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Solution conformations of proline rings in proteins studied by NMR spectroscopy

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Summary

Three different conformations of proline rings in a protein in solution, *Up*, *Down* and *Twist*, have been distinguished, and stereospecific assignments of the pyrrolidine β-, γ- and δ-hydrogens have been made on the basis of ^{1}H-^{1}H vicinal coupling constant patterns and intraresidue NOEs. For all three conformations, interhydrogen distances in the pairs α-β^3, β^3-γ^3, β^2-γ^2, γ^2-δ^2, and γ^3-δ^3 (2.3 Å) are shorter than those in the pairs α-β^2, β^2-γ^3, β^3-γ^2, γ^2-δ^3, and γ^3-δ^2 (2.7–3.0 Å), resulting in stronger NOESY cross peaks. For the *Up* conformation, the β^3-γ^2 and γ^2-δ^3 spin–spin coupling constants are small (< 3 Hz), and weak cross peaks are obtained in a short-mixing-time (10 ms) TOCSY spectrum; all other vicinal coupling constants are in the range 5–12 Hz, and result in medium to strong TOCSY cross peaks. For the *Down* form, the α-β^2, β^2-γ^3, and γ^3-δ^2 vicinal coupling constants are small, leading to weak TOCSY cross peaks; all other couplings again are in the range 5–12 Hz, and result in medium to strong TOCSY cross peaks. In the case of a *Twist* conformation, dynamically averaged coupling constants are anticipated. The procedure has been applied to bovine pancreatic trypsin inhibitor and *Cucurbita maxima* trypsin inhibitor-V, and ring conformations of all prolines in the two proteins have been determined.

Introduction

Stereospecific assignments of diastereotopic hydrogens of amino acid side chains considerably improve the accuracy and precision of the NMR-determined three-dimensional solution structure of a protein, as they reduce uncertainties associated with the distance constraints utilized (Basus, 1989; Güntert et al., 1989; Clore et al., 1990,1991). Methods are available for the stereospecific assignments of β-methylene hydrogens (Wagner et al., 1987), valine methyl groups (Zuiderweg et al., 1985), and leucine methyl groups (Weber et al., 1988; Güntert et al., 1989; Neri et al., 1989; Griesinger and Eggenberger, 1992; Ostler et al., 1993; Constantine et al., 1994). Recently, we have demonstrated a simple means of obtaining stereospecific assignments of γ- and δ-methylene hydrogens (Cai et al., 1995a). χ^1 torsional angle constraints, obtainable in the process of stereospecific assignments, help to determine side-chain conformations with accuracy.

To date, much research effort has been expended toward the stereospecific assignments of amino acid residues other than proline. Proline is unique, because its ring structure imposes conformational restraints on the secondary structure of a protein – its occurrence produces a turn and breaks helical and sheet structures (Anteunis and Sleeckx, 1987). The pyrrolidine ring is, however, not completely rigid. From the crystal structures (Thomasson and Applequist, 1990), NMR coupling constant measurements (Haasnoot et al., 1981; De Leeuw et al., 1983; Mádi et al., 1990), conformational energy computations (Némethy et al., 1992), and molecular dynamics simulations of some proline-containing peptides (Brunne et al., 1993; Schmidt et al., 1993), it has been established that the pyrrolidine ring exists essentially in two distinct puckered conformations, in which the C^β and C^γ atoms are displaced in opposite directions from the mean plane of the ring (Fig. 1). In the *Up* conformer (Fig. 1, left), the C^γ atom is positioned above the plane containing the other

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Supplementary Material: A listing of proteins chosen for the survey of crystal structure coordinates of prolines, and TOCSY and NOESY maps of BPTI and CMTI-V can be obtained from the authors (6 pages).

