Chain Reversals in Model Peptides: Studies of Cystine-Containing Cyclic Peptides. 3. Conformational Free Energies of Cyclization of Tetrapeptides of Sequence Ac-Cys-Pro-X-Cys-NHMe

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Abstract: Six tetrapeptides of amino acid sequence Ac-Cys-Pro-X-Cys-NHMe, where X = Asn, Gly, Ser, Phe, Val, and Aib, respectively, were synthesized, and the molecular structures of the cyclic forms of two of them (with X = Ser and Val, respectively) were determined by X-ray diffraction. These two cyclic peptides were found to adopt a type I β-turn conformation centered at Pro-X. The molecular structures of two of the cyclic peptides (with X = Gly and Ser, respectively) were examined by two-dimensional NMR spectroscopy in aqueous solution. The Gly peptide exhibited a type II β-turn at Pro-Gly, and Ser peptide exhibited a structure similar to the X-ray structure, with evidence of about 10% of a different conformation. The chemical equilibrium between the oxidized (cyclic) and reduced (acyclic) forms of this series of peptides was examined in aqueous solution at 25 °C and pH 8 to obtain quantitative information about the β-turn-forming potential for each Pro-X pair. The method of disulfide exchange was employed together with automated HPLC for the analysis of the thiol/disulfide equilibrium mixture. The peptide with X = Aib was used as the formal oxidant. These tetrapeptides provide experimental information indicating that residue X leads to a decreasing tendency in the order Asn, Aib, Gly, Ser, Phe, and Val for the Pro-X fragment to form a β-turn.

Introduction

As an aid in predicting protein conformation, it is very useful to obtain experimental information about the intrinsic tendency of each of the 20 naturally occurring amino acid residues to adopt specific conformations *in water*. The intrinsic tendency reflects the interactions between the atoms of the side chains of a residue and those of its own backbone. We have recently completed the acquisition of this information for the α-helical tendency from studies of the helix-coil transition in binary random host-guest copolymers[1] and have begun a series of investigations to determine the tendencies toward formation of β-turns and β-sheets.[2,3]

The basis for these newer studies is earlier work by Laskowski and Scheraga[4] on the influence of noncovalent interactions on the reactivity of primary valence bonds. Such noncovalent interactions can affect the *observed* equilibrium constants for the hydrolysis of peptide bonds or the reduction of disulfide bonds in proteins. For example, whereas the intrinsic tendency for peptide bond hydrolysis is to go to completion, local interactions in a protein can prevent hydrolysis from going to completion and can even facilitate *synthesis* of peptide bonds by proteolytic enzymes.[5] Likewise, the equilibrium constants (or redox potentials) for reduction of disulfide bonds in proteins can reflect the local environment in the molecule.

To determine the tendencies toward formation of β-turn and β-sheet conformations in chain reversals, we make use of the cyclization process[2,3] shown in Figure 1, which involves the effect of noncovalent interactions on the reactivity of disulfide bonds. The standard free energy change for this process consists of three additive components: (i) the intrinsic free energy of formation of a disulfide bond, as in the formation of cystine from cysteine, (ii) the free energy of formation of a β-turn from a statistical ensemble of conformations in the dipeptide portion B-C, and (iii) the free energy of formation of an extended conformation from a statistical ensemble of conformations in residues A and D, leading to a hairpinlike antiparallel β-sheet structure which also involves interactions between residues A and D. It is the free energies in (ii) and (iii) that will influence the *observed* equilibrium constants in the reactions in Figure 1 and that are of interest in this series of papers. We previously focussed on hexapeptides (Figure 1a),[2,3] and, in this paper, we consider tetrapeptides (Figure 1b). The reaction in Figure 1b provides information about the tendency toward formation of a β-turn in B-C (component ii) insofar as it influences the equilibrium constant for cyclization. In contrast to the hexapeptides investigated previously,[2,3] which are able to accommodate three sequentially distinct β-turns,[2] tetrapeptides have a higher probability of forming a β-turn at the B-C position. In this initial study on tetrapeptides, we consider those in which B-C is Pro-X, where X is, in turn, Asn, Gly, Ser, Phe, Val, and Aib.

Chain reversals have been the subject of many experimental and theoretical studies because of their involvement in the initiation of protein folding.[6-10] In the early stages of the folding process, chain segments can adopt β-turn conformations that are determined mainly by the local amino acid sequence.[6-10] These conformations of finite but very low stability can act as initiation sites for the folding process, leading to a restriction of the conformational space of the rest of the chain as it folds. As indicated above, medium- and long-range interactions, such as disulfide bonds,[4,11] can stabilize chain reverals in proteins. Protein frag-

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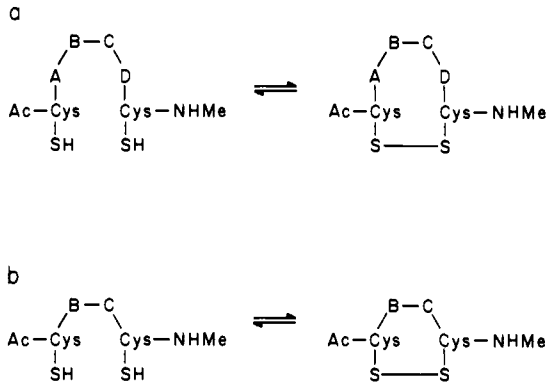

Figure 1. Representation of the equilibria between the acyclic and cyclic forms of (a) a hexapeptide and (b) a tetrapeptide. The terminal blocking groups Ac and NHMe are CH_3CO– and $-NHCH_3$, respectively. A, B, C, and D are amino acid residues.

ments and linear model peptides, that lack these stabilizing medium- and long-range interactions, usually adopt only a small population of ordered conformations in aqueous solution. Thus, early attempts to detect ordered structures in peptides in aqueous solution were often unsuccessful or led to results that were difficult to interpret.[12-15] This was probably due to the relatively insensitive methods used. Cyclic peptides have been used widely as models for chain reversals because a higher proportion of ordered structure is present in solution in such relatively restricted conformations; e.g., a disulfide bond can stabilize a small cyclic peptide into a well-defined compact structure[16,17] thereby providing a model for characterizing chain reversals.

Various theoretical approaches have also been applied to study the formation of chain reversals at the central dipeptide portion of a tetrapeptide.[6,18-25] However, disparities among the results of these methods indicated the need for an experimental approach to the problem. Sato et al.[26] were the first to develop a method to estimate the bend-forming tendency of a peptide. They measured the exciton transfer between terminal chromophores of a linear tetrapeptide in several nonaqueous solvents. Boussard and Marraud[27] examined the infrared absorption spectra of blocked dipeptides in apolar solvents and estimated the proportion of the peptide population in the folded state. The aforementioned experimental method[2,3] was developed in this laboratory to take advantage of cyclization to stabilize chain reversals *in aqueous solution.* For our initial studies of tetrapeptides, we chose the Pro-X sequence shown in Figure 1b because of its high probability to form a β-turn.[24] Cysteine has a high probability of occurrence at positions 1 and 4 of β-turns[23] and, indeed, a high probability of occurrence at these positions when a prolyl residue occurs at position 2.[28] Experimental evidence for bend formation in cyclic

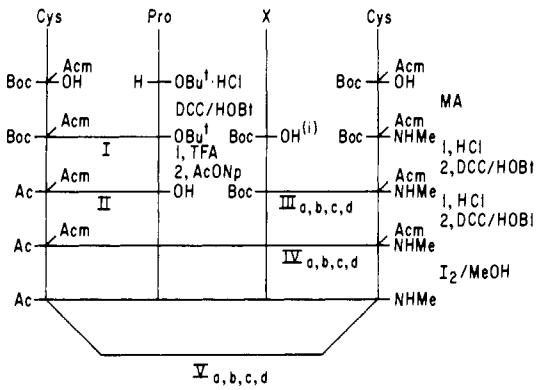

Figure 2. Schematic representation of the syntheses of the cyclic disulfide-containing tetrapeptides: a, X = Gly; b, X = Phe; c, X = Asn; and d, X = Val. (i) Boc-X-OH was used for all residues except Asn. Boc-Asn-ONp was used when X = Asn.

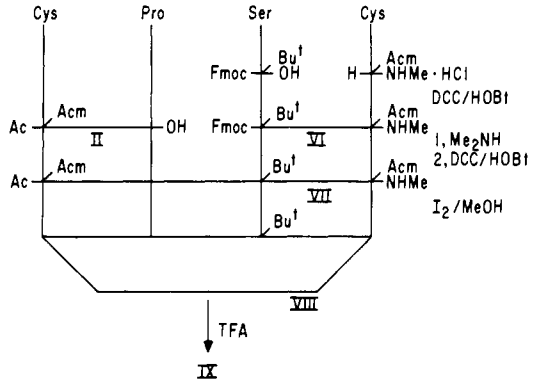

Figure 3. Schematic representation of the synthesis of the cyclic disulfide-containing peptide with X = Ser.

tetrapeptides of sequence Cys-Pro-X-Cys in apolar solution has been reported,[16,17] and the crystal structure of the cyclic tetrapeptide with X = Aib shows formation of a type III β-turn;[29] such a type III β-turn has also been observed for this compound in DMSO solution.[30] [It has been pointed out that type I and type III β-turns form a continuous class.[31]] Our choice as to which Pro-X pairs to consider in this initial study was based on the conformational energy calculations of Zimmerman and Scheraga.[24] The pairs Pro-Phe, Pro-Asn, Pro-Val, Pro-Ser, and Pro-Gly were selected to represent a wide distribution of probabilities of formation of β-turns. The pair Pro-Aib, which is sterically constrained into a β-turn conformation, was included to provide a relatively rigid peptide useful for comparative purposes.

Experimental Section

Materials and Methods. (1) Peptide Synthesis. The four cyclic disulfide-containing tetrapeptides Ac-Cys-Pro-X-Cys-NHMe where X = Gly(Va), Phe (Vb), Asn (Vc), and Val (Vd) were synthesized according to the scheme in Figure 2. The *tert*-butyloxycarbonyl (Boc) group was employed for α-amino protection and the acetamidomethyl (Acm) function for thiol protection. All peptide couplings were mediated by dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBT).[32] At first, the N-terminal peptide I was prepared. Deprotection by trifluoroacetic acid (TFA), followed by acetylation with *p*-nitrophenyl acetate (AcONp), yielded the common fragment II. The C-terminal dipeptides (IIIa–d) were synthesized by starting with Boc-Cys(Acm)-NHMe.[2] Prior to coupling, removal of the Boc groups was accomplished by HCl/EtOAc. The *S*-Acm protected linear tetrapeptides (IVa–d) were

