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Pressure-stability of phospholipid bicelles: Measurement of residual dipolar couplings under extreme conditions

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Abstract

High-pressure NMR of proteins in solutions currently gains increasing interest. 3D structure determination of proteins under high pressure is, however, so far impossible due to the lack of NOE information. Residual dipolar couplings induced by the addition of magnetically orienting media are known to be capable of replacing NOE information to a very high extent. In the present contribution we study the pressure-stability of dimyristoylphosphatidylcholine (DMPC)/ dihexanoylphosphatidylcholine (DHPC) bicelles and demonstrate the feasibility of measuring residual dipolar couplings in proteins under high pressure.

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DHPC, dihexanoylphosphatidylcholine; 3D, three-dimensional; PTFE, polytetrafluoroethene; BPTI, basic pancreatic trypsin inhibitor; *Tm*Csp, cold-shock protein from *Thermotoga maritima*; CTAB, hexadecyl(cetyl)trimethylammonium bromide; EDTA, ethylenediaminetetraacetic acid.

The pressure response of proteins provides valuable insights in folding and stability of proteins (Gross and Jaenicke, 1994; Jonas and Jonas, 1994; Heremans and Smeller, 1998). The development of high-pressure intrumentation for NMR spectroscopy opened a new field of research (Jonas, 1972; Yamada, 1974) which has gained more and more importance with the increased sensitivity of modern high-field spectrometers. In combination with two-dimensional NMR spectroscopy it allows to observe pressure-induced structural changes of proteins in atomic detail (see, e.g., Akasaka et al., 1999; Iwadate et al., 2000; Orekhov et al., 2000; Kalbitzer et al., 2000). A major problem in high-pressure NMR spectroscopic studies of protein solutions, however, is the strongly restricted sample volume (usually less than 50 μl) together with the low filling factor of the coil. As the concentration of many protein solutions is limited to values of 1 to 3 mM, the detection of NOESY spectra of sufficient quality for structural work is impossible in most cases due to the relatively poor signal-to-noise ratio. Especially the important long-range NOE contacts are often too weak to be detected in high-pressure experiments. So far, the resulting lack of NOE information prevents the determination of high-pressure 3D NMR structures of proteins. On the other hand, NMR spectroscopic protein structure determination is currently being revolutionized by the use of so-called residual dipolar couplings (Tjandra and Bax, 1997; Delaglio et al., 2000; Fowler et al., 2000; Meiler et al., 2000; Mueller et al., 2000). Residual dipolar couplings are capable of replacing NOE information to a very high extent (Clore et al., 1999). In solutions of diamagnetic proteins they are commonly induced by the addition of magnetically orienting media to the solutions. A frequently used orienting medium are phospholipid bicelles formed, e.g., in mixtures of dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC). In the present contribution we, therefore, investigate the pressure stability of this medium and we demonstrate the

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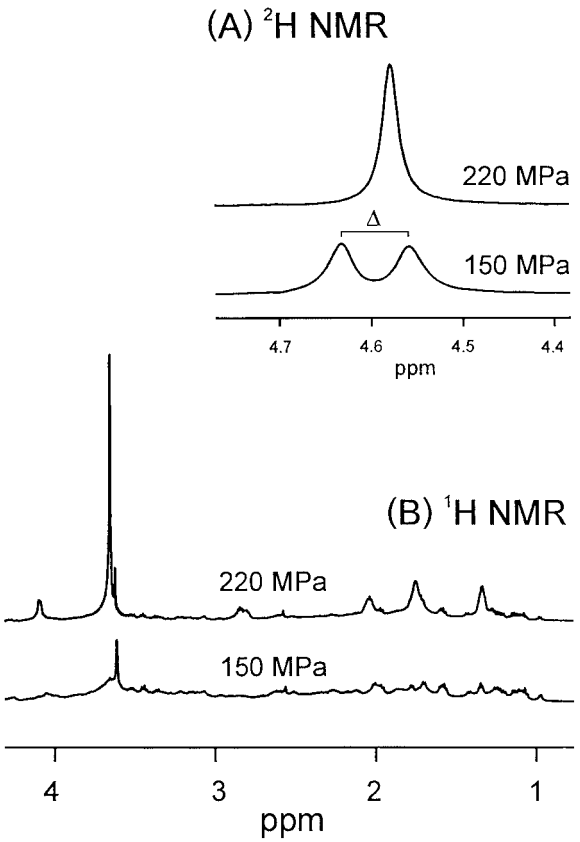