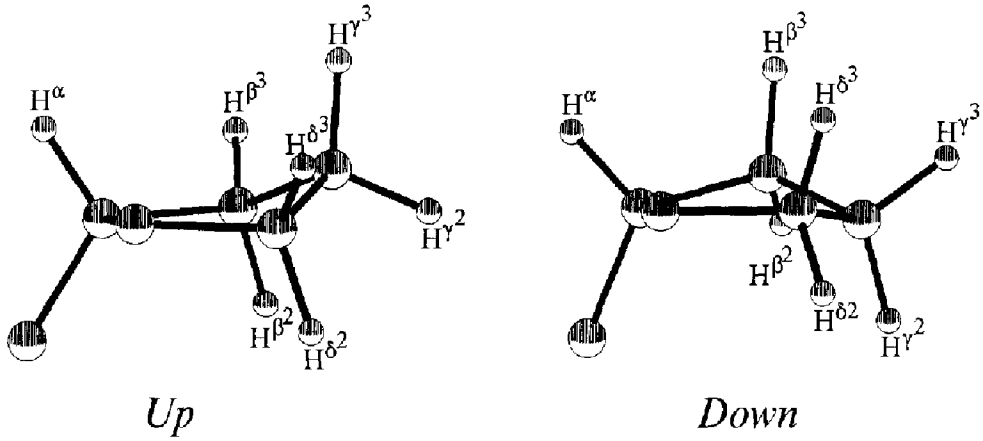


Fig. 1. *Up* and *Down* conformations of the proline ring, generated from the X-ray coordinates of BPTI (Wlodawer et al., 1987). The *Up* conformation is preferred by Pro^2, with the torsional angles χ^1, χ^2 and χ^3 at –15.5°, 29.5° and –31°, respectively. The *Down* conformation is preferred by Pro^9, with the corresponding torsional angles at 28.8°, –30.3° and 21.9°, respectively. In each of the conformations, the β and γ carbon atoms are displaced in opposite directions from the mean plane of the ring. Any in-between conformation is considered to be a *Twist* form. The pyrrolidine ring hydrogens are stereospecifically marked, according to the IUPAC-IUB Commission on Biochemical Nomenclature (1970).

four atoms, i.e., C^α, C^β, C^δ and N, whereas in the *Down* form (Fig. 1, right), the C^γ atom is located below the plane. The computed potential energy surface for the ring atoms yields a double minimum with an energy barrier of 2–3.5 kcal/mol, corresponding to these two conformers (Madison, 1977). Based on the observation of dynamically averaged coupling constants and longer $^{13}C^\gamma$ longitudinal relaxation times (De Leeuw et al., 1983; Schmidt et al., 1993), *Twist* conformations are considered to be interconverting *Up* and *Down* forms.

Recently, Milner-White et al. (1992) analyzed high-resolution X-ray crystallographic structures of 57 proteins, and found that 297 prolines, mostly trans, could be unambiguously assigned the *Up* or *Down* pucker. A complementary survey of the atomic coordinates of 225 prolines from 33 recently published high-resolution protein crystal structures (Supplementary Material) has allowed us to classify these prolines into three groups: the first group, comprising 95 prolines, has χ^1, χ^2 and χ^3 torsional angles at $-22° \pm 15°$, $33° \pm 15°$, and $-31° \pm 15°$, respectively, and is identified with the *Up* form (Fig. 1, left). The second group of 90 prolines has the corresponding torsional angles at $26° \pm 15°$, $-33° \pm 15°$ and $26° \pm 15°$, respectively, and is identified with the *Down* form (Fig. 1, right). The remaining 40 prolines have dihedral angles that are in-between those of the *Up* and *Down* forms, and are identified as *Twist* forms. The broad ranges of χ^1 torsional angles given here for the *Up* and *Down* forms encompass those of both cis and trans prolines described by Milner-White and co-workers (1992).

Earlier conformational studies of proline-containing peptides involved measurements of ^1H-^1H vicinal coupling constants and ^{13}C longitudinal relaxation times (Haasnoot et al., 1981). Such an approach is hardly applicable to proteins because of cross-peak overlap, increased line widths, and poor sensitivity and low natural abundance of the ^{13}C nucleus. In this paper we illustrate a simple ^1H NMR strategy to determine the ring conformations of all the prolines in BPTI (bovine pancreatic trypsin inhibitor) and in CMTI-V (*Cucurbita maxima* trypsin inhibitor-V).

