Rapid internal dynamics of BPTI is insensitive to pressure

^{15}N spin relaxation at 2 kbar

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Abstract Pressure effects on the backbone dynamics of a native basic pancreatic trypsin inhibitor (BPTI) have been measured by ^{15}N spin relaxation and chemical shifts at 30 and 2000 bar. The experiments utilized the on-line variable pressure cell nuclear magnetic resonance system on ^{15}N-uniformly labeled BPTI at a proton frequency of 750.13 MHz at 36°C. Longitudinal (R_1) and transverse (R_2) ^{15}N relaxation times and (^{1}H)–^{15}N nuclear Overhauser effects were measured for 41 protonated backbone nitrogens at both pressures. The model free analysis of the internal dynamics gave order parameters for individual H–N vectors at both pressures. The results indicate that rapid internal dynamics in the ps–ns range for the polypeptide backbone is not significantly affected by pressure in the range between 30 bar and 2 kbar. The result is consistent with the linear pressure dependence of ^{1}H and ^{15}N chemical shifts of BPTI, which suggests that local compressibilities and amplitudes of associated conformational fluctuation are nearly invariant in the same pressure range. Overall, we conclude that at 2 kbar BPTI remains within the same native ensemble as at 1 bar, with a small shift of population from that at 1 bar.

Key words: High pressure nuclear magnetic resonance; Basic pancreatic trypsin inhibitor; ^{15}N spin relaxation; Chemical shift; Internal dynamics

1. Introduction

The application of pressure to a globular protein generally leads to a more compact structure within the folded manifold [1–5]. Reduction of the volume of a folded protein is accomplished by decreasing inter atomic distances or 'free space' between atoms, and therefore will tend to reduce the mobility of protein atoms. The effect of reduced mobility of atoms is an issue of general interest with regard to the internal structure and dynamics of proteins as well as to the mechanism by which pressure affects protein function. Available information on pressure effects on atomic fluctuations in proteins is limited; B factors of a lysozyme crystal are slightly decreased at 1 kbar as compared with those at 1 bar [2]. Molecular dynamics calculations of basic pancreatic trypsin inhibitor (BPTI) in solution show that backbone atom fluctuations are decreased by about one third at 10 kbar from those at 1 bar [3]. On the other hand, Brunne and van Gunsteren [4] found that the rms fluctuation in ϕ,ψ angles remain the same at 5 kbar as at 1 bar in their MD calculation. However, the effect of pressure on rapid internal motions of proteins in solution has not been explored by a direct experiment.

Internal mobility of atoms for the entire polypeptide backbone in a protein in ps–ns ranges can be studied most conveniently by spin relaxation of a ^{15}N nucleus in a ^{15}N-uniformly labeled protein [6]. Application of the same technique to a protein under pressure is expected to shed light on the general issue of pressure effect of internal dynamics. Recently, we introduced an on-line variable pressure cell technique to a high field nuclear magnetic resonance (NMR) spectrometer system, which opens the full capability of modern NMR spectroscopy to the study of proteins under pressure at least up to 2 kbar [7–10]. The active volume of the sample solution for the NMR signal detection is small ($\sim$10 μl) because of the limited cell volume, making the sensitivity of signal detection considerably low compared to measurements under normal pressure. Nonetheless the use of a high field spectrometer (e.g. 17.6 T or 750 MHz for proton) is expected to overcome the sensitivity problem to a significant extent. Our purpose here is to investigate pressure effects on ns–ps backbone dynamics of a globular protein at individual residue basis for the first time using ^{15}N spin relaxation.

We chose BPTI as a model target system. As a globular protein with 58 amino acid residues, it has three disulfide bridges 5–55, 14–38 and 30–51, of which the bond 14–38 joins flexible loops in the reactive site segment. The N terminal segment from Asp-3 to Leu-6 forms a 3_{10} helix, and is followed by an antiparallel β-sheet from Ile-18 to Asn-24 and from Leu-29 to Tyr-35 joined by a four residues turn, while the C terminal segment from Ala-48 to Thr-54 is an α helical structure [11,12]. Our previous studies with high pressure NMR showed that BPTI remains fully folded at least up to 2 kbar (36°C, pH 4.6) [7,9,10]. The same work also shows that pressure leads to compaction of the folded structure, including shortening of most intra-molecular hydrogen bonds [7] and increased side chain packing [9] along with changes in torsional angles of the polypeptide backbone, all of which occur in site-specific fashions [10]. Since a positive compressibility is associated with volume fluctuation [13], the above result indicates that the conformation of BPTI is fluctuating within its folded structure. B factors [11,12], ^{15}N spin relaxation by Szyperski [14] and molecular dynamics calculation carried out by Kitchen et al. [3] show the presence of internal motions in the ps–ns time range in the BPTI backbone. In particular, the latter work shows a substantial decrease in the atomic mobility at high pressure throughout the molecule. These results

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further motivated us to investigate the effect of pressure on rapid backbone dynamics of BPTI monitored by ^{15}N spin relaxation.

2. Materials and methods

2.1. Sample preparation

^{15}N-labeled BPTI was prepared as previously reported [10]. The lyophilized protein was dissolved to a concentration of 5 mM in water (90% $^{1}H_2O$, 10% $^{2}H_2O$) containing 100 mM acetate buffer, pH 4.6.

2.2. NMR measurements and data analysis

Our high pressure NMR system combines the commercial Bruker DMX-750 with a homemade on-line high pressure quartz cell whose inner diameter was about 0.8 mm [15,16]. The spin relaxation measurements of the uniformly ^{15}N-labeled BPTI were carried out on a Bruker 5 mm inverse-detected triple-resonance probe with field gradient and deuterium field-frequency lock for the same sample in the pressure cell at 30 and 2000 bar. The choice of 30 bar instead of 1 bar for the low pressure measurement was purely for technical reasons. The spectrum at 30 bar was essentially the same as the spectrum at 1 bar. Longitudinal ($R_1 = 1/T_1$) and transverse ($R_2 = 1/T_2$) ^{15}N relaxation rates and steady-state (^{1}H)–^{15}N nuclear Overhauser effect (NOE) were measured at 309 K (36°C) at a proton frequency of 750.13 MHz and a nitrogen-15 frequency of 76.02 MHz on two-dimensional ^{1}H–^{15}N NMR spectroscopy. The inversion recovery and spin echo pulse sequences designed by Farrow et al. [17] were used with 11 and 12 relaxation delays (τ), respectively, with a recycle delay of 1.8 s prior to each scan. Spectra were acquired in 2435 Hz along F_1 (^{15}N) and in 10 000 Hz along F_2 (^{1}H) with 1024 points in the t_1 domain and 256 points in the t_2 domain. The total measurement times required for T_1 and T_2 were about 45 and 28 h, respectively. For measurements of (^{1}H)–^{15}N NOE, signals were recorded with and without presaturation of the amide protons for 1.5 s with a relaxation delay of 1 s.

All the data were processed with the NMRPipe package [18]. The time domain signals were baseline corrected and processed with 90° shift sine apodization function in t_2 (^{1}H) and a 90° shifted sine-squared function in t_1 (^{15}N). Signal intensities were fitted to single exponential functions of the relaxation delay τ. The standard error values in R_1 and R_2 data were determined by the scatter of the data points around the fitted exponential decaying curves. NOE values were obtained by ratios of peak heights with and without proton saturation. The relaxation parameters obtained were analyzed with the formalism of Lipari and Szabo [19] by using a program of Palmer, A.G., Model Free version 3.1.

3. Results

Complete ^{15}N resonance assignments of BPTI at 1 bar were reported by Glushka et al. [20]. Extension of these assignments to 2000 bar was straightforward by noting the linearity of shifts with pressure on the ^{1}H–^{15}N HSQC spectra measured at 500 bar intervals [10]. A total of 41 protonated backbone nitrogen signals were identified. Some representative plots of ^{15}N T_1 and T_2 decays at 30 bar (solid lines, filled squares) and 2000 bar (dotted lines, open circles) are shown in Fig. 1 respectively.

Experimentally determined longitudinal ($R_1 = 1/T_1$) and transverse ($R_2 = 1/T_2$) ^{15}N relaxation rates times are plotted for backbone amide groups in Fig. 2 (A: R_1) and (B: R_2) at 30 bar (solid lines) and at 2000 bar (dotted lines). R_1 values at 30 bar show relatively little variation ($1.29 \pm 0.77 \sim 2.41 \pm 0.67$ s^{-1}) against amino acid sequence, with an average value of 1.95 ± 0.15 s^{-1}. Deviations of R_1 slightly above the average were noted particularly in the N-terminal helix (residues 5–7), and below the average (1.38 ± 0.021 s^{-1}) in C-terminal region (residues 53 and 58). The relaxation data contained large errors in the loop regions, particularly for residue 15 and residue 42, which are omitted from the figure.

