Journal of Biomolecular NMR, **17:** 257–263, 2000.
KLUWER/ESCOM

Pressure effect on the dynamics of an isolated α-helix studied by ^{15}N-^{1}H NMR relaxation

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Received 20 December 1999; Accepted 8 May 2000

Key words: alpha-helix, bacteriorhodopsin, chemical shift anisotropy (CSA), dynamics, high pressure, hydrogen bond, spin relaxation

Abstract

Dynamics and structure of (1–36)bacteriorhodopsin solubilized in chloroform/methanol mixture (1:1) were investigated by ^{1}H-^{15}N NMR spectroscopy under a hydrostatic pressure of 2000 bar. It was shown that the peptide retains its spatial structure at high pressure. ^{15}N transverse and longitudinal relaxation times, ^{15}N{^{1}H} nuclear Overhauser effects, chemical shifts and the translation diffusion rate of the peptide at 2000 bar were compared with the respective data at ambient pressure [Orekhov et al. (1999) *J. Biomol. NMR*, **14**, 345–356]. The model free analysis of the relaxation data for the helical 9–31 fragment revealed that the high pressure decreases the overall rotation and translation diffusion, as well as apparent order parameters of fast picosecond internal motions (S_f^2) but has no effect on internal nanosecond motions (S_s^2 and τ_s) of the peptide. The decrease of translation and overall rotation diffusion was attributed to the increase in solvent viscosity and the decrease of apparent order parameters S_f^2 to a compression of hydrogen bonds. It is suggested that this compression causes an elongation of H-N bonds and a decrease of absolute values of chemical shift anisotropy (CSA). In particular, the observed decrease of S_f^2 at 2000 bar can be explained by 0.001 nm increase of N-H bond lengths and 10 ppm decrease of ^{15}N CSA values.

Introduction

Pressure, applied to biopolymers, is a variable the response to which provides valuable information on a system under study (Gross and Jaenicke, 1994; Jonas and Jonas, 1994; Heremans and Smeller, 1998). NMR spectroscopy was introduced into this field after appropriate high-pressure instrumentation had been developed (Jonas, 1972; Yamada, 1974; Jonas et al., 1993; Yamada et al., 1994). At the very beginning paramagnetism-induced chemical shifts (Morishima et al., 1980; Morishima and Hara, 1982) detected local structure changes in proteins under pressure, far from the denaturing conditions. More recently, pressure effects on the protein structure were studied by nuclear Overhauser effects, spin-spin coupling constants (Peng et al., 1993; Urbauer et al., 1996; Li et al., 1999) and diamagnetic chemical shifts (see Akasaka et al., 1999 and references therein). The thermodynamic parameters of pressure-induced protein unfolding were also studied by NMR spectroscopy (Samarasinghe et al., 1992; Peng et al., 1993; Yamaguchi et al., 1995; Prehoda et al., 1998).

Only few studies were devoted to pressure effects on protein dynamics. Until now the most detailed information on high-pressure effects on protein inter-

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Supplementary material: The relaxation rates, NOEs, and the model-free parameters of (1–36)BR at 2000 bar are available from the authors upon request.

nal dynamics was provided by NMR, which revealed remarkable retardation of the flip rates of aromatic rings within the hydrophobic core of BPTI (Wagner, 1980, 1983; Li et al., 1999). According to Carter et al. (1978), pressure initially slows down deuterium exchange rates of amide protons in lysozyme and ribonuclease A but has the opposite effect at elevated pressures, i.e. initially proteins become more rigid while further pressure increase induces protein unfolding. Similar results were reported in phosphorescence quenching studies of protein dynamics under high pressure (Cioni and Strambini, 1999).

Pressure-induced changes in the internal dynamics of a protein might be put into effect by two mechanisms. First, high pressure promotes hydration of non-polar side chains in proteins (e.g. see Yamaguchi et al., 1995). As a result, the equilibrium between folded and unfolded states of a protein could be shifted toward unfolded ones (see Kharakoz, 1997 and references therein). This should be manifested in the apparent motional amplitudes on a broad time scale. Second, pressure decreases the size of internal cavities (Wagner, 1980; Akasaka et al., 1997), thus the rate of structural fluctuations might slow down. However, the resulting effect of pressure on a protein is hard to understand as the two mechanisms act in opposite directions. To make the problem more tractable, it would be useful to avoid hydration effects and to study the effect of pressure on protein dynamics in an organic solvent. Further reduction of the problem would be a study of not a whole protein but its constituents, such as β-sheet or α-helix.

In the present report the (1–36) bacteriorhodopsin ((1–36)BR) solubilized in chloroform–methanol mixture, in which residues 9–31 of the peptide are helical (Pervushin and Arseniev, 1992), has been chosen as a well-studied model to examine the effect of pressure on the internal dynamics of α-helix by ^{1}H-^{15}N NMR relaxation techniques. A set of ^{15}N relaxation data for (1–36)BR has been recently obtained at the three spectrometer frequencies of 500, 600 and 750 MHz at ambient pressure (Orekhov et al., 1999). In addition, the molecular dynamics simulation of (1–36)BR is also available at ambient pressure (Korzhnev et al., 1999a). As a result, intramolecular motions on pico- and nanosecond time scales have been detected in the peptide. Nanosecond motions with an average order parameter of ca. 0.6 in the helical part of the peptide were attributed to helix–coil equilibrium transitions (Korzhnev et al., 1999b).

Materials and methods

The uniformly ^{15}N-labelled (1–36)BR was obtained as described previously (Orekhov et al., 1995). All NMR measurements were performed on a Bruker DMX-750 spectrometer operating at a ^{1}H frequency of 750.13 MHz with deuterium field-frequency lock. The on-line variable pressure NMR sample cell technique (Yamada, 1974; Yamada et al., 1994) was employed without modification of the hardware. The cell was made of quartz with an inner diameter of about 1 mm, which was connected to a high-pressure kerosene line. The pressure can be chosen and maintained for several days at any desired value between 1 and 2000 bar with a hand pump located remote from the 17.6 Tesla magnet (Japan Magnet Technology). The cell was placed in a 5 mm triple resonance probe with inverse ^{1}H detection and *x,y,z*-pulse field gradients provided by Bruker Co. In all NMR experiments special attention was paid to the temperature of the sample. The temperature control unit was calibrated to a temperature of 30 °C using a methanol sample at 1 bar as in the previous study of (1–36)BR dynamics (Orekhov et al., 1999). All spectra were processed and quantified by using VNMR software (Varian Associates Inc.; NMR Instruments).

Chemical shifts of the amide protons and nitrogens were measured in the ^{1}H-^{15}N PFG sensitivity enhanced HSQC spectra (Kay et al., 1992; Zhang et al., 1994). Chemical shifts of other protons were determined in the ^{15}N decoupled TOCSY (Bax and Davis, 1985) spectra. Assignments of ^{1}H and ^{15}N signals at 1 bar (Orekhov et al., 1995) were used to assign signals at 2000 bar, which was straightforward due to the relatively small changes in the chemical shifts by pressure. Uncertainties of the chemical shifts were within 0.01 ppm both for ^{1}H and ^{15}N. Chemical shifts of individual protons were referenced to the residual proton signal of the methyl group of 99.8% deuterated methanol $C^{2}H_{3}O^{1}H$, 3.26 ppm downfield from the tetramethylsilane signal. For ^{15}N chemical shift reference, the procedure of indirect referencing for ^{15}N chemical shifts was used (Wishart et al., 1995) at both pressures.

For the diffusion measurements, a slightly modified version of the spin-echo experiment described by Altieri et al. (1995) was used. A standard Bruker PFG power supply on the DMX-750 instrument was used along with a field gradient coil on the probe. Prior to the diffusion experiments the temperature of the sample was allowed to equilibrate for at least 1 h. No

precise calibration of the gradient strength was performed, since we were interested only in the relative changes in the self-diffusion rates at 1 and 2000 bar. The encoding/decoding pulse field gradient was varied 64 times up to ca. 50 Gs/cm. Delays for the diffusion of 30 and 100 ms were used in the experiments at 1 and 2000 bar, respectively. A relaxation delay of 2 s was used prior to each scan. Signals of the peptide methyl groups, the residual protons of deuterated chloroform and the methanol methyl group were used for the diffusion measurements of (1–36)BR. Self-diffusion rates and their uncertainties were obtained in a three-parameter least squares fit of the signal exponent decays versus the square of the gradient strength. Introduction of the offset parameter into the fit was caused by the technical aspects of our self-diffusion experiments and was statistically proven by an F-test.

Longitudinal and transverse relaxation rates and heteronuclear $^{15}N\{^1H\}$ NOEs were measured with the pulse sequences of Farrow et al. (1994). A T_2 experiment was recorded with a delay of 1 ms between ^{15}N 180° pulses in the CPMG sequence. T_1 and T_2 relaxation times of the backbone nitrogens were obtained with relaxation delays of 0.02, 0.03, 0.07, 0.13, 0.20, 0.26, 0.40, 0.53, 0.79, 1.05, 1.58 and 0.02, 0.03, 0.05, 0.06, 0.08, 0.09, 0.11, 0.13, 0.14, 0.16, 0.17 and 0.20 s, respectively. A delay of 2 s was used prior to each scan in the T_1 and T_2 experiments. For the $^{15}N\{^1H\}$ NOE experiment, a relaxation delay of 1 s was used before each scan and the length of the proton presaturation period was 3 s. The total acquisition time for each relaxation or NOE measurement was about 1 day. Fitting of the exponential relaxation decays and model-free analysis were performed with the homebuilt software DASHA (Orekhov et al., 1996). Measurements were performed for the same set of backbone amides as in the previous study at 1 bar (Orekhov et al., 1999). A two-parameter exponential fit was used to obtain the longitudinal and transverse relaxation rates. The inclusion of the offset parameter into the fit was shown to be statistically not meaningful for all amide nitrogens on the basis of the F-test with a confidence level of 0.1. Uncertainties of the relaxation rates were obtained from a covariance matrix after the least squares fit of the relaxation decays. These uncertainties correspond well to those obtained by Monte Carlo simulation. To account for the possible small systematic bias in the experimental data a minimal relative uncertainty level of 2% was chosen for T_1 and T_2. Uncertainties in the $^{15}N\{^1H\}$ NOEs were estimated from the signal/noise ratio and the lowest absolute uncertainty level was set to 0.05. Uncertainties of the model free parameters were obtained by Monte Carlo calculations with 500 minimizations starting from the simulated pseudo experimental relaxation rates normally distributed within the experimental uncertainties.

Results and discussion

Chemical shifts

Backbone $C^\alpha H$ proton chemical shifts are known to be an indicator of the secondary structure of polypeptides (Szilagyi and Jardetzky, 1989; Wishart et al., 1991, 1992). The formation of an α-helix is accompanied by a 0.3–0.4 ppm upfield shift of $C^\alpha H$ proton signals from the respective values in a random coil (Jimenez et al., 1987; Szilagyi and Jardetzky, 1989; Williamson, 1990; Wishart et al., 1991). At ambient pressure the $C^\alpha H$ protons from the α-helical part of (1–36)BR showed a value of 0.38 ± 0.13 ppm. A pressure of 2000 bar induced small changes in the $C^\alpha H$ proton chemical shifts of (1–36)BR, resembling random variations near zero (Figure 1). We consider this as evidence that the secondary structure of the peptide is conserved under pressure.