Materials and Methods

CMTI-V was isolated from pumpkin seeds and purified by reversed-phase high performance liquid chromatography (RP-HPLC), as described before (Krishnamoorthi et al., 1990). BPTI was purchased from Sigma chemical company and purified by RP-HPLC. The yield of the pure protein amounted to about 30%. An ~3 mM sample of CMTI-V was prepared by dissolving the lyophilized protein in 0.5 ml 99.96% D_2O containing 0.2 M KCl, pD ~5.4. An ~5 mM BPTI sample, pD ~4.8, was prepared by dissolving the purified protein in 0.5 ml 99.96% D_2O containing 50 mM KCl. pH measurements were done at room temperature (22 °C), using a Fisher pH meter (model 815 MP) in combination with a glass microelectrode. pH adjustments were made by addition of small amounts of 0.2 M NaOD and/or 0.2 M DCl.

2D NMR experiments were performed with a Varian UNITYplus spectrometer, operating at a proton frequency of 499.496 MHz. The temperature was maintained at 30 °C or at 36 °C for the CMTI-V and BPTI samples, respectively. Data sets were collected and processed by using the Varian NMR software VNMR (v. 4.2) on a Silicon Graphics workstation. A P.E.COSY (Mueller, 1987) experiment was recorded with 1024 increments of 4K data points. TOCSY (Bax and Davis, 1985) and NOESY (Anil Kumar et al., 1980) experiments were recorded with 256 increments of 2K data points. The TOCSY experiments were performed at mixing times of 10, 20, 30 and 70 ms, using a clean MLEV16+60° spin-lock sequence with an effective locking field strength of

6.9 KHz. NOESY experiments were carried out at three mixing times, 30, 50 and 100 ms. Spectral widths of 7000 Hz were used in both dimensions for all experiments. The t_1 dimension was zero-filled to 4K for P.E.COSY experiments, and to 2K for all other experiments. All data were collected in hypercomplex phase-sensitive mode.

Sequential proton resonance assignments for BPTI were taken from Wagner et al. (1987). Sequential resonance assignments of CMTI-V, including its three-dimensional solution structure determination, are presented elsewhere (Cai et al., 1995b).

$^3J_{H\alpha H\beta}$ coupling constants were determined from peak separations in P.E.COSY maps, and were also estimated from TOCSY cross-peak intensities (Cai et al., 1995a). Other coupling constants were estimated from TOCSY cross-peak intensities. NOE intensities were measured in NOESY experiments with mixing times of 30 and 50 ms. Earlier studies used relative intensities of COSY cross peaks to deduce the sugar pucker in individual nucleotides (Hosur et al., 1986; Chary et al., 1988).

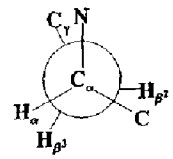
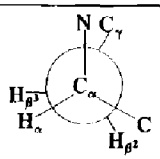
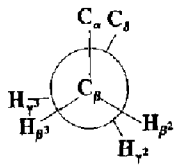
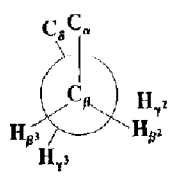
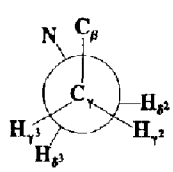
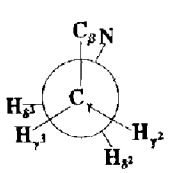
Results and Discussion

Determination of proline ring conformation on the basis of vicinal coupling constant patterns

The *Up* and *Down* conformations of the proline ring (Fig. 1) are distinct from each other in terms of the torsional angles χ^1, χ^2 and χ^3, and hence the corresponding vicinal coupling constants also differ. Table 1 presents the vicinal coupling constants calculated via the Karplus equation (Demarco et al., 1978), using the average torsional angles obtained from our survey of prolines in high-resolution crystal structures of proteins (Supplementary Material). Also given are the predicted cross-peak intensities in a short-mixing-time (10 ms) TOCSY experiment.