Figure 1. (A) ^{2}H NMR spectra of D_2O added to a solution containing 5 wt.-% CTAB-doped DMPC/DHPC bicelles and 1.5 mM BPTI measured at 333 K at 150 MPa and 220 MPa. Note the slight pressure-dependent shift of the signals (see Chen et al., 1999). (B) ^{1}H NMR spectra measured at 150 MPa and 220 MPa. Note the appearance of narrow signals at 220 MPa indicating the onset of rapid and isotropic reorientation of the lipid molecules. In the presence of magnetically oriented bicelles (150 MPa), the ^{1}H NMR signals of the lipid molecules are broadened.

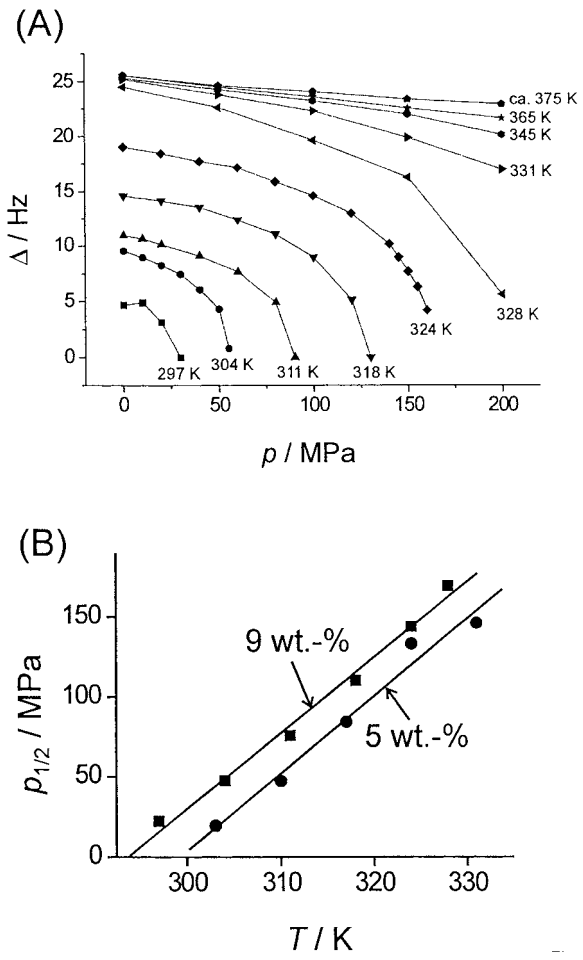

Figure 2. (A) ^{2}H NMR doublet splitting, Δ, as a function of pressure, p, observed for D_2O in a mixture containing 9 wt.-% DMPC/DHPC bicelles ($q = 3.3$, CTAB-doped) measured at various temperatures. (B) 'Decay pressure', $p_{1/2}$, at which the ^{2}H NMR doublet splitting, Δ, drops down to $\frac{1}{2}$ of its initial value as a function of temperature for 5 wt.-% (filled circles) and 9 wt.-% (filled squares) total lipid concentration.

possibility to measure residual dipolar couplings in high-pressure experiments.

The experiments were carried out on Bruker DRX-600 and DMX-500 spectrometers. The high-pressure apparatus used in this study (Price and Lüdemann, 1997) and the technical details are described in a recent paper by Arnold et al. (2001). The protein solution was inserted into a sample cell of the type suggested by Yamada (1974). The sample cell is made from a borosilicate glass capillary. The outer diameter of the end of the sample cell is adapted to the dimensions of the 8 mm high-resolution probes of Bruker DRX-600 and DMX-500 spectrometers. The active volume of the capillary (inner diameter: 1 mm, outer diameter: 5 mm, length of the active part: 35–40 mm) amounts to ca. 40 µl. Note the low filling factor of the coil resulting from the geometry of this apparatus which is the reason for the above-mentioned sensitivity problems in high-pressure experiments. Before use, each capillary is pressure checked at least up to 250 MPa (some of the capillaries withstand pressures up to 400 MPa). The maximum pressure used in these experiments was 220 MPa. The temperature of the sample inside the capillary was carefully calibrated making use of the well-known temperature-dependence of the chemical shift difference between the methyl and hydroxyl ^{1}H NMR signal of ethylene glycol (Raiford et al., 1979). Solutions con-