The 2000 bar pressure-induced changes in the amide proton chemical shifts of (1–36)BR exhibit a downfield trend of 0.032 ± 0.037 ppm (Figure 1b). A slightly larger (0.05–0.06 ppm) pressure effect on the chemical shifts of hydrogen bonded amide protons was reported for globular proteins dissolved in water (Inoue et al., 1998; Li et al., 1998). The amide ^{15}N signals of (1–36)BR exhibit significant shifts at 2000 bar with a mean value of 0.48 ± 0.24 ppm (Figure 1c), which is comparable to the 0.47 ppm for BPTI (Akasaka et al., 1999). The considerable variance among pressure-induced ^{15}N chemical shifts of (1–36)BR suggests specific changes of the local environment for the amide groups.

Self-diffusion data and overall molecular rotation

(1–36)BR is a rod-like molecule (Pervushin and Arseniev, 1992), thus the anisotropy of overall molecular tumbling has to be properly accounted for in the regular analysis of NMR relaxation data. However, the NH vectors of the α-helical part of (1–36)BR nearly coincide with the longest axis of the diffusion tensor. This enables one to use a single effective correlation time of the overall molecular tumbling (τ_R) for all NH vectors of the (1–36)BR α-helical part (Orekhov

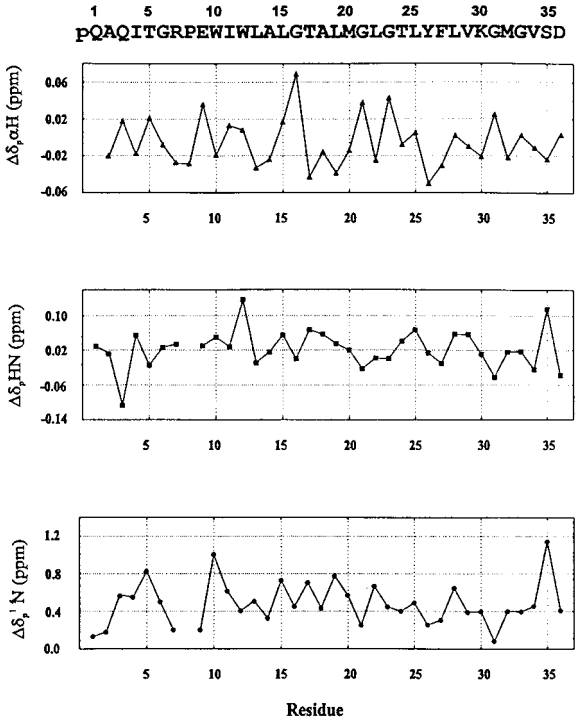

Figure 1. Pressure-induced changes in the chemical shifts of Hα protons (mean value for Gly residues) ($\Delta\delta_p$Hα), HN-protons ($\Delta\delta_p$HN) and ^{15}N nuclei ($\Delta\delta_p{}^{15}$N) in (1–36)BR.

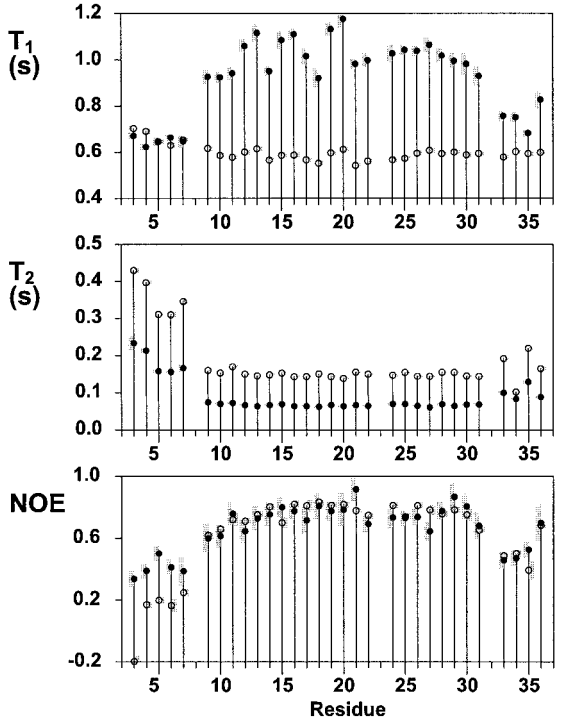

Figure 2. Experimental longitudinal and transverse ^{15}N relaxation times and heteronuclear ^{15}N{^{1}H} steady-state NOEs of (1–36)BR at 2000 bar (filled circles). Corresponding values obtained at 1 bar (Orekhov et al., 1999) are presented for comparison (open circles). Uncertainties of the experimental values are shown as grey bars to the left and right (1 bar) from the filled and open circles, respectively.