The *Up* and *Down* forms are seen to possess distinctive $^3J_{H\alpha H\beta 2}$ values, i.e., > 5 Hz for the *Up* form and < 3 Hz for the *Down* form, and are anticipated to produce medium to strong and weak TOCSY cross peaks, respectively (Cai et al., 1995a). Similarly, the anticipated TOCSY cross-peak intensity patterns are different for some of the β-γ and γ-δ hydrogen pairs in the two conformations. For any dynamically averaged *Twist* form, both $^3J_{H\alpha H\beta 2}$ and $^3J_{H\alpha H\beta 3}$ are expected to be around 7 Hz (Mádi et al., 1990), and medium TOCSY cross peaks are expected. However, it is difficult to distinguish unambiguously between the *Up* and *Twist* forms solely from $^3J_{H\alpha H\beta}$ values, because of experimental errors. For the *Twist* form, all vicinal coupling constants, being the weighted averages of those for the *Up* and *Down* forms, are found in a narrow range of values and result in medium to strong TOCSY

TABLE 1
CALCULATED COUPLING CONSTANTS AND TOCSY INTENSITY PATTERNS FOR PROLINE RING *UP* AND *DOWN* CONFORMATIONS[a]

Dihedral angle[b]	*Up* form: $\chi^1=-22°$, $\chi^2=33°$, $\chi^3=-31°$				*Down* form: $\chi^1=26°$, $\chi^2=-33°$, $\chi^3=26°$			
		θ (°)	$^3J_{HH}$[c] (Hz)	I_{TOCSY}[d]		θ (°)	$^3J_{HH}$[c] (Hz)	I_{TOCSY}[d]
$\theta_{\alpha\beta^2}=\chi^1-120$		−142 ± 15	7–11	M to S		−94 ± 15	2–3	W
$\theta_{\alpha\beta^3}=\chi^1$		−22 ± 15	7–10	M to S		26 ± 15	6–10	M to S
$\theta_{\beta^2\gamma^2}=\chi^2$		33 ± 15	5–9	M to S		−33 ± 15	5–9	M to S
$\theta_{\beta^2\gamma^3}=\chi^2+120$		153 ± 15	8–12	S		87 ± 15	2–3	W
$\theta_{\beta^3\gamma^2}=\chi^2-120$		−87 ± 15	2–3	W		−153 ± 15	8–12	S
$\theta_{\beta^3\gamma^3}=\chi^2$		33 ± 15	5–9	M to S		−33 ± 15	5–9	M to S
$\theta_{\gamma^2\delta^2}=\chi^3$		−31 ± 15	5–9	M to S		26 ± 15	6–10	M to S
$\theta_{\gamma^2\delta^3}=\chi^3+120$		89 ± 15	2–3	W		146 ± 15	7–12	M to S
$\theta_{\gamma^3\delta^2}=\chi^3-120$		−151 ± 15	8–12	S		−94 ± 15	2–3	W
$\theta_{\gamma^3\delta^3}=\chi^3$		−31 ± 15	5–9	M to S		26 ± 15	6–10	M to S

[a] β^2, β^3, γ^2, γ^3, δ^2 and δ^3 positions are defined according to the IUPAC-IUB convention (1970).

[b] θ_{ij} represents the dihedral angle between the planes containing two hydrogens attached to carbon atoms i and j. The dihedral angle ranges given here apply to both cis and trans prolines (Milner-White et al., 1992).

[c] Coupling constants are calculated according to the following Karplus equation (Demarco et al., 1978): $^3J_{HH}=9.5*\cos^2\theta-1.6*\cos\theta+1.8$.

[d] I_{TOCSY} represents predicted cross-peak intensities in a short-mixing-time TOCSY experiment, where W, M and S indicate weak, medium and strong cross peaks, respectively.

