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ARTICLE

Measuring residual dipolar couplings at high hydrostatic pressure: robustness of alignment media to high pressure

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Abstract Among other perturbations, high hydrostatic pressure has proven to be a mild yet efficient way to unfold proteins. Combining pressure perturbation with NMR spectroscopy allows for a residue-per-residue description of folding reactions. Accessing the full power of NMR spectroscopy under pressure involves the investigation of conformational sampling using orientational restraints such as residual dipolar couplings (RDCs) under conditions of partial alignment. The aim of this study was to identify and characterize stable and pressure resistant alignment media for measurement of RDCs at high pressure. Four alignment media were tested. A $C_{12}E_5$/*n*-hexanol alcohol mixture remains stable from 1 to 2,500 bar, whereas Pf1 phage and DNA nanotubes undergo a reversible transition between 300 and 900 bar. Phospholipid bicelles are stable only until 300 bar at ambient temperature. Hence, RDCs can be measured at high pressure, and their interpretation will provide atomic details of the structural and dynamic perturbations on unfolded or partially folded states of proteins under pressure.

Keywords High hydrostatic pressure · NMR spectroscopy · Residual dipolