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High pressure NMR study of a small protein, gurmarin

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Abstract

The effect of pressure on the structure of gurmarin, a globular, 35-residue protein from *Gymnema sylvestre*, was studied in aqueous environment (95% ^1H_2O/5% ^2H_2O, pH 2.0) with an on-line variable pressure NMR system operating at 750 MHz. Two-dimensional TOCSY and NOESY spectra were measured as functions of pressure between 1 and 2000 bar at 40 °C. Practically all the proton signals of gurmarin underwent some shifts with pressure, showing that the entire protein structure responds to, and is altered by, pressure. Most amide protons showed different degrees of low field shifts with pressure, namely 0–0.2 ppm with an average of 0.051 ppm at 2000 bar, showing that they are involved in hydrogen bonding and that these hydrogen bonds are shortened by pressure by different degrees. The tendency was also confirmed that the chemical shifts of the amide protons exposed to the solvent (water) are more sensitive to pressure than those internally hydrogen bonded with carbonyls. The pressure-induced shifts of the H^α signals of the residues in the β-sheet showed a negative correlation with the 'folding' shifts (difference between the shift at 1 bar and that of a random coil), suggesting that the main-chain torsion angles of the β-sheet are slightly altered by pressure. Significant pressure-induced shifts were also observed for the side-chain protons (but no larger than 10% of the 'folding' shifts), demonstrating that the tertiary structure of gurmarin is also affected by pressure. Finally, the linearity of the pressure-induced shifts suggests that the compressibility of gurmarin is invariant in the pressure range between 1 and 2000 bar.

Abbreviations: NOESY, nuclear Overhauser effect spectroscopy; ppm, parts per million; TOCSY, total correlation spectroscopy; TPPI, time proportional phase incrementation; TSP, 3-trimethylsilyl-(2,2,3,3,-^{2}H)-propionate-d_4.

Introduction

Interest in pressure effects on the structure and dynamics of proteins has increased recently, not only as a means for understanding the molecular basis of biological effects of pressure, but also for understanding the basic nature of the folded protein structure and stability (Gekko and Noguchi, 1979; Wagner, 1980; Morishima, 1987; Kitchen et al., 1992; Jonas and Jonas, 1994; Yamaguchi et al., 1995; Takeda et al., 1995; Chalikian et al., 1995; Markley et al., 1996; Urbauer et al., 1996; Goossens et al., 1996; Paci and Marchi, 1996; Akasaka et al., 1997; Li et al., 1998; Panick et al., 1998). So far, however, pressure effects on protein structure have been studied little at atomic scale detail. We recently introduced a new technique that utilizes an on-line high pressure sample cell system in conjunction with a high-resolution NMR spectrometer operating at 17.6 T (Yamada et al., 1997). With this new technique, one can, in principle, trace structural changes of a small protein in solution at each atom site in the pressure range from 1 bar to 2000 bar. So far, we have shown a preferential compression of the hydrophobic core in lysozyme

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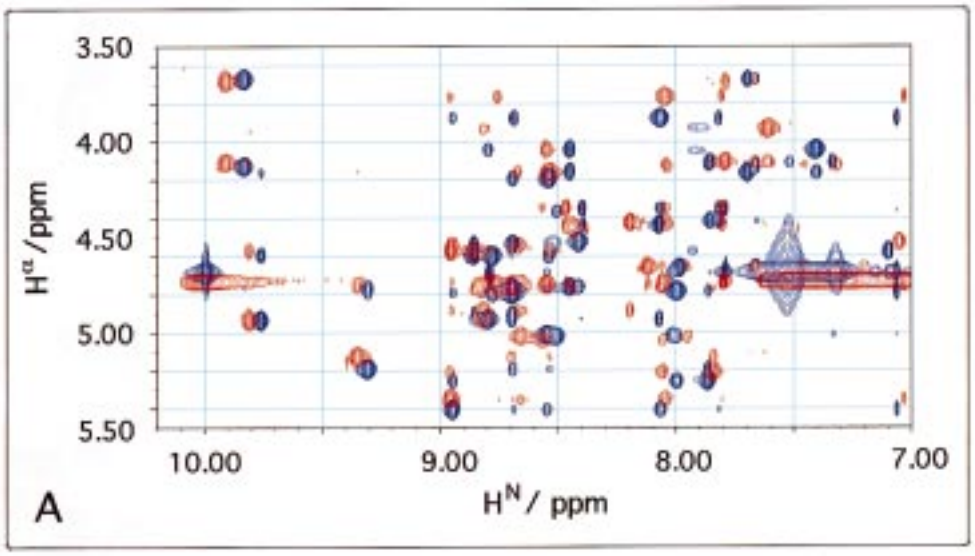
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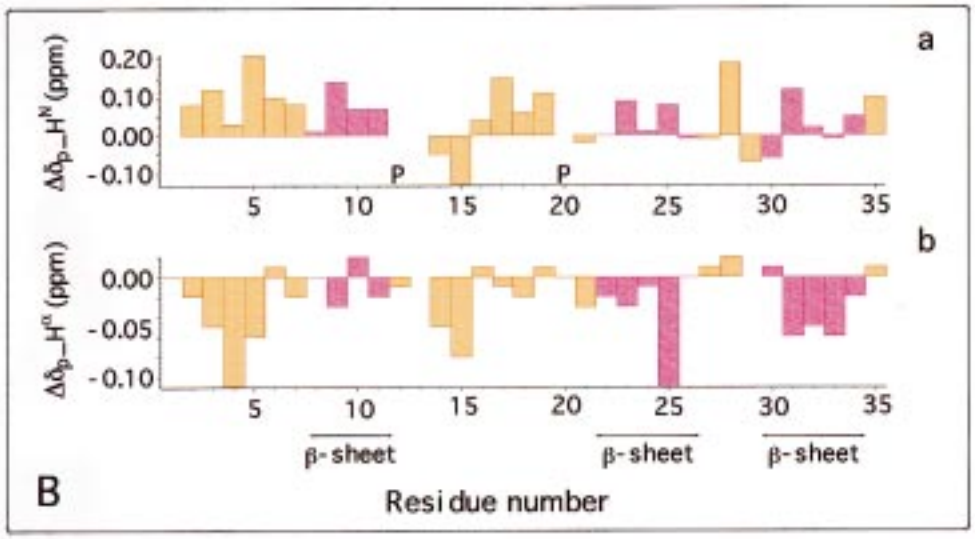
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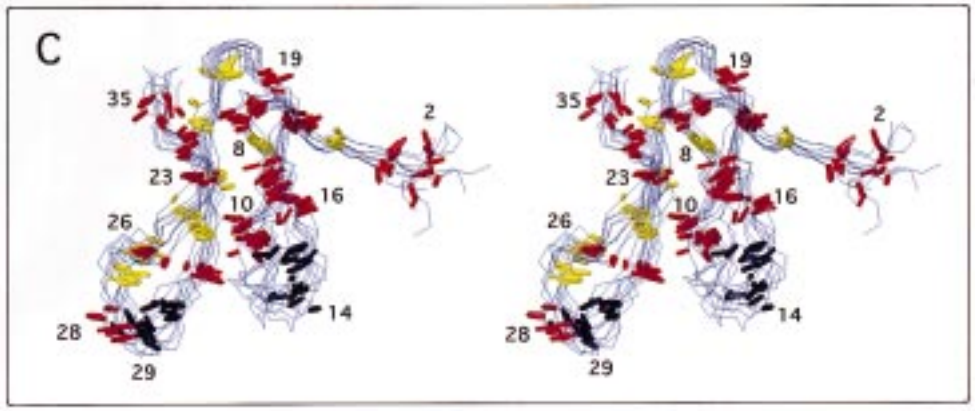
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Figure 1. A. The fingerprint (H^N-H^α) region of the NOESY spectra of gurmarin at 1 bar (blue) and 2000 bar (red), measured at 750 MHz at 40 °C. All expected H^N-H^α cross peaks except for that of Tyr^{13} (35 residues minus 2 Pro residues) were assigned to individual residues by reference to the literature (Arai et al., 1995). Gurmarin was dissolved to a concentration of 9.7 mM in 150 mM maleate-2,3-d_2 buffer in 95% ^1H_2O/5% ^2H_2O, pH 2.0. B. Histograms of pressure-induced shifts $\Delta\delta_p$ (δ at 2000 bar minus δ at 1 bar) at 40 °C for individual amide protons (a) and C^α protons (b). C. Cross-eyed stereo view of the backbone atoms with positions and directions of the NH bonds, derived from 10 NMR structures (Arai et al., 1995) which were taken from the Brookhaven Protein Data Bank and drawn using the program MOLMOL. The three colors represent different ranges of pressure-induced shifts ($\Delta\delta_p$) of the NH protons: $\Delta\delta_p > 0.03$ ppm (red), $-0.03 \leq \Delta\delta_p \leq 0.03$ ppm (yellow) and $\Delta\delta_p < -0.03$ ppm (blue).