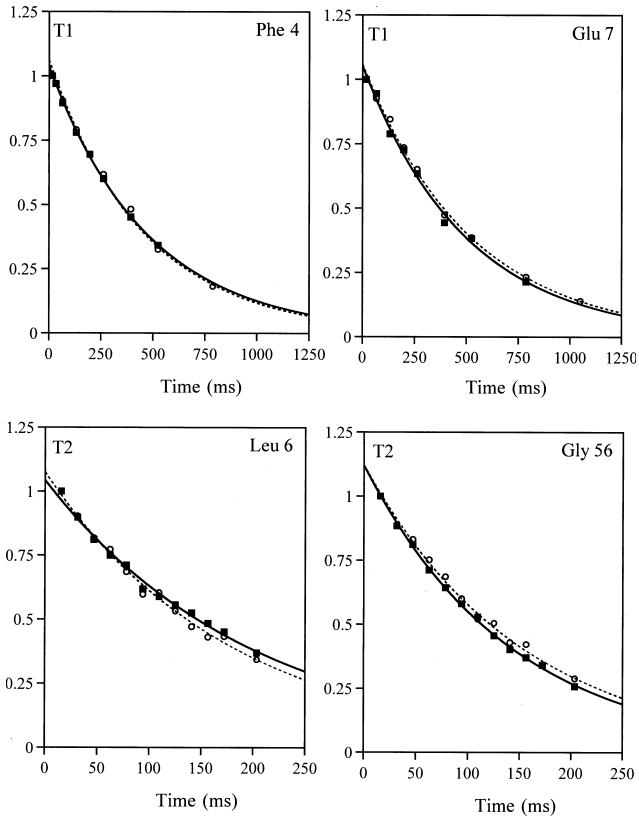

Fig. 1. Plots of cross peak intensity versus delay time in ^{15}N T_1 and T_2 experiments at 30 bar (solid lines, filled squares) and 2000 bar (dotted lines, open circles) for selected residues of BPTI with single exponential least-squares fit curves.

In contrast, R_2 values at 30 bar show a larger variation, namely ($2.80 \pm 0.021 \sim 12.73 \pm 0.69$ s^{-1}) about the average value of 5.61 ± 0.16 s^{-1}. Values significantly larger than the average are in two loop regions (around residues 12–16 and residue 38 and 39) (an average of 7.99 ± 0.36 s^{-1}). Values significantly smaller than the average are in the C-terminal region (residues 53 and 58) (an average of 5.00 ± 0.21 s^{-1}). These features of R_1 and R_2 variations along the sequence at 30 bar coincide qualitatively with those found previously at 1 bar at a lower frequency (500 MHz for proton) by Szyperski et al. [14] for uniformly ^{15}N labeled BPTI under similar conditions at 1 bar. Briefly, the smaller R_2 values in the C-terminal region suggest the presence of internal motions, whereas the larger R_2 values in the loop regions show the existence of some mechanism of line broadening.

At 2000 bar (Fig. 2, solid lines), the R_1 and R_2 values range between 1.28 ± 0.76–2.28 ± 0.12 s^{-1} for R_1 and 3.24 ± 0.17–10.49 ± 0.84 s^{-1} for R_2. The average values of R_1 and R_2 are 1.91 ± 0.13 s^{-1} and R_2 5.66 ± 0.27 s^{-1}, respectively, which almost coincide with 1.95 ± 0.15 s^{-1} and 5.61 ± 0.16 s^{-1} at 30 bar within experimental errors. The (^{1}H)–^{15}N NOE values (data not shown) show larger scattering of data than R_1 and R_2 values due to limited signal-to-noise ratio, with variations in the range 0.50–0.96 at 30 bar with an average of 0.77 ± 0.10 and in the range 0.53–0.98 at 2000 bar with an