TABLE 2
COMPARISON OF PROLINE RING CONFORMATIONS OF BPTI IN THE SOLID STATE AND IN SOLUTION[a]

^{1}H-^{1}H pair	Data type	Pro^2	Pro^8	Pro^9	Pro^{13}
H^{α}-$H^{\beta 2}$	$^3J_{X\text{-ray}}$[b]	7.8	1.9	1.8	1.7
	$^3J_{NMR}$[c]	7	7	3	3
	I_{TOCSY}[d]	M	M	W	W
H^{α}-$H^{\beta 3}$	$^3J_{X\text{-ray}}$	9.1	7.8	8.7	6.6
	$^3J_{NMR}$	7	9	10	7
	I_{TOCSY}	S	S	S	S
$H^{\beta 2}$-$H^{\gamma 2}$	$^3J_{X\text{-ray}}$	7.6	7.8	7.7	6.5
	I_{TOCSY}	S	d[e]	n.d.[f]	n.d.
$H^{\beta 2}$-$H^{\gamma 3}$	$^3J_{X\text{-ray}}$	10.2	1.8	1.8	1.8
	I_{TOCSY}	S	d	W	n.d.
$H^{\beta 3}$-$H^{\gamma 2}$	$^3J_{X\text{-ray}}$	1.8	10.3	10.4	11.4
	I_{TOCSY}	W	d	S	S
$H^{\beta 3}$-$H^{\gamma 3}$	$^3J_{X\text{-ray}}$	7.6	7.8	7.7	6.5
	I_{TOCSY}	S	d	n.d.	n.d.
$H^{\gamma 2}$-$H^{\delta 2}$	$^3J_{X\text{-ray}}$	7.4	8.7	9.1	8.3
	I_{TOCSY}	M	d	S	S
$H^{\gamma 2}$-$H^{\delta 3}$	$^3J_{X\text{-ray}}$	1.8	8.6	8.9	9.3
	I_{TOCSY}	W	d	S	M
$H^{\gamma 3}$-$H^{\delta 2}$	$^3J_{X\text{-ray}}$	10.5	2.3	2.2	2.1
	I_{TOCSY}	S	d	W	W
$H^{\gamma 3}$-$H^{\delta 3}$	$^3J_{X\text{-ray}}$	7.6	8.7	9.1	8.3
	I_{TOCSY}	M	d	M	M
H^{α}-$H^{\beta 2}$	$R_{X\text{-ray}}$[g]	3.0	2.7	2.7	2.7
	NOE	M	W	M	W
H^{α}-$H^{\beta 3}$	$R_{X\text{-ray}}$	2.3	2.3	2.3	2.3
	NOE	S	M	S	M
Proline ring form	X-ray	*Up*	*Down*	*Down*	*Down*
	NMR	*Up*	*Up*	*Down*	*Down*

[a] The crystal structure coordinates were taken from Wlodawer et al. (1987).
[b] Coupling constants (Hz) were calculated from the following Karplus equation (Demarco et al., 1978): $^3J_{HH} = 9.5 * \cos^2\theta - 1.6 * \cos\theta + 1.8$, with the dihedral angles taken from the BPTI crystal structure (Wlodawer et al., 1987).
[c] Coupling constants (Hz) were measured from a P.E.COSY map.
[d] Cross-peak intensities of a 10-ms TOCSY experiment, where W, M and S represent weak, medium and strong cross peaks, respectively. The contour levels of W, M and S are in the ranges 0–3, 4–7 and ≥8, respectively.
[e] d = not determined due to degeneracy of γ-hydrogens.
[f] n.d. = not determined due to cross-peak overlap.
[g] Distances (Å) were obtained from the crystal structure (Wlodawer et al., 1987).

cross peaks. On the other hand, for the *Up* form, $^3J_{H\beta 3H\gamma 2}$ and $^3J_{H\gamma 2H\delta 3}$ are small (< 3 Hz), and weak TOCSY cross peaks are expected.