et al., 1999). τ_R cannot be obtained from the T_1/T_2 ratio, as all backbone amides of the α-helical part of (1–36)BR are involved in nanosecond time scale motions (Orekhov et al., 1995, 1999). An effective τ_R of 5.8 ns for NH vectors of the α-helical part of (1–36)BR at ambient pressure was obtained using the relaxation data set measured at three spectrometer frequencies (Orekhov et al., 1999). Due to technical reasons the relaxation measurements at 2000 bar were not possible on three spectrometers. Therefore, the pressure effect on the τ_R value was estimated from the change in the sample viscosity, which was measured by pulse field gradient NMR.

Coefficients of the translation self-diffusion of (1–36)BR, chloroform and methanol were measured at 1 and 2000 bar for the (1–36)BR sample in chloroform-methanol mixture (see Materials and methods). It was found that for all three ingredients of the sample the ratio of the self-diffusion coefficients measured at 2000 and 1 bar is the same within experimental error (0.325 ± 0.005, 0.330 ± 0.008 and 0.335 ± 0.005 for (1–36)BR, chloroform, and methanol, respectively). This suggests that from the hydrodynamic point of view only the viscosity of the solution increased at 2000 bar by a factor of 1/(0.325±0.005). As the effective τ_R for a rigid particle of arbitrary shape is in direct proportion to the viscosity, the effective τ_R for NH vectors of the α-helical part of (1–36)BR at 2000 bar was estimated to be 5.8 ns/0.325±0.005 = 17.8 ± 0.3 ns.

Relaxation data and their analysis

Experimental T_1, T_2 and ^{15}N{^{1}H} NOE values (at 750 MHz) and their uncertainties (ca. 3% for T_1, T_2 and 0.08 for ^{15}N{^{1}H} NOEs) for (1–36)BR at 2000 bar are summarised in Figure 2. Corresponding values measured at 1 bar (at 750 MHz) are presented for comparison. The considerable difference in the relaxation rates at 1 and 2000 bar can be explained by the significant increase of the solvent viscosity and therefore the overall rotational correlation time at high pressure.

Previous study of (1–36)BR at ambient pressure showed that an extended model-free approach (Clore et al., 1990) has to be applied for all backbone amides of the peptide. Amplitudes of the

pico- and nanosecond motions for amides in the α-helical region (residues 9–32) are characterised by the mean order parameters of $<S_f^2>_{9-32} = 0.84$ and $<S_s^2>_{9-32} = 0.60$, respectively and the mean correlation time of nanosecond motions, $<\tau_s>_{9-32}$ is 3.0 ns. Nanosecond motions in the α-helical region of (1–36)BR were explained by helix-coil equilibrium transitions spanning a wide range of time scales, from 10^{-10} to 10^{-5} s (Korzhnev et al., 1999a,b). For the comparison with the results of the current study, it is important to know whether the order parameters at ambient pressure would be different if only the relaxation data measured at 17.6 Tesla are used in the model-free analysis. It turned out that for the α-helical part of the peptide neither S_f^2 nor S_s^2 were changed within their uncertainties (data not shown) from the values calculated using the full data set acquired at all three magnetic fields. This result merely confirms the known fact (Orekhov et al., 1995; Korzhnev et al., 1997) that measurements at several magnetic fields are mostly important to resolve ambiguity in the selection of the dynamic model and parameters of the overall rotational diffusion. If those are known a priori, then the values of dynamic parameters can be reliably obtained at one magnetic field.