(Akasaka et al., 1997) and shortening of individual hydrogen bonds in BPTI (Li et al., 1998).

In the present work, the effects of pressure on the conformation of gurmarin, a 35-residue polypeptide from *Gymnema sylvestre* (Imoto et al., 1991), was studied in aqueous environment. Gurmarin was discovered as an inhibitor of the sweet taste sensation in rat in *Gymnema sylvestre*, a plant used from old times in India as a remedy for diabetes (Imoto et al., 1991). The protein has the primary sequence EQCVKKDELCIPYYLDCCEPLECKKVN WWDHKCIG-COOH with three disulfide bridges (Arai et al., 1995). The structure contains an antiparallel β-sheet (β1: residues 8–11, β2: residues 22–26, and β3: residues 30–34) and loops, and its folding topology shares commonality with ω-conotoxin, a neurotoxin from marine snails (Nishiuchi et al., 1986), and MCTI-II, a serine protease inhibitor from bitter gourd seeds (Hara et al., 1989). Because of its small size, we were able to follow most of the proton signals of gurmarin as a function of pressure. Moreover, practically all the proton signals of gurmarin have been assigned, and its average structure has been determined on the basis of NOE distance and *J* coupling constraints (Arai et al., 1995).

Materials and methods

Sample preparation

Gurmarin (Imoto et al., 1991) was purified from leaves of *Gymnema sylvestre*. For two-dimensional NMR measurements, the samples were dissolved into 95% ^1H_2O/5% ^2H_2O containing 150 mM maleic-2,3-d_2-anhydride (Isotec Inc.) to make a 9.7 mM solution of gurmarin for NMR measurements. The pH of the maleate buffer solution was adjusted to 2.0 by adding small amount of NaOH or HCl. Among carboxylate buffers, maleic acid (pKa = 1.96) is known to have one of the smallest volume changes ($\Delta V = -6.7$ ml/mol) (Isaacs, 1981) upon proton dissociation, and thus this buffer minimizes pressure-dependent pH changes. For chemical shift reference, trace amounts of 3-trimethylsilyl-(2,2,3,3-^{2}H)-tetradeuteropropionic acid sodium salt (TSP-d_4) and dioxane were added to the sample solution.

High pressure NMR apparatus

The design principle of the on-line high pressure NMR cell system used in the present study was reported earlier (Yamada, 1974). The protein solution, contained in a quartz tube cell (inner diameter 1 mm, outer diameter 3 mm), was connected to a pressure mediator (kerosene) via a frictionless piston (Teflon) in a separator cylinder made of BeCu. The cell was positioned in a commercial NMR probe for 5 mm sample tube (Bruker), and the pressure in the cell was regulated to any chosen value between 1 and 2000 bar with a remote hand-pump.