Stereospecific assignments of proline hydrogens

For the *Up* and *Down* forms (Fig. 1) as well as the *Twist* form of proline, the distances between hydrogens in the pairs α-β^3, β^3-γ^3, β^2-γ^2, γ^2-δ^2, and γ^3-δ^3 are shorter (2.3 Å) than between those in the pairs α-β^2, β^2-γ^3, β^3-γ^2, γ^2-δ^3, and γ^3-δ^2 (~2.8 Å). The ratio of NOESY cross-peak intensities for the two groups is estimated to be ~3, as NOEs depend on r^{-6}, where r is the internuclear distance. Therefore, stereospecific assignments can, in principle, be made according to relative cross-peak intensities in a short-mixing-time NOESY map. However, NOESY cross peaks due to the β- and γ-hydrogens cannot always be identified unambiguously, because they usually occur close to the diagonal, and also in a crowded, overlapping spectral region. On the other hand, vicinal ^{1}H-^{1}H coupling constant patterns are sufficiently different for the *Up* and *Down* forms to be distinguishable from each other (Table 1). These differences, readily manifested as weak and strong cross peaks in a 10-ms TOCSY experiment, aid in the stereospecific assignments of the pyrrolidine ring hydrogens: the α-β^2 cross peak should appear much weaker than the α-β^3 cross peak for the *Down* form, whereas both cross peaks are expected to be strong for the *Up* form; similarly, among the four γ-δ cross peaks, the γ^2-δ^2 cross peak is weak for the *Down* form, and the γ^2-δ^3 cross peak is weak for the *Up* form. In the case of a *Twist* form, the vicinal coupling constants are dynamically averaged and cannot be used to make any stereospecific assignments; in favorable cases, NOESY cross-peak intensity patterns may be used instead.

Conformations and stereospecific assignments of proline rings in BPTI

BPTI possesses four prolines (Wlodawer et al., 1987), i.e., Pro^2, Pro^8, Pro^9 and Pro^{13}. Table 2 presents various proton vicinal coupling constants that have been calculated by means of the Karplus equation (Demarco et al., 1978), using the dihedral angles from the crystal structure of the protein (Wlodawer et al., 1987). These values are compared with those determined by a P.E.COSY experiment. Also given are the relative intensities of relevant TOCSY cross peaks. As expected, a one-to-one correla-

TABLE 3
STEREOSPECIFIC ASSIGNMENTS OF PROLINE HYDROGENS IN BPTI AND CMTI-V[a]

Residue	H^{α}	$H^{\beta 2}$	$H^{\beta 3}$	$H^{\gamma 2}$	$H^{\gamma 3}$	$H^{\delta 2}$	$H^{\delta 3}$
BPTI							
Pro^2	4.33	0.91	2.03	1.60	1.88	3.60	3.73
Pro^8	4.63	1.83	2.44	d[b]	d	d	d
Pro^9	3.71	0.23	0.09	1.26	0.17	3.34	2.93
Pro^{13}	4.55	2.10	2.18	1.99	2.10	3.63	3.59
CMTI-V							
Pro^4	4.53	1.89	2.26	d	d	d	d
Pro^{10}	4.19	1.68	2.28	1.57	1.94	3.83	3.35
Pro^{29}	4.87	2.08	2.47	2.08	1.97	3.56	3.81
Pro^{41}	4.57	1.95	2.34	2.03	2.18	4.18	3.80
Pro^{64}	4.34	2.14	1.95	2.35	2.01	3.77	3.90
Pro^{65}	4.74	1.85	2.08	1.79	2.10	4.40	4.27

[a] Chemical shifts are given in ppm. Sequential proton resonance assignments of BPTI were taken from Wagner et al. (1987). γ-Hydrogens of Pro^{13} in BPTI have been reassigned according to the DQF-COSY δ-γ cross peaks. Sequential resonance assignments of CMTI-V are given elsewhere (Cai et al., 1995b).
[b] d = not determined due to degeneracy of γ-hydrogens.