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Table I. Analytical Data for Cystine-Containing Cyclic Tetrapeptide

peptide	amino acid analysis[a]	FABMS $(M + H)^+$ (m/z)[b]
CPGC	Cys, 1.96; Pro, 1.00; Gly, 0.99	432 (432)[c]
CPFC	Cys, 2.21; Pro, 1.00; Phe, 0.99	522 (522)
CPVC	Cys, 1.50; Pro, 1.00; Val, 1.10	474 (474)[d]
CPNC	Cys, 1.96; Pro, 1.00; Asn, 1.09	489 (489)
CPSC	Cys, 1.60; Pro, 1.00; Ser, 0.98	462 (462)
CPAibC	Cys, 1.38; Pro, 1.00; Aib, 1.00	460 (460)

[a] Normalized to proline. Amino acid analyses were carried out at the Biotechnology Analytical and Synthesis Facility at Cornell University. [b] Fast-atom bombardment mass spectrometric ratio of mass to charge for the protonated molecular ion $(M + H)^+$, including the Ac– and NHMe– terminal groups. [c] The theoretical mass-to-charge ratio of the protonated molecular ion is given in parentheses. Mass spectral determinations were carried out at the Cornell Mass Spectrometry Facility. [d] An additional peak of m/z = 948 (948) in the chromatogram of an equilibrium mixture of CPVC and CPAibC, due to a cyclic dimer, was observed.

purified by passage of an aqueous solution through a mixed-bed ion exchange resin and recrystallized from appropriate solvents. The identity of all the intermediates was confirmed by proton NMR spectroscopy, and their purity was determined by TLC and melting point determinations. Cyclization was carried out with I_2/MeOH. The cyclic peptides were isolated by chromatography on a Dowex I (acetate form) column followed by preparative RP-HPLC on a C_{18} column with a linear gradient elution of acetonitrile in water, both as 0.09% TFA solutions.

The synthesis of the cyclic Ser peptide (IX) is outlined in Figure 3. The 9-fluorenylmethyloxycarbonyl (Fmoc) group was used for α-amino protection and the *tert*-butyl (Bu^t) group for hydroxy protection for Ser. Peptide assembly was carried out with DCC/HOBt, as above. The Fmoc group was removed by dimethylamine/*N*,*N*′-dimethylformamide. After cyclization the Bu^t protected cyclic peptide (VIII) was isolated and purified. Upon treatment with TFA, IX was obtained.

The cyclic Aib peptide (XIII) was assembled as portrayed in Figure 4. The central fragment Boc-Pro-Aib-OH was prepared according to Schmitt and Jung.[33] This dipeptide acid, with a sterically hindered Aib residue,[33] was preactivated with DCC/HOBt at 40 °C before coupling with the amino component to produce the tripeptide X. After deprotection with HCl/EtOAc, DCC/HOBT was again employed for the formation of the tetrapeptide XI. After deprotection, XI was acetylated with acetic anhydride and cyclized in the same manner.

The yields of the monomeric species from oxidative cyclization varied from 46–85%. This is rather unexpected compared to the yields (30–54%) of our previous work on a series of cyclic disulfide-containing hexapeptides.[2] Ravi and Balaram reported the synthesis of a similar series of Boc protected cyclic cystine tetrapeptides.[17] These authors used completely different strategies and obtained yields of only 12–25% from cyclization.

The identity and purity of the cyclic disulfide-containing tetrapeptides were established by analytical RP-HPLC, TLC, amino acid analysis, proton NMR spectroscopy, and FAB mass spectrometry. The physical constants and analytical data are presented in Table I. The bis(thiol)-acyclic tetrapeptides were prepared from the cyclic disulfides by reduction with DL-dithiothreitol (DTT)[2] and isolated by RP-HPLC. The detailed experimental procedures are available in the supplementary material.

(2) X-ray Diffraction of Single Crystals of Cyclic Peptides. Single crystals of Vd (from CH_3CN–H_2O) and IX (from H_2O) were grown for X-ray diffraction analysis. All single-crystal X-ray diffraction measurements were made with a Nicolet R3M diffractometer controlled by a Micro-Vax II computer. Preliminary X-ray photographs and a fast data collection at low resolution established that both crystals belonged to the orthorhombic crystal system, and the pattern of systematic extinctions ($h00$, $h = 2n+1$; $0k0$, $k = 2n+1$; $00l$, $l = 2n+1$) uniquely determined the space group as $P2_12_12_1$ for both crystals. The unit cell dimensions reported in Table II were determined in each case by least-squares fitting of 15 diffractometer-measured reflections with moderate values of 2θ ($25° < 2\theta < 40°$ for Cu Kα radiation).

All unique data with $2\theta \leq 112°$ were collected using θ:2θ scans at ambient temperature. Each crystal was sealed in Lindemann capillaries with mother liquor. Periodic monitoring of the intensities of three check reflections indicated that no serious decomposition of the crystals occurred during the collection of X-ray intensity data. Empirical absorption corrections (ψ-scans) were made based on reflection intensity measurements at different azimuthal angles.

Table II. Single-Crystal Data

	serine peptide	valine peptide
crystallization solvent	H_2O	CH_3CN/H_2O (1/9)
size of crystal, mm	0.2 × 0.5 × 0.3	0.2 × 0.3 × 0.2
space group	$P2_12_12_1$	$P2_12_12_1$
formula of asymmetric unit	$C_{17}H_{27}N_5O_6S_2{\cdot}4H_2O$	$C_{19}H_{31}N_5O_5S_2{\cdot}H_2O$
formula wt[a]	533.6	491.6
unit cell dimensions (Å) ($\alpha = \beta = \gamma = 90°$)		
a	9.752 (3)	8.378 (3)
b	13.577 (4)	13.339 (5)
c	19.134 (6)	21.954 (6)
calcd density, g/cm^3	1.39	1.33
radiation used, λ, Å	Cu Kα, 1.54178	Cu Kα, 1.54178
no. of unique data obsd[b]/total	1584/1911	1590/1844
fraction obsd, %	82.8	86.2
R factors for final X-ray model $R^c/R_{rms}{}^d$	0.0618/0.067	0.0371/0.0371

[a] These values differ from those in Table I because of the water of crystallization. [b] $|F_o| > 3\sigma(F_o)$. [c] $R = \{\sum[|F_o| - |F_c|]\}/\sum|F_o|$. [d] $R_{rms} = [\{\sum w(|F_o| - |F_c|)^2\}/\sum w|F_o|^2]^{1/2}$ where F_o = observed structure factor, F_c = calculated structure factor, w = weight based on intensity.