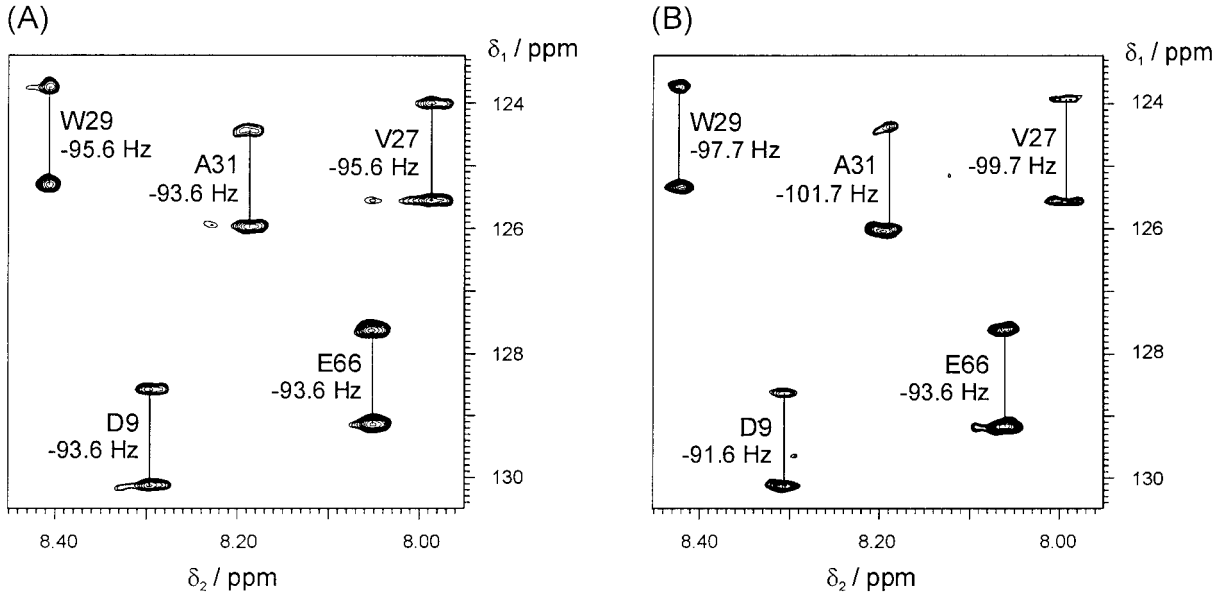

Figure 3. Selected region from the ^{1}H-coupled ^{1}H-^{15}N HSQC spectrum of 1 mM *Tm*Csp in isotropic solution (A) and in a solution (B) containing 5 wt.-% DMPC/DHPC bicelles ($q = 3.0$, CTAB-doped) measured at 336 K and a pressure of 150 MPa. The effective ^{1}H-^{15}N coupling constant giving rise to the doublet-splitting in indirect spectral dimension (δ_1) is given together with the assignment of the signals. Note the changes in the effective ^{1}H-^{15}N coupling constant caused by residual dipolar couplings in the oriented phase.

taining a bicelle-forming mixture of phospholipids DMPC and DHPC (purchased from Larodan Fine Chemicals, Malmø, Sweden) were prepared as described by Losonczi and Prestegard (1998). Hexadecyl(cetyl)trimethylammonium bromide (CTAB) was added in order to stabilize the bicelles (Losonczi and Prestegard, 1998) so that the mixtures finally contained DMPC, DHPC, and CTAB in a molar ratio of $q : 1 : 0.1$ ($3 \leq q \leq 3.5$). The total lipid concentration (DMPC + DHPC) of the thus prepared stock solution was 15 wt.-%. Basic Pancreatic Trypsin Inhibitor (BPTI), purchased from FLUKA Chemie AG, was dissolved in 50 mM phosphate buffer and then admixed to the lipid-containing stock solution (final protein concentration: 1.5 mM). The ^{15}N-labeled cold-shock protein from *Thermotoga maritima* (*Tm*Csp) was prepared according to the procedure described by Kremer et al. (2001). This sample was dissolved in a buffer containing 50 mM sodium phosphate, 20 mM NaCl, and 0.2 mM EDTA and was then admixed to the lipid-containing stock solution (final protein concentration: 1 mM).