TABLE 4
DETERMINATION OF PROLINE RING CONFORMATIONS IN CMTI-V

	Pro^4	Pro^{10}	Pro^{29}	Pro^{41}	Pro^{64}	Pro^{65}
$^3J_{H\alpha H\beta 2}$ (Hz)	7	2	6	7	5	10
$^3J_{H\alpha H\beta 3}$ (Hz)	10	11	10	10	11	7
$I_{H\alpha H\beta 2}$ [a]	M	W	S	M	M	S
$I_{H\alpha H\beta 3}$	S	S	S	S	S	M
$I_{H\beta 2H\gamma 2}$	d[b]	n.d.[c]	n.d.	n.d.	M	n.d.
$I_{H\beta 2H\gamma 3}$	d	W	n.d.	M	n.d.	n.d.
$I_{H\beta 3H\gamma 2}$	d	S	n.d.	W	W	n.d.
$I_{H\beta 3H\gamma 3}$	d	M	M	n.d.	n.d.	n.d.
$I_{H\gamma 2H\delta 2}$	d	M	S	M	M	M
$I_{H\gamma 2H\delta 3}$	d	S	W	W	W	W
$I_{H\gamma 3H\delta 2}$	d	W	S	M	M	M
$I_{H\gamma 3H\delta 3}$	d	M	M	M	M	M
$NOE_{H\alpha H\beta 2}$ [d]	M	W	M	W	W	M
$NOE_{H\alpha H\beta 3}$	S	M	S	M	M	S
Ring form	*Up*	*Down*	*Up*	*Up*	*Up*	*Up*

[a] I_{ij} represent cross-peak intensities in a 10-ms TOCSY experiment. Cross-peak intensities are measured in terms of contour levels, where W indicates 0–3, M indicates 4–7 and S indicates 8–11 observed contour levels.

[b] d = not determined due to degeneracy of γ-hydrogens.

[c] n.d. = not determined due to cross-peak overlap.

[d] NOE_{ij} represent NOESY cross-peak intensities. W, M and S indicate weak, medium and strong NOE cross peaks, as classified in footnote a for TOCSY cross peaks.

tion is observed between the magnitude of the coupling constant and the intensity of the corresponding TOCSY cross peak. The NMR-determined ring conformation is compared with that deduced from the X-ray structural data for each of the prolines in the bottom rows of Table 2: Pro^2 prefers an *Up* form, whereas Pro^9 and Pro^{13} exist as *Down* forms, in both the solid and the solution phases; in the case of Pro^8, the measured α-β coupling constants (7 and 8 Hz) appear to rule out a *Down* conformation as found in the crystal structure (Wlodawer et al., 1987). Whether Pro^8 exists completely in an *Up* form, or is dynamically averaged between a major *Up* form and a minor *Down* form, could not be resolved.

Table 2 also lists relative NOESY cross-peak intensities and distances between α- and β-hydrogens for the four prolines in the crystal structure (Wlodawer et al., 1987). The NOESY intensity patterns are consistent with the stereospecific assignments made. Table 3 lists stereospecific assignments made for all resolved ring hydrogens of the four prolines in BPTI, utilizing 10- and 20-ms mixing time TOCSY data (Supplementary Material). Our assignments are found to be in agreement with the automated stereospecific assignments reported for Pro^2, Pro^8 and Pro^9 (Berndt et al., 1992); no automated stereospecific assignments were reported for Pro^{13}.

A perusal of Table 2 reveals that the classification of the α-β^2 NOESY cross peaks apparently does not follow the r^{-6} dependence: Pro^2 shows a medium α-β^2 cross peak with a corresponding distance of 3.0 Å in the crystalline phase (Wlodawer et al., 1987), whereas Pro^8, with a value of 2.7 Å, shows a weak NOESY α-β^2 cross peak. This anomaly may be attributed to differences in spin-diffusion effects (Borgias and James, 1988; Majumdar and Hosur, 1990) as well as internal dynamics (Bax, 1989). Alternatively, conformational differences may exist between the solution and crystalline phases for Pro^2. A similar situation is also noted for the comparative intensities of the α-β^2 and α-β^3 NOESY cross peaks of Trp^9 and Trp^{54} in CMTI-V (Supplementary Material). However, for any given proline or tryptophan residue, the α-β^3 NOE is *always* stronger than the α-β^2 NOE.