Both crystal structures were solved by SHELXTL PLUS,[34] an automatic version of SHELXS 86.[35] The structures were refined by full-matrix least-squares using $1/\sigma^2(F_o)$ weighting. The positions of hydrogen atoms were calculated by using idealized bond geometry and an assumed C–H bond length of 0.98 Å. The full-matrix least-squares refinements led to satisfactory discrepancy indices for both crystals.

(3) NMR Spectroscopy. A 15 mM solution of CPGC (pH 2.8) and a 4 mM solution of CPSC (pH 3.6), both in 90% H_2O/10% D_2O, were used for the NMR experiments.

The temperature dependences of the chemical shifts of the amide protons were determined using a Bruker AF-300 spectrometer. One-dimensional spectra were acquired at 20, 25, 29 °C, and the chemical shifts at each temperature were measured by using the chemical shift of DSS (2,2-dimethyl-2-silapentane-5-sulfonate, 0.015 ppm) as a reference. Then, the temperature dependence of the chemical shift for each amide NH proton was calculated by using a linear least-squares fit.

NOESY[36] and DQF-COSY[37] spectra of CPGC and CPSC were acquired using a Varian XL-400 spectrometer. A mixing time of 400 ms was used for the NOESY experiments. In these experiments, the water signal was suppressed by irradiation with a weak rf field at the frequency of the H_2O peak.

A ROESY[38] spectrum of CPSC was obtained using a GE GN-500 spectrometer. In this experiment, selective excitation of the desirable part of the spectrum was carried out using the JR-echo[39] method. In this pulse-sequence, the excitation has a null at the frequency of the water signal; therefore, spectra of aqueous solutions can be acquired without irradiation at the water frequency (to overcome the problem of saturation transfer). A mixing time of 300 ms was used to acquire the ROESY spectrum. All of the NOESY and ROESY spectra were obtained at 25 °C.

(4) Reverse-Phase HPLC. Analytical and preparative scale HPLC of the product mixture from the synthesis of the oxidized and reduced tetrapeptides was carried out with a Spectra Physics SP 8000 liquid chromatograph coupled to a 770 spectrophotometric detector. Analytical scale RP-HPLC chromatograms were obtained with a Waters RCM 100 Radial Compression Module (Water Associates) loaded with a Nova-PAK C_{18} 5 mm i.d. cartridge. These chromatograms were generated with both spectrophotometric detection at 210 nm and detection with the automated disulfide/thiol detection system (DTDS);[36] thus, disulfide/thiol-containing fractions were identified among those visualized at 210 nm.

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Table III. Linear Composition Gradients of RP-HPLC Eluant Used in the Analysis of the Disulfide-Exchange Equilibrium Mixture

peptide mixture	initial composition, %CH_3CN (v/v)	final composition, %CH_3CN (v/v)	gradient duration (min)	gradient flow (mL/min)
CPAibC + CPNC	5	15	50	1
CPAibC + CPGC	5	15	50	1
CPAibC + CPSC	5	15	50	1
CPAibC + CPFC	7	30	50	1
CPAibC + CPVC	8	18	100	0.6

Preparative chromatography was carried out with a Rainin Dynamax Macro C_{18} column (Rainin Instrument Co.) with spectrophotometric detection at 220 nm.

The flow rates were 0.5 and 20.0 mL/min for the analytical and preparative scale chromatograms, respectively. Linear gradients of acetonitrile in water, both as 0.09% TFA solutions, were used to achieve the elution of the tetrapeptides. The linear gradient, expressed as proportion of acetonitrile (v/v), used for both analytical and preparative scale elution of CPVC, CPNC, CPFC, and CPAibC was 10–50%, the one used for CPGC was 2–50% and for CPSC was 5–50%. The duration of the gradient was 40 min for the analytical runs and 45 min for the preparative scale separations.

(5) Disulfide-Exchange Equilibrium. The apparent observed equilibrium constants for the process

$$\{\text{peptide-X}\}_r + \{\text{peptide-Y}\}_{ox} \underset{}{\overset{K'_{8.0}}{\rightleftharpoons}} \{\text{peptide-X}\}_{ox} + \{\text{peptide-Y}\}_r \quad (1)$$

was measured at 25 °C, pH 8, and used to study the relative stability of chain reversals. The subscripts r and ox denote oxidized and reduced species respectively, where

$$K'_{8.0} = [X_{ox}][Y_r]_0/[X_r]_0[Y_{ox}] \quad (2)$$

for the equilibrium observed at pH 8.0 and $[X_r]$ and $[Y_r]$ are the analytical concentrations of the respective reduced peptides.[2] The zero subscript on $[Y_r]$ and $[X_r]$ indicates the total (analytical) concentration of all neutral and ionized forms of the reduced species (see eq 3 of ref 2).