NMR measurements and data analysis

One- and two-dimensional NMR spectra at various pressures were measured on a Bruker DMX-750 spectrometer operating at a proton frequency of 750.13 MHz. All NMR spectra were obtained with spectral windows of 10 kHz. TOCSY experiments (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) with mixing times of 55 ms and 80 ms and NOESY (Jeener et al., 1979; Macura et al., 1981) experiments with a mixing time of 100 ms were performed. All the two-dimensional spectra were obtained with 256 complex points in the t_1 domain and 4096 complex points in the t_2 domain, with TPPI (Redfield and Kunz, 1975; Marion and Wüthrich, 1983) for phase sensitive detection in the t_1 domain. Water suppression was accomplished with the pulsed field gradient WATERGATE technique (Piotto et al., 1992) incorporating the 3–9–19 pulse sequence (Sklenar et al., 1993). At all pressures, chemical shifts were measured relative to dioxane added as an internal reference. At 1 bar, dioxane resonated at 3.763 ppm downfield from the methyl signal of 3-trimethylsilyl-(2,2,3,3-^{2}H)-propionate-d_4 (TSP). The mutual separa-

tion of the two signals decreased only slightly with pressure, i.e., by 0.007 ppm at 2000 bar. Data were processed with the XWIN-NMR package (Bruker) running on a Silicon Graphics Indigo2 workstation. The time domain data were zero-filled to 2048 and 4096 complex points in the t_1 and t_2 domains, respectively, and apodized with a quadratic sine-bell window function in both dimensions.

Results

Two-dimensional ^{1}H TOCSY and NOESY spectra were measured at 500 bar intervals from 1 bar to 2000 bar at 40 °C. Figure 1A shows a superposition of two NOESY spectra measured at 1 bar (blue) and at 2000 bar (red) in the fingerprint (H^N-H^α) region. The two spectra differ considerably from each other on both the amide proton and C^α proton axes. Chemical shifts of individual proton signals changed continuously with pressure, while their line widths remained invariant, allowing determination of their chemical shifts at all pressures based on the signal assignments previously performed at 1 bar (Arai et al., 1995). In Figure 1B, the pressure-induced shifts $\Delta\delta_p$ (δ at 2000 bar minus δ at 1 bar) are plotted as histograms for individual amide protons (a) and C^α protons (b). Figure 1C visualizes the distribution of the pressure-induced shifts of the amide protons on the three-dimensional structure of gurmarin determined at 1 bar (Arai et al., 1995). The chemical shift changes are color-coded as (1) $\Delta\delta_p > 0.03$ ppm (red), (2) $-0.03 \leq \Delta\delta_p \leq 0.03$ ppm (yellow) and (3) $\Delta\delta_p < -0.03$ ppm (blue). For the C^α protons, we plot the pressure-induced shifts against their 'folding' shifts ($\Delta\delta_f$), defined as shifts in the folded state at 1 bar minus shifts in the random coiled state at 1 bar (Wüthrich, 1986), in Figure 2.

Pressure-induced shifts $\Delta\delta_p$ at 2000 bar for most protons of gurmarin, including those of the side-chain protons, are listed in Table 1. Shifts of some of the side-chain protons could not be traced with pressure due to the overlap of signals. We note that practically all the protons undergo measurable shifts with pressure, indicating that the entire protein structure responds to a new pressure. Chemical shift changes of individual proton signals are shown as a function of pressure in Figure 3 at 500 bar intervals; (a) for amide protons, (b) for C^α protons and (c) for side-chain protons. We note that all these shifts are surprisingly linear with pressure in the pressure range studied. Moreover, these shifts were fully reversible with pressure, showing that the structural change responsible for the shifts is reversible.

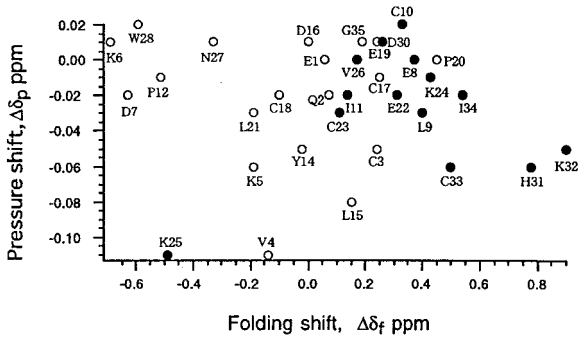

Figure 2. Plot of pressure-induced shifts of individual C^α protons $\Delta\delta_p$ (δ at 2000 bar minus δ at 1 bar) at 40 °C against their 'folding' shifts ($\Delta\delta_f$), defined as shifts in the folded state at 1 bar minus shifts in random coiled state at 1 bar. The filled circles show C^α protons in the β-sheet region.