Conformations and stereospecific assignments of proline rings in CMTI-V

CMTI-V comprises six prolines (Krishnamoorthi et al., 1990), i.e., Pro^4, Pro^{10}, Pro^{29}, Pro^{41}, Pro^{64} and Pro^{65}. Figure 2 shows representative portions of TOCSY and P.E.COSY maps that contain the α-β cross peaks for Pro^{10} and Pro^{41}. It can be seen that for Pro^{10}, the small $^3J_{H\alpha H\beta 2}$ coupling constant (2 Hz) gives rise to a weak TOCSY cross peak, whereas the large $^3J_{H\alpha H\beta 3}$ coupling constant (11 Hz) results in a strong cross peak, consistent

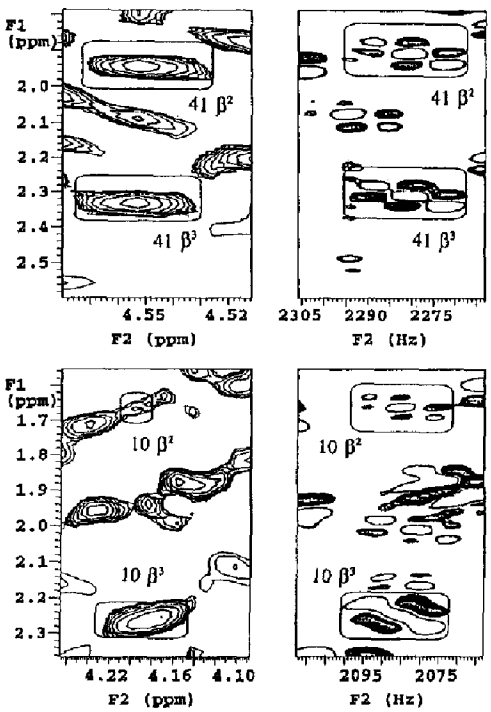


Fig. 2. Portions of 500 MHz TOCSY and P.E.COSY maps of CMTI-V (left and right panels, respectively), showing cross peaks from the α- and β-hydrogens of Pro^{10} and Pro^{41}. Large and small coupling constants result in strong and weak TOCSY cross peaks, respectively. Pro^{41} exists in an *Up* conformation, as indicated by its two large α-β coupling constants (upper panel), whereas Pro^{10} exists in a *Down* conformation, as indicated by its weak α-β^2 coupling.

with a *Down* conformation for the pyrrolidine ring. For Pro^{41}, the corresponding TOCSY cross peaks are of medium to strong intensities and are consistent with the coupling constants (7 and 10 Hz) measured from the P.E.COSY map. These results point out an *Up* conformation for Pro^{41}. Table 4 lists the vicinal coupling constants and TOCSY cross-peak intensities determined for the ring hydrogens of the six prolines in CMTI-V. With the exception of Pro^{10}, which adopts a *Down* form, the remaining five prolines all exist as *Up* conformers in solution. In the case of Pro^{4}, due to chemical shift degeneracy of the γ-hydrogens, TOCSY cross peaks of β-γ and γ-δ pairs could not be stereospecifically identified. However, the α-β coupling constant, $^{3}J_{H\alpha H\beta 2}$, rules out a *Down* conformation.

Stereospecific assignments of prolines in CMTI-V are included in Table 3.

Conclusions

The results presented here demonstrate that solution conformations of proline rings in proteins can be characterized in a simple and reliable manner by the estimation of various ^{1}H-^{1}H vicinal coupling constants from cross-peak intensities in a short-mixing-time (10 ms) TOCSY map. Thus, ring conformations and stereospecific assignments of the four prolines in BPTI (M_r 6500) and the six prolines in CMTI-V (M_r 7100) have been reported.

The procedure may be extended to slightly larger proteins by means of 3D NMR experiments, provided that NOEs are not dominated by spin-diffusion effects.

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