It was demonstrated[2] that the formal (pH-independent) equilibrium constants K', at a standard state of unit proton activity can be extrapolated from the macroscopic acid dissociation constants for the peptides. The relative standard free energy of cyclization $(\Delta G°)'$ is then calculated from the value of K'. This standardization procedure corrects for any effect that different residues X might have on the ionization of the thiols in the cysteine residues and hence on the cyclization process. Since the corrections to obtain K' from $K'_{8.0}$ are generally small,[2] we chose to investigate the relative stabilities of chain-reversals based on the values of the apparent equilibrium constants obtained at pH 8.0, i.e., $K'_{8.0}$.

(6) Equilibration of Cyclic and Acyclic Peptides. All solutions were prepared and the disulfide-exchange equilibrations were carried out as described in our previous paper.[2] The disulfide-exchange reaction was quenched by lowering the pH to 2.0 with aqueous TFA (0.3 M). The concentrations of the stock solutions of both the reduced and oxidized peptides were determined by using the photometric NTSB assay.[40] Sets of four experiments were carried out using both {peptide-X} and {peptide-Y} as the actual reductant in two differing mole ratios, i.e., equilibrium was approached from both directions. We chose to employ the cyclization of the peptide CPAibC as the standard reference process. Thus, the Aib-containing tetrapeptide corresponds to {peptide-Y} in eq 1.

The equilibrium mixtures were analyzed by elution from a Waters RCM 100 radial compression module loaded with a Nova PAK C_{18} cartridge. The flow rates and the 0.09% TFA acetonitrile/water gradients employed are given in Table III. The column effluent was split into two streams: one, with a flow of 0.3 mL/min, was led to a reaction coil with a total reaction time of 20 min, during which light was excluded, before reaching the DTDS, and the other stream was led to the spectrophotometer for detection at 210 nm.

The concentrations of the peptides at equilibrium were obtained by coupling the DTDS to the analytical RP-HPLC system (vide supra).

Results

(1) X-ray Structures. The structures of terminally blocked cyclic CPSC and CPVC are shown in Figures 5 and 6, respectively.

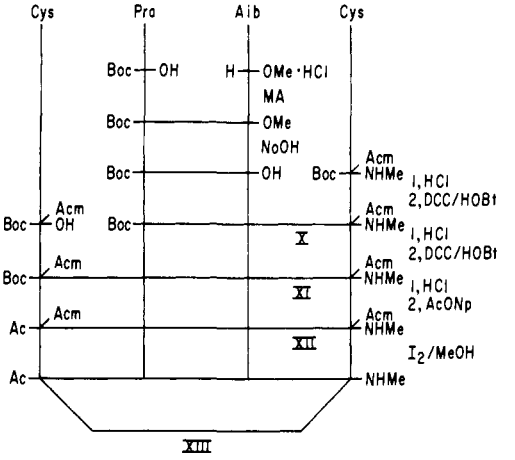

Figure 4. Schematic representation of the synthesis of the cyclic disulfide-containing peptide with X = Aib.

The dihedral angles (in the IUPAC-IUB convention[41]) of the backbones of these peptides, are reported in Tables IV and V, respectively. The two peptides possess a trans Cys-Pro peptide group and a backbone hydrogen bond between the Cys (1) C=O and the Cys (4) N–H. For both peptides, the dihedral angles ϕ and ψ for the Pro and X residues (in positions 2 and 3) are very close to the values expected for a type I β-turn.[18] From previous work, it was already known that bends with Pro at position 2 followed by amino acid residues other than Gly[42] and Aib[29] are almost exclusively of type I.

(2) Conformational Analysis in Solution. The ^1H NMR assignments for CPSC in 90% H_2O/10% D_2O were obtained using DQF-COSY and NOESY spectra. In addition, we also obtained a ROESY spectrum. The NOESY spectrum was acquired by using rf irradiation to saturate the water signal, and the ROESY spectrum was acquired using selective excitation. In spite of the differences in the methods used for water-suppression, the pattern of NOEs in the ROESY and NOESY spectra was essentially the same. The only major difference between the two spectra was that one extra NOE peak was observed in the ROESY spectrum (this peak was the Cys-1 NH-C$^\alpha$H peak and was not observed in the NOESY spectrum because it is very close to the chemical shift of water).

This peptide has at least two different conformations which interconvert slowly, i.e., there were exchange cross peaks between these two conformations in the NOESY spectrum. Some of the ^1H resonances of the minor conformation (~10%) are well resolved from the ^1H resonances of the major conformation. The minor conformation might arise from the existence of a population of conformers with a cis Cys-Pro peptide bond. The conformational analysis described below pertains to the major conformation. A strong NOE was observed between the Cys-1 C$^\alpha$H and Pro-2 C$^\delta$H; therefore, the Cys-Pro peptide bond is trans in the major conformation. Cys-1 C$^\alpha$H to Cys-4 C$^\alpha$H NOEs are expected for a cis Cys-Pro peptide bond; such an NOE was not observed.

The temperature dependence of the chemical shift of the Cys-4 amide proton is small (less than 4.5 ppb/°C). All other amide protons showed a larger temperature dependence of their chemical shifts (>7 ppb/°C). These data suggest that either the Cys-4 NH is involved in a hydrogen bond or the Cys-4 NH is in a hydrophobic environment protected from the solvent.

An NOE was observed between Ser-3 NH and Cys-4 NH. In addition, NOEs were observed between the following pairs of protons: Pro-2 C$^\alpha$H and Ser-3 NH, Ser-3 C$^\alpha$H and Ser-3 NH, and Ser-3 C$^\alpha$H and Cys-4 NH. This pattern of NOEs and the small temperature dependence of the chemical shift of Cys-4 NH can be explained by the existence of a type-I turn involving residues

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(41) IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry* **1970**, *9*, 3471–3479.