Discussion

In general, the deviations of ^{1}H chemical shifts of a folded protein (without a prosthetic group) from the corresponding 'random coil' chemical shifts, i.e., the 'folding' shifts, are on the order of 0.1–1 ppm (Pardi et al., 1983; Wüthrich, 1986). Most pronouncedly, the shifts are caused by diamagnetic shielding of the external magnetic field by ring currents of nearby aromatic groups (Perkins, 1982), by magnetic susceptibility anisotropy of neighboring peptide groups (Szilagyi and Jardetzky, 1989; Wishart et al., 1991; Ösapay and Case, 1994), and by the effect of nearby charged groups (Williamson and Asakura, 1993). Namely, any changes in the distance from the proton in question to either the aromatic ring, the peptide bond, or the charge in the neighborhood will lead to the change in the chemical shift of this proton. Our experimental results indicate that practically all the main-chain and side-chain proton signals change their chemical shift positions with pressure (Figure 1B and Figure 3). This result is a clear demonstration that pressure-induced structural changes occur over the entire protein molecule. The degree of structural change, however, should be minor, since the pressure-induced shifts are about an order of magnitude less than the 'folding' shifts.

Most amide protons showed shifts to low field with increasing pressure, the average for the entire NH groups being 0.051 ppm (Figure 1B). This tendency which was observed with basic pancreatic trypsin inhibitor (BPTI) was attributed to the shortening of in-