The pressure stability of DMPC/DHPC bicelles was monitored by measuring the ^{2}H NMR signals of D_2O added to the solutions (Finer and Darke, 1974). The presence of magnetically oriented bicelles is indicated by the characteristic doublet splitting, Δ, of the ^{2}H NMR signal (see Figure 1A). The dependence of the ^{2}H NMR signal on the pressure is demonstrated in Figure 1A for a sample containing 5 wt.-% DMPC/DHPC bicelles ($q = 3$) and 1.5 mM BPTI at 333 K. The oriented phase remains stable up to a pressure of ca. 200 MPa. The disappearance of the quadrupole splitting, Δ, in the ^{2}H NMR spectra at 220 MPa is accompanied by the appearance of narrow ^{1}H NMR signals indicating the onset of rapid and isotropic reorientation of the lipid molecules (see Figure 1B). This behavior is a further evidence for the decay of the magnetically oriented bicelles at 220 MPa. As the stability of DMPC/DHPC bicelles depends strongly on the temperature (Ottiger and Bax, 1998; Losonczi and Prestegard, 1998) we have measured Δ for a mixture with $q = 3.3$ as a function of pressure at various temperatures. The result of this measurement is summarized in Figure 2. To evaluate the pressure stability of bicelles, we define the 'decay pressure', $p_{1/2}$, at which the ^{2}H NMR doublet splitting, Δ, drops down to $\frac{1}{2}$ of its initial value (see Figure 2B). Note, that the pressure stability of the bicelles at 5 wt.-% total lipid concentration is somewhat lower than at 9 wt.-%. It is remarkable that the pressure stability of the bicelles strongly increases with the temperature. For $T > 335$ K, the oriented phase remains stable up to ca. 200 MPa and, seem-

ingly, even higher for both lipid concentrations. It is, furthermore, interesting to note that the bicelles are extremely temperature-stable in our experiments (up to ca. 375 K, the maximum temperature applied in our experiments). This is somewhat unexpected. In the experiments carried out by Ottiger and Bax (1998), pure DMPC/DHPC bicelles remained stable only up to ca. 318 K. Beyond this temperature, the solutions became isotropic. The most likely reason for the extraordinarily high temperature-stability of DMPC/DHPC bicelles observed in our experiments is the addition of CTAB to the solutions (the mixtures studied by Ottiger and Bax were CTAB-free).

In order to demonstrate the feasibility of measuring residual dipolar couplings under high pressure, we have detected ^{1}H-coupled ^{1}H-^{15}N HSQC experiments of *Tm*Csp in a bicellar solution. Figures 3A and 3B show a selected region of the ^{1}H-coupled ^{1}H-^{15}N HSQC spectrum of 1 mM *Tm*Csp in isotropic and bicellar solution, respectively, measured at 336 K and a pressure of 150 MPa. For each individual FID, 128 scans were added resulting in a signal-to-noise ratio of ca. 5–10 for characteristic signals of the spectrum in bicellar solution. The residual ^{1}H-^{15}N dipolar couplings are the difference between the effective coupling constants observed in isotropic and bicellar solution. The presence and long-term stability of magnetically oriented phospholipid bicelles was detected by ^{2}H NMR spectroscopy (see above) before and after the acquisition of the HSQC spectrum. The doublet splitting, Δ, remained constant within the experimental error.

In summary, we can state that the common phospholipid bicelles are highly pressure-stable especially at elevated temperatures. This allows to make use of residual dipolar couplings for the study of structural changes induced by high pressure in proteins. Since NOEs are usually difficult to observe in high-pressure NMR spectroscopy, the measurement of the residual dipolar couplings can provide an independent source of direct quantitative structural information. A further surprising observation is the extremely high temperature-stability of DMPC/DHPC bicelles in the presence of small amounts of CTAB. This is of special interest for high-temperature studies of proteins originating from hyperthermophilic organisms such as cold-shock proteins from *Thermotogae*.

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