(42) Kolaskar, A. S.; Ramabrahmam, V.; Soman, K. V. *Int. J. Peptide Protein Res.* **1980**, *16*, 1–11.

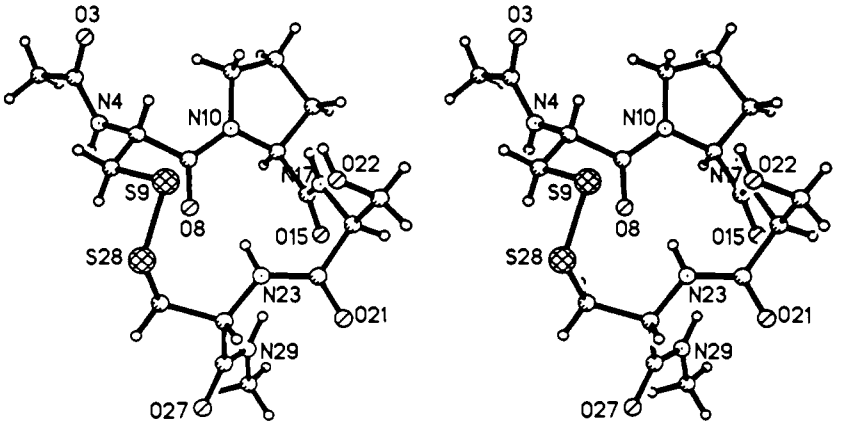

Figure 5. Stereodiagram of crystal structure of cyclic CPSC.

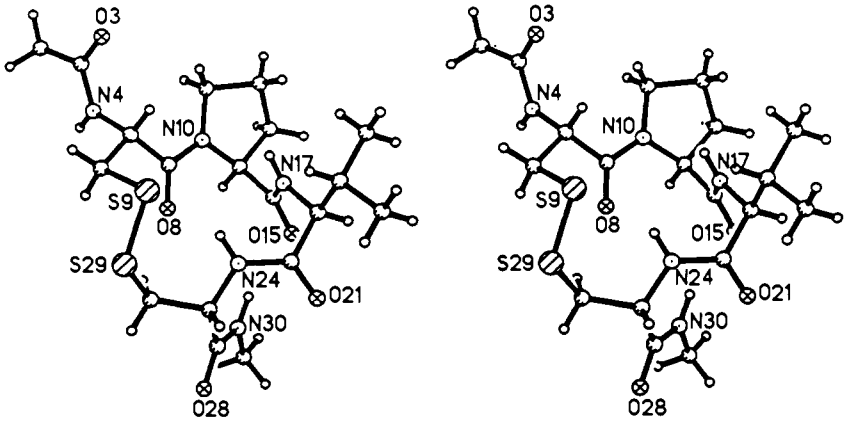

Figure 6. Stereodiagram of crystal structure of cyclic CPVC.

Table IV. Dihedral Angles[a] in Ac-Cys-Pro-Ser-Cys-NHMe (Cyclic)

dihedral angle		(deg)	dihedral angle		(deg)
C2–N4–C5–C7	(ϕ_{Cys1})	−129.6	C20–N23–C24–C26	(ϕ_{Cys4})	−81.0
N4–C5–C7–N10	(ψ_{Cys1})	91.0	N23–C24–C26–N29	(ψ_{Cys4})	−13.5
C5–C7–N10–C12	(ω_{Cys1})	−177.1	C24–C26–N29–C30	(ω_{Cys4})	−179.6
C7–N10–C12–C14	(ϕ_{Pro2})	−57.0	N4–C5–C6–S9	(χ^1_{Cys1})	179.1
N10–C12–C14–N17	(ψ_{Pro2})	−45.7	C5–C6–S9–S28	(χ^2_{Cys1})	−148.6
C12–C14–N17–C18	(ω_{Pro2})	−177.6	N23–C24–C25–S28	(χ^1_{Cys4})	−65.2
C14–N17–C18–C20	(ϕ_{Ser3})	−70.7	C24–C25–S28–S9	(χ^2_{Cys4})	82.7
N17–C18–C20–N23	(ψ_{Ser3})	−13.0	C6–S9–S28–C25	(χ_{SS})	70.3
C18–C20–N23–C24	(ω_{Ser3})	−178.6			

[a] In the IUPAC-IUB convention.[41]

Table V. Dihedral Angles[a] in Ac-Cys-Pro-Val-Cys-NHMe (Cyclic)

dihedral angle		(deg)	dihedral angle		(deg)
C2–N4–C5–C7	(ϕ_{Cys1})	−135.1	C20–N24–C25–C27	(ϕ_{Cys4})	−72.7
N4–C5–C7–N10	(ψ_{Cys1})	72.7	N24–C25–C27–N30	(ψ_{Cys4})	−15.5
C5–C7–N10–C12	(ω_{Cys1})	174.9	C25–C27–N30–C31	(ω_{Cys4})	−176.3
C7–N10–C12–C14	(ϕ_{Pro2})	−60.1	N4–C5–C6–S9	(χ^1_{Cys1})	−169.5
N10–C12–C14–N17	(ψ_{Pro2})	−29.4	C5–C6–S9–S29	(χ^2_{Cys1})	−142.0
C12–C14–N17–C18	(ω_{Pro2})	−178.7	N24–C25–C26–S29	(χ^1_{Cys4})	−66.3
C14–N17–C18–C20	(ϕ_{Val3})	−71.5	C25–C26–S29–S9	(χ^2_{Cys4})	74.2
N17–C18–C20–N24	(ψ_{Val3})	−18.0	C6–S9–S29–C26	(χ_{SS})	77.5
C18–C20–N24–C25	(ω_{Val3})	173.4			