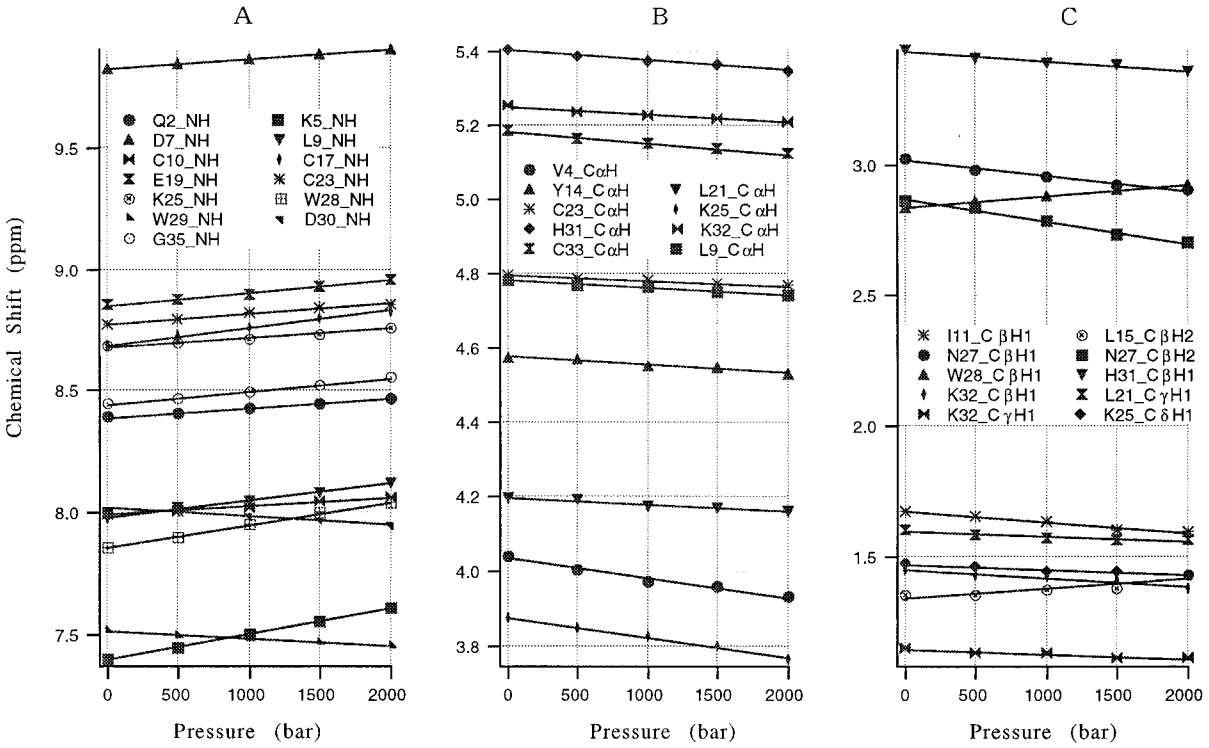

Figure 3. Plot of chemical shifts of selected proton signals of gurmarin against pressure. (A) NH protons, (B) C^{α} protons and (C) side-chain protons. Gurmarin was dissolved to a concentration of 9.7 mM in 150 mM maleate-2,3-d_2 buffer in 95% ^1H_2O/5% ^2H_2O, pH 2.0.

dividual hydrogen bonds (Li et al., 1998). The present observation confirms that this is a general phenomenon for the peptide NH protons in proteins under pressure.

In Figure 1C, one notices further a general tendency for amide groups directed outwardly to the solvent (water) to show larger low field shifts (red) and for those directed inwardly to show much smaller shifts (yellow). The larger tendency for low field shifts of amide protons exposed to solvent is attributable to favorable hydrogen bonding interactions of amide protons with surrounding water molecules (Li et al., 1998). The amide groups showing distinctly small shifts (residues 21, 22, 24, 26, 27, 32 and 33) are localized in the β2 and β3 strands, and coincide almost perfectly with the amide groups that have distinctly slow rates of hydrogen exchange measured at 1 bar (residues 21, 22, 24, 26, 32, 33 and 34; Arai et al., 1995). The coincidence indicates that both quantities reflect well the degree of exposure of the amide group. However, this coincidence is not good for the slowly exchanging amide protons in the β1 strand (residues 8–11; Arai et al., 1995), for which pressure-induced shifts are relatively large except for residue 8 (Figure 1B(a)). This discrepancy may indicate the fluctuating nature of the very short β1 strand.

Recently, pressure dependence of hydrogen exchange rate was found to be different for individual hydrogen bonded amide protons. The result was interpreted in terms of volumes of activation for exchangeable local structures (Hitchens and Bryant, 1998). However, in view of our finding that individual hydrogen bonds are shortened to different degrees by pressure, the exchange rate may also be affected by pressure-strengthening of hydrogen bonds. How the shortening of hydrogen bond affects the exchange rate is an interesting question to be explored in future investigations.

We also note that those in the turn regions show shifts to high field. The high field shifts are rather unusual for amide protons, and may represent a special feature of amide groups in turns, namely pressure-induced rupture of hydrogen bonds.

We notice in Figure 1B that increasing pressure causes different degrees of shifts for different C^{α} protons. In order to explore the origin of these shifts, we plot the pressure-induced shifts against the 'folding' shifts for all the C^{α} protons in Figure 2. We note,

Table 1. Pressure-induced ^{1}H chemical shifts $\Delta\delta_p$ (shifts at 2000 bar minus shifts at 1 bar) in gurmarin[a]