Being interested in the possible change in the dynamic parameters of pico- and nanosecond motions under pressure, we apply here the same extended model-free form of the spectral density function as in our previous study of (1–36)BR dynamics (Orekhov et al., 1999). However, amide groups of the flexible N-terminal and C-terminal residues of (1–36) BR were excluded from the analysis because their orientations in the molecular coordinate frame are unknown. Furthermore, at ambient pressure, the dynamics of most of these HNs were parameterised by more than three parameters, which is not possible with the measurements at one magnetic field. Direct fit of the model to the experimental relaxation rates and NOEs for the α-helical part of the peptide (residues 9–32) with the effective τ_R value being fixed to 17.8 ns provides mean values $<S_f^2>_{9-32} = 0.75$, $<S_s^2>_{9-32} = 0.62$, and $<\tau_s>_{9-32} = 2.8$ ns (Figure 3). While the parameters of nanosecond motions S_s^2 and τ_s exhibit almost no change within their uncertainties, the order parameters of fast picosecond motions S_f^2 drop significantly for all α-helical residues at 2000 bar. Formally, this points to a significant increase in the amplitudes of motions with characteristic times less than ca. 10 ps at 2000 bar.

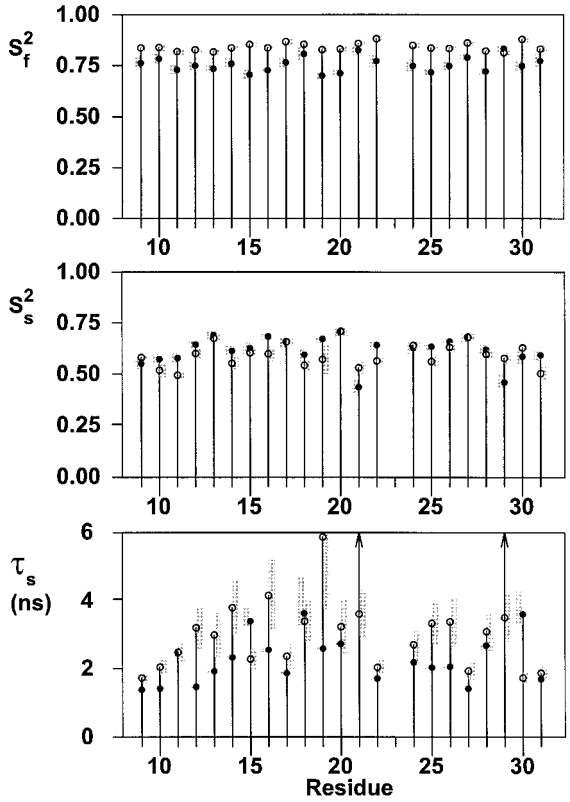

Figure 3. Results of model-free calculations for the α-helical part of (1–36)BR. Order parameters of the pico- (S_f^2) and nanosecond (S_s^2) motions obtained in the model-free analysis at 2000 bar (filled circles). For all NH vectors the calculations were performed using an effective overall rotation correlation time of $\tau_R = 17.8$ ns, a CSA value of 170 ppm, and an H-N distance of 0.102 nm. Corresponding values at 1 bar (Orekhov et al., 1999) are presented for comparison (open circles). Uncertainties of the dynamic parameters are shown as grey bars to the left and right from the filled and open circles, respectively.

However, this suggestion is in contradiction to the commonly accepted standpoint. Most intramolecular dynamic processes are related to the formation and collapse of small temporal voids. This inevitably requires a decrease in the fluctuation amplitudes under pressure. The drop of fast motional fluctuations was observed in a molecular dynamics simulation of bovine pancreatic trypsin inhibitor at 5000 bar (Brunne and van Gunsteren, 1993). The small decrease in the temperature B-factors was found in an X-ray study of lysozyme crystals at 1000 bar (Kundrot and Richards, 1987).