[a] In the IUPAC-IUB convention.[41]

CPSC, with Pro and Ser at positions i+1 and i+2, respectively, of the turn, with a hydrogen bond involving the carbonyl of Cys-1 and NH of Cys-4. If such a type-I turn exists, then, in addition to these NOEs, we would also expect an NOE between Pro-2 $C^{\delta}H$ and Ser-3 NH. We cannot confirm the existence of such an NOE, because the chemical shifts of the Pro-2 $C^{\delta}H$ protons and one of the two Ser-3 $C^{\beta}H$ protons are identical. A comparison of the NMR data (in solution) with the crystal structure of CPSC shows that all the NOEs corresponding to interproton distances less than 3.5 Å were observed (if there was no overlap of peaks). This shows that the major conformation in solution is very similar to the conformation adopted by the peptide in the solid state. In addition, the observed coupling constant for Cys-4 NH-$C^{\alpha}H$ (6.9 Hz) is exactly the same as that expected for the dihedral angle ϕ of −81.0° observed in the crystal structure. The only major discrepancy between the NMR data and the X-ray structure was that the observed coupling constant for Ser-3 NH-$C^{\alpha}H$ (8.2 Hz) corresponding to a value of ϕ of −92.5° was closer to that of an ideal type I turn (−90.0°)[6] than that observed in the crystal structure (ϕ = −70.7°).

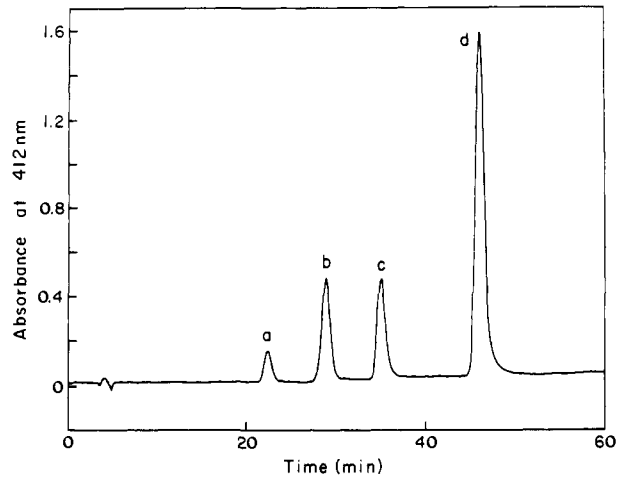

Figure 7. HPLC chromatogram from a disulfide/thiol analysis of the equilibrium mixture established between $(CPGC)_r$ and $(CPAibC)_{ox}$. The peptide identities are as follows: a, oxidized CPGC; b, reduced CPGC; c, oxidized CPAibC; and d, reduced CPAibC.

In a similar manner, the ^1H NMR assignments for CPGC were obtained using DQF-COSY and NOESY spectra. Each proton in the peptide could be assigned to a *single* resonance; therefore, we conclude that, if this peptide can adopt more than one conformation in solution, the conformations interconvert at a fast rate compared to the NMR time scale. A strong NOE was observed between Cys-1 $C^{\alpha}H$ and Pro-2 $C^{\delta}H$; therefore, the Cys-Pro peptide bond is trans. We did not observe any peaks corresponding to the cis conformation.

The temperature dependence of the chemical shift of the Cys-4 amide proton is small (less than 4 ppb/°C). All other amide protons showed a larger temperature dependence of chemical shift (>8 ppb/°C). These data suggest that either the Cys-4 NH proton is involved in a hydrogen bond or the Cys-4 NH is in a hydrophobic environment protected from the solvent.

A strong NOE was observed between Gly-3 NH and Cys-4 NH. A very strong NOE was observed between Pro-2 $C^{\alpha}H$ and Gly-3 NH. An NOE was also observed between Gly-3 $C^{\alpha}H$ and Cys-4 NH, and this NOE was weaker than the Pro-2 $C^{\alpha}H$- Gly-3 NH NOE. This pattern of NOEs and the small temperature dependence of the chemical shift of the Cys-4 NH can be explained by the existence of a type-II turn involving residues CPGC, with Pro and Gly at positions i+1 and i+2, respectively, of the turn, with a hydrogen bond involving the carbonyl of Cys-1 and NH of Cys-4.

(3) Cyclization Equilibria. A typical chromatogram of an equilibrium mixture is shown in Figure 7. The reduced forms of CPGC, CPSC, CPNC, and CPAibC eluted after the respective oxidized forms. In contrast, the reduced forms of CPFC and CPVC eluted before the oxidized forms. No dimer formation was detected for all the tetrapeptides with the exception of CPVC (see Table I). Presumably, the low tendency toward cyclization (i.e., $K'_{8.0} \ll 1$, see Table VI), implying a preference for an extended conformation, contributes to dimer formation.

The numerical output from digital integration of the chromatogram was substituted directly into eq 2, yielding the apparent equilibrium constants, $K'_{8.0}$. The mean values of $K'_{8.0}$ that were used to calculate standard Gibbs free energy changes $(\Delta G°)'$ are shown in Table VI. The error expressed in the value $K'_{8.0}$ is the 95% confidence interval.

Discussion

The X-ray structures in Figures 5 and 6 and the NMR structures of CPSC and CPGC confirm our expectation that the B–C portions of the cyclic tetrapeptides examined here have a high probability of forming a β-turn. Therefore, the series of CPXC tetrapeptides is a useful one for obtaining β-turn propensities.

