D–C_{2D} 1.761 (16) Å, C_{1D}–S_D–O_D 103.5 (6)°, C_{2D}–S_D –O_D 107.5 (5)°, and C_{1D}–S_D–C_{2D} 92.1 (8)°. These values compare well with an earlier determination of a 1:1 Me_2SO solvate of 2-(bromotelluro)benzamide.[31] The parameters determined for Me_2SO in a recent study of a pentadecapeptide crystal containing solvent molecules are less accurate because of considerable solvent disorder.[32]

Conclusions

The cyclic peptide disulfide **1** adopts a highly folded, compact conformation in the solid state, with an S–S bridge linking residues 1 and 4 of an incipient 3_{10} helix. This conformation appears to be largely favored in solution, though a slight destabilization of the Aib(3)-Cys(4) β turn may contribute to the relatively high $d\delta/dT$ value and H–D exchange rate of the methylamide NH group. Alternatively, steric accessibility of the terminal NH may affect the observed NMR parameters, in solution. The stereo-

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chemical constraints, imposed by the Pro and Aib residues, make **1** a useful, conformationally rigid model for the study of the spectroscopic properties[33] of peptide disulfides.

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Supplementary Material Available: Tables of structure factor amplitudes, thermal parameters of non-hydrogen atoms, and positional parameters of hydrogen atoms (26 pages). Ordering information is given on any current masthead page.

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^{14}N and ^{1}H ENDOR of Nitrosylhemoglobin

Mathias Höhn,[†] Jürgen Hüttermann,*[†] James C. W. Chien,[‡] and L. Charles Dickinson[‡]

Contribution from the Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, Postfach 397, D-8400 Regensburg, Germany, and Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01002. *Received February 11, 1982*

Abstract: ^{1}H and ^{14}N ENDOR spectra were obtained from NO derivatives of human hemoglobin, its separate α and β subunits, myoglobin, and hemin dimethyl ester. ^{14}N couplings of approximately 17 MHz are found for N_ϵ of the proximal histidine (F8), showing substantial anisotropy. Small but systematic differences exist in the N_ϵ couplings of the different compounds. Of the proton interactions, one is assigned to a meso proton ($\sim$0.4 MHz) and another one to a methyl proton of the valine (E11) on the distal side of the hemepocket ($\sim$3.8 MHz maximum coupling). One H-D exchangeable proton coupling ($\sim$1.9 MHz) is attributed to the N_{ϵ_2} proton of the distal histidine (E7). Distinct differences between α and β chains are observed upon r-t transitions. The β-chain couplings show no discernible change between coupling in both states, indicating that none of the amino acids (F8, E7, E11) are involved. For the α chains, both the N_ϵ-histidine and the distal side interactions (E7) are lost upon a transition from r to t. The quaternary R and T states of HbNO reflect the behavior of the subunits with slight but significant differences in the coupling size of several interactions, showing the influence of the quaternary state on the subunits.

Extensive electron spin resonance (ESR) studies on NO-ligated hemoglobins (HbNO)[1] and their derivatives both in single crystal form and as frozen solutions have yielded detailed information on the stereochemical and electronic structure of the nitrosyl heme group.[2-6] Since HbNO undergoes a quaternary R-T transition effected by a change in pH and by addition of inositol hexaphosphate (IHP), it has been considered a useful model for the elucidation of the allosteric behavior of hemoglobin upon oxygenation.[7,8]

The quaternary R and T states in HbNO are manifest by two distinctly different ESR spectra, both being of rhombic symmetry with respect to the iron ion. The R state corresponds to a hexacoordinated heme iron in which, in the frozen solution powder spectra used typically, the ligand NO and the proximal histidine (His F8) appear to contribute to a hyperfine structure via ^{14}N interactions for at least one g turning point, usually denoted g_z. The spectrum observed in the T state is characterized by a high-field shift of the g_z resonance that again carries a well-expressed ^{14}NO hyperfine substructure. An interaction with the His (F8) nitrogen cannot be resolved.[5,9,10]

Isolated NO-ligated subunits of Hb exhibit different ESR spectra that, in addition, react differently with respect to changes of pH. Whereas the spectra of the β chains, within the limits of the ESR resolution, appear to experience no change between basic and acidic conditions,[11,12] there is a drastic effect for the α chains, which exhibit features comparable to those of the quaternary R and T state spectra, respectively, when going from alkaline to acid pH.[11,12,13] Since the spectra of the tetramer HbNO were shown to be a linear superposition of the subunit spectra for both the R and the T state,[14] the conclusion of these and several related findings with hybrids[11,15] and natural hemoglobin mutants[9,10,16,17] is that the α chains undergo a distinct tertiary structure change well sensed at the heme upon an R-T transition. The β chains,

[†] Universität Regensburg.
[‡] University of Massachusetts.

(1) Abbreviations used: hemoglobin, Hb; myoglobin, Mb; hemin dimethyl ester, hemin DME; nitrosyl derivatives are written with NO used as suffix; inositol hexaphosphate, IHP; *p*-hydroxymercury benzoate, pMB; electron spin resonance, ESR; electron-nuclear double resonance, ENDOR.

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