One could suggest that the observed decrease of the order parameters S_f^2 is due to a shift of the helix–coil equilibrium. However, helix–coil transitions for an α-helix in organic solvent occur on the time scale

from 10^{-10} to 10^{-5} s (see Korzhnev et al., 1999b and references cited therein), which is one or two orders away from the time scale relevant to the order parameter S_f^2. In addition, no pressure effect on the helix-coil equilibrium and dynamics (manifested respectively in the chemical shifts of $C^{\alpha}H$ protons and in the S_s^2 and τ_s parameters of the nanosecond motions) was observed. Therefore we conclude that pressure change in the range from 1 to 2000 bar has no effect on the helix–coil transition.

To explain the apparent decrease in the S_f^2 values, attention was paid to the physical parameters of the model-free analysis. It was noted that S_f^2 is strongly correlated with the values of the chemical shift anisotropy (CSA) of ^{15}N nuclei and H-N distances. At the same time parameters of nanosecond motions (S_s^2 and τ_s) are almost insensitive to a small change in CSA and H-N distance. Thus, the mean order parameter $<S_f^2>_{9-32}$ at 2000 bar would match the value obtained at 1 bar (0.102 nm and 170 ppm were used), if at 2000 bar the H-N distance is increased by 0.002 nm or the CSA decreased by 25 ppm. Smaller adjustment of both parameters has a similar effect. For example, the use of a CSA value of 160 ppm and an H-N distance of 0.103 nm results in $<S_f^2>_{9-32} = 0.83$, which is very close to the value observed at 1 bar.

The pressure effect on the H-N bond length is not unexpected. Indeed, pressure can contract distances between the non-covalent bonded atoms. In particular, we may anticipate the contraction of hydrogen bonds. An X-ray diffraction study (Taylor et al., 1984) and ab initio molecular orbital calculations (Kuroki et al., 1990) suggested an elongation of the H-N bonds with the contraction of the O...N distance in a hydrogen bond. Solid-state NMR study of oligopeptides (X-Gly-Gly) revealed strong correlation between H-N bond length and ^{15}N chemical shift of the NH group (Kuroki et al., 1990); namely, 0.001 nm elongation of the H-N bond causes a ca. 0.40 ppm downfield shift of the amide ^{15}N signal. Thus pressure-induced downfield shift of ^{15}N signals of (1–36)BR (ca. 0.48 ppm at 2000 bar) suggests that H-N bonds of (1–36)BR are on average ca. 0.001 nm longer at 2000 bar. Therefore, low apparent values of S_f^2, observed in this work for the α-helix of (1–36)BR at 2000 bar, can be partially explained by the increase of H-N distances associated with the overall contraction of the hydrogen bonds.

It is known that ^{15}N CSA of peptide amide groups spans quite a wide range and is very sensitive to the geometry of hydrogen bonds (Harbison et al., 1983; Kuroki et al., 1991). Both theoretical (Kuroki et al., 1991; Sitkoff and Case, 1998) and experimental (Ashikawa et al., 1999) studies suggest that a decrease of the hydrogen bond distance N...O is accompanied by a decrease of the CSA absolute values. Thus the apparent decrease of S_f^2 can be partially explained by a decrease of the CSA absolute values accompanying contraction of the hydrogen bonds.

Conclusions

In this study we have shown that the rates of overall rotation and translation diffusion of (1–36)BR decreased by a factor of 3 at 2000 bar due to the change in solvent viscosity. However, the peptide exhibited no detectable changes in its overall structure and internal dynamics on the nanosecond time scale. The observed pressure-induced changes in the order parameters of fast picosecond motions are explained by a contraction of the hydrogen bonds accompanied by an elongation of the H-N bonds or/and a decrease of the ^{15}N CSA absolute values.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (given to K.A.), Russian Foundation for Basic Research (Research grants 96-04-50893 to O.V.Yu. and 96-04-00054 to A.A.S), and a grant ‘Pressure effect’ of The Ministry of Science and Technology of the Russian Federation.

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