As observed in our previous experiments,[2,3] the apparent equilibrium constants $K'_{8.0}$ (measured at 25 °C, pH 8.0) are insensitive to the solute mole ratio and the peptide concentrations. This demonstrates an absence of kinetic barriers to equilibration or significant perturbing solute–solute interactions.

Table VI. Apparent Equilibrium Constants and Standard Gibbs Free Energies for Peptide Cyclizations at 25 °C and pH 8.0

reductant[a]	$K'_{8.0}$[b]	$(\Delta G°)'$ kcal mol^{-1}
$CPNC_r$	1.03 (0.11)	−0.02
$CPGC_r$	0.91 (0.02)	0.05
$CPSC_r$	0.61 (0.02)	0.29
$CPFC_r$	0.42 (0.05)	0.51
$CPVC_r$	0.11 (0.02)	1.31

[a] The oxidant in the equilibrium mixtures was $CPAibC_{ox}$. [b] The values in parentheses are the errors in the values of $K'_{8.0}$, expressed as the 95% confidence interval.

Table VI shows small but significant differences in the conformational free energies of cyclization among the tetrapeptides. CPNC appears to cyclize more readily than the other peptides, with CPVC being the most resistant to cyclization. As anticipated from steric considerations, CPAibC exhibits facile cyclization, and the value of $(\Delta G°)'$ for this reference process is 0 by definition. Therefore, the propensity of the tetrapeptides to undergo cyclization follows the order CPNC > CPAibC > CPGC > CPSC > CPFC > CPVC.

In comparing our experimental results with some of the available statistical and theoretical models, we considered only those models that are based on studies of Pro-containing β-turns, namely, those of Ananthanarayan et al.,[28] Kolaskar et al.,[42] and Zimmerman and Scheraga.[24] None of these authors considered the residue Aib because it is not usually found in proteins; therefore, there are not enough data for an analysis of its propensity to form a β-turn, and CPAibC is not included in this comparison.

Ananthanarayanan and co-workers[28] carried out an analysis of the positional preferences of the individual amino acid residues in the β-turn conformation of linear tetrapeptides of the type Z-Pro-Y-X, where these sequences were obtained from the crystallographic data of 34 globular proteins. The criterion used for assigning the β-turn conformation to the Z-Pro-Y-X sequence was that the virtual dihedral angle formed by the four consecutive C^{α} atoms should lie between 0° and ±60°. Only 66 of the 252 Z-Pro-Y-X fragments found in the proteins examined were seen to adopt a β-turn conformation. The order of preference of the amino acid residues of interest here, in the B–C portion of the Z-Pro-Y-X β-turns, was found to be Gly > Asn > Ser > Val > Phe.

The study of Kolaskar and co-workers[42] is also based on the detection of β-turn conformations from crystal structure data. Using the criterion of Lewis et al.,[6,19] they considered that a β-turn was formed if the distance between the first and fourth C^{α} atoms is ≤7.0 Å. These authors found 609 pentapeptide sequences adopting chain-reversal conformations in 21 globular proteins and calculated the frequency of occurrence of pairs of amino acids in β-turns. They found a higher frequency for the pair Pro-Asn (63%) than for the pair Pro-Gly (38%), followed by the pair Pro-Ser (31%) and Pro-Val (8%). Surprisingly, they found a high frequency of occurrence for the pair Pro-Phe (50%).

Zimmerman and Scheraga[24] carried out conformational energy minimization for the *N*-acetyl-*N'*-methylamides of several Pro-X dipeptides and calculated the propensity of each peptide to form a β-turn conformation. The highest probability for β-turn formation was found for X = Gly, followed by Ser, Val, Asn, and Phe. However, whereas the statistical analyses of Ananthanarayanan et al.[28] and Kolaskar et al.[38] implicity included the effect of hydration, this effect was not taken into account in the early calculations of Zimmerman and Scheraga.[24]

An experimental approach was adopted by Dyson and co-workers[8] who obtained NMR parameters for peptides of the series Tyr-Pro-X-Asp-Val. They assumed that a low temperature coefficient of the chemical shift of the amide-proton resonance of Asp suggests the presence of a β-turn stabilized by an intra-

molecular hydrogen bond. These coefficients can provide a measure of the β-turn populations in aqueous solution that can be correlated directly with the β-turn probabilities determined from protein crystal structures. The lowest temperature coefficient for Asp, indicating the presence of the highest β-turn population in solution, was measured for the pentapeptide with X = Gly. Increasing temperature coefficients were found for X = Asn, Phe, Ser, and Val in that order.

It is clear that the agreement among the results obtained from the different analyses is not perfect. In some cases, the statistical analyses are limited by the low occurrence of certain residues and even the lower occurrence of pairs of residues in the available crystal structures.[42] The experimental method used by Dyson and co-workers[8] requires that the linear peptide under observation have a relatively high β-turn population in solution in order to be able to measure the NMR parameters.

By measuring the standard free energies of cyclization of tetrapeptides, we have been able to determine directly the propensity for a given sequence to adopt a β-turn conformation. These propensities presumably arise because of short-range interactions in the formation of chain reversals.

Acknowledgment. This work was supported by a research grant from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (DK-08465) of the National Institutes of Health. Support was also received from the National Foundation for Cancer Research. We thank D. M. Rothwarf for helpful discussions.

Supplementary Material Available: Tables of fractional coordinates, thermal parameters, bond distances, and bond angles for the cyclic serine and valine peptides (5 pages). Ordering information is given on any current masthead page.