Residue	H^N	H^α	H^β	H^γ	H^δ	H^ε	γMe (Ile)
E1		0.00	−0.05	−0.01			
			−0.02	−0.02			
Q2	0.08	−0.02	−0.03	0.01			
			0.00				
C3	0.12	−0.05	0.03				
			−0.08				
V4	0.03	−0.11	−0.02	−0.01			
				−0.01			
K5	0.21	−0.06	−0.04	0.00	0.00	−0.01	
			−0.06	0.01			
K6	0.10	0.01	−0.02	−0.02		−0.02	
D7	0.08	−0.02	0.00				
			−0.05				
E8	0.01	0.00	0.00	0.00			
L9	0.14	−0.03	−0.02	−0.02			
			0.00				
C10	0.07	0.02	0.00				
I11	0.07	−0.02	−0.08		−0.02		−0.06
P12		−0.01	−0.02	−0.08	0.03		
			0.00		−0.05		
Y13			−0.05				
			−0.05				
Y14	−0.05	−0.05	0.03				
L15	−0.13	−0.08	0.00	0.00			
			0.08				
D16	0.04	0.01	0.00				
C17	0.15	−0.01	−0.04				
			0.02				
C18	0.06	−0.02	0.00				
			0.01				
E19	0.11	0.01	0.01	−0.03			
P20		0.00	−0.03	0.00	0.00		
					0.01		
L21	−0.02	−0.03	−0.06	−0.04			
			−0.04				
E22	0.00	−0.02	0.01	0.01			
				0.01			
C23	0.09	−0.03	−0.03				
			−0.02				
K24	0.01	−0.01	−0.01	−0.03		−0.01	
			−0.01	−0.03			
K25	0.08	−0.11	−0.03	0.02	−0.04	0.00	
			−0.03				
V26	−0.01	0.00		−0.01			
				−0.03			
N27	−0.01	0.01	−0.13				
			−0.16				
W28	0.19	0.02	0.09				
			0.03				
W29	−0.07						
D30	−0.06	0.01	0.02				
			0.05				
H31	0.12	−0.06	−0.08				
			−0.04				
K32	0.02	−0.05	−0.07	−0.04	−0.03	−0.02	
C33	−0.01	−0.06	−0.05				
			−0.01				
I34	0.05	−0.02	−0.02	0.03			−0.01
G35	0.10	0.01					

[a] Measured at 40 °C and pH 2.0 with dioxane as chemical shift reference common to two pressures. Experimental error, +/−0.01 ppm.

disregarding the point for K25, a fairly good negative correlation between the pressure-induced shifts and the folding shifts for the C^α protons of the β-sheet (correlation coefficient, −0.61), suggesting that origin of the two shifts may be related. Calculation with the program MOLMOL showed that aromatic ring current contributions to the folding shifts are negligible. Thus the folding shifts of the C^α protons, and hence the pressure-induced shifts as well, may arise primarily from the anisotropic magnetic susceptibility effect from the nearby peptide bonds, i.e. they may be determined by the spatial orientations of the C^α protons to the neighboring peptide bonds. This means that the main-chain torsion angles of the β-sheet may be slightly altered by pressure. Although, in principle, *J* coupling constants such as $^3J_{H^N C^\alpha}$ can also be used for the torsion analysis of the main chain, our experience indicates that they are relatively insensitive to pressure than chemical shifts. Moreover, the signals were relatively broad under the present experimental condition, which prohibited analyses of coupling constants as functions of pressure.

Pressure also affected the chemical shifts of most side-chain protons of gurmarin, generally within 10% of the folding shift at 2000 bar (Table 1). Considerable shifts occurred not only in the C^β protons close to the main-chain, but also in γ, δ and ε protons remote from the peptide bonds. Considerable shifts in δ and ε protons are expected to occur only associated with a change in the tertiary structure, although the detail of the structural change is not known at present.

Finally, a striking linearity was noted in the chemical shift changes for practically all the proton signals with pressure (Figure 3). A similar observation was previously made for some side-chain protons of hen lysozyme (Akasaka et al., 1997). Throughout the pressure range, the signals remained as apparently homogeneous Lorentzian lines, indicating that the signals result from the dynamic average of an ensemble of conformers. The effect of pressure would, therefore, be to change the ensemble of conformers in the conformational space of the protein, thereby causing a change in the average of the conformations and conformational dependent chemical shifts. Granting that the chemical shift changes are linearly dependent on small changes in the average conformation or interatomic distances, the observed linearity of chemical shifts would suggest that the compressibility of gurmarin is practically invariant in the pressure range between 1 bar and 2000 bar.

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