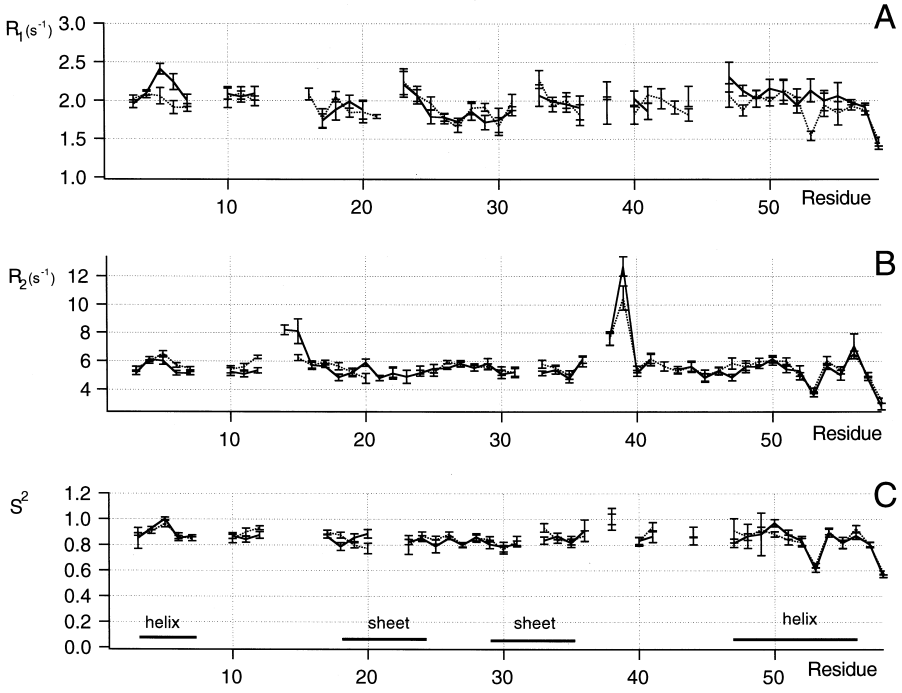

Fig. 2. Plot of experimentally determined (A), longitudinal ($R_1 = 1/T_1$), (B) transverse ($R_2 = 1/T_2$) ^{15}N relaxation rates and (C) order parameters (S^2) for backbone amide groups at 30 bar (solid lines) and at 2000 bar (dotted lines). The error bars are calculated from the S.D. of the data points in the determination of relaxation rates. Measurements were made at 36°C in 5 mM aqueous (90% ^1H_2O/10% ^2H_2O) solution of BPTI, pH 4.6 at a proton frequency of 750.13 MHz by using the on-line variable pressure cell technique.

average of 0.75 ± 0.10. Average NOE values at two pressures are very close to each other.

The model free analysis by Lipari and Szabo [19] was performed on the R_1 and R_2 and NOE data. The result gave the overall rotational correlation time of $\tau_c = 3.28 \pm 0.03$ ns at 30 bar and $\tau_c = 3.48 \pm 0.03$ ns at 2 kbar, with the order parameters plotted in Fig. 2C. The average order parameters for all residues are 0.85 ± 0.08 at 30 bar and 0.85 ± 0.08 at 2 kbar.

4. Discussion

Contrary to our naive expectation, changes in the R_1, R_2 and NOE values at 2000 bar from the corresponding values at 30 bar were quite small. The patterns of variation in R_1 and R_2 values against the amino acid sequence in Fig. 2 depict the same tendencies for R_1 and R_2 at both pressures. These observations indicate straightforward that the effect of pressure on the motional dynamics of the polypeptide backbone of BPTI in the ps–ns range is not large in the range between 30 and 2000 bar. Let us examine the difference in more detail below.

First, under the present experimental condition, the change in the overall rotational correlation time of the protein by pressure is very small (3.28 ± 0.03 ns at 30 bar and 3.48 ± 0.03 ns at 2 kbar). Obviously, this is due to the compensation between the two opposing effects, i.e. the hydrogen-bond breaking and the hydrogen-bond contraction of water by pressure, under the present experimental condition [21]. Thus any significant differences in the R_1 and R_2 values for individual residues between the two pressures would suggest changes in internal dynamics by pressure.

At 30 bar, distinctly larger R_2 values, namely the extra broadening of signals, than the average are noted for the two loop regions (around residues 12–16 and residues 38, 39). The extra broadening can be explained by the existence of an exchange phenomenon between different conformations in the two loop regions. Since the loops are connected by the disulfide bridge 14–38, the broadening is likely to result from multiple conformations of the disulfide bridge [22] that are mutually exchanging slowly in the NMR time scale. At 2000 bar, the R_2 values for the loop regions show some decreasing tendency (from 12.73 ± 0.69 s^{-1} at 30 bar to 10.49 ± 0.84 s^{-1} at 2000 bar for the second loop (residue 39)), suggesting that the exchange phenomenon is slightly affected by pressure.

On the other hand, the distinctly smaller R_2 values in the C-terminal part at 30 bar suggests the presence of rapid internal motions in this region. The R_2 values remained the same at 2 kbar, indicating that the internal motions in this region are not affected by pressure. The result is in qualitative agreement with the result of a molecular dynamics calculation at 5 kbar [4].

Except the regions mentioned above, there are no significant changes in R_1 and R_2 values with pressure. The result of the model free analysis shows that there are practically no differences in the order parameters between the two pressures (Fig. 2C). At first, this result may look puzzling, since the application of pressure to a globular protein generally leads to a more compact structure [1–5,7–10] and is expected to restrain internal motions. We will discuss, in the following, the results of the spin relaxation in the light of the previously obtained pressure-induced chemical shifts of BPTI [7,9,10]. Practically all the ^1H and ^{15}N signals of BPTI change their chemical shifts continuously, linearly and reversibly with pressure between 1 bar and 2 kbar. No conformational transition (i.e. denaturation) takes place within 2 kbar. Thus BPTI remains 'elastic' below 2 kbar [23]. The continuous changes in

chemical shifts indicate that the average structure of folded BPTI changes continuously with pressure within the folded manifold. Namely, the effect of pressure is to shift the population slightly within the native ensemble, and the resultant ensemble average gives the linear chemical shift changes.

The distinct linearity of pressure-dependent shifts in BPTI indicates that the conformational change involving small changes in non-covalent bond distances and torsion angles can be approximated as linear functions of pressure. The linearity of local conformational response to pressure may be expressed as pressure-independent local compressibility. Since compressibility is closely related to the amplitude of volume fluctuation [13], a pressure-independent local compressibility would indicate that the amplitude of local volume fluctuation is also pressure-independent. This would give the basis for pressure-independent atomic fluctuations at all frequencies and therefore for the resultant pressure-independent spin relaxation as observed.

In reality, the linear chemical shift with pressure is expected when the shift of population within the native ensemble is small with a small resultant change in the average conformation. Indeed, our estimate based on ^{15}N pressure shifts indicates that the average change in ψ angles in the polypeptide backbone of BPTI is only 1–2° at 2 kbar for the β-sheet part and slightly larger but comparable magnitudes of change are expected for other regions [10]. It means that the structural changes induced in the backbone of BPTI at 2 kbar are small on average. On the other hand, from the observed average order parameters 0.85, we estimate the average angle of fluctuation of the N–H vector to be about of $\pm 20°$ for both pressures. Since the angle of fluctuation is a measure of the distribution of the native ensemble, the notion that pressure causes a comparatively small shift of population at 2 kbar within the native ensemble appears to be justified. The application of pressure at 2 kbar would not significantly affect the average dynamic behavior of BPTI including ^{15}N spin relaxation that represents average backbone dynamics in the ps–ns time range.

In conclusion, rapid internal dynamics in the ps–ns range of the polypeptide backbone of a folded globular protein BPTI is not significantly affected by pressure in the range 30 bar to 2 kbar. The result is not surprising in view of the linear pressure dependence of ^{1}H and ^{15}N chemical shifts which shows small shifts of population in the native ensemble in the same pressure range. Since similar linearity in pressure shifts up to 2 kbar has been observed also in lysozyme and gurmarin, all with disulfide bonds [5,7–10], the same notion will probably apply to these proteins. However, this does not mean that generally pressure has little effect on protein dynamics below 2 kbar. On the contrary, pressure dramatically affects rare motions in proteins which occur outside the range of ps–ns, e.g. ring flip motions in the ms range [9,24] and unfolding-refolding reactions in the s range [25,26]. This is because the latter are rare events requiring large activation volumes, while chemical shifts and spin relaxation represent average dynamic properties of the native ensemble involving little activation volumes. The whole picture of dynamics required to understand protein function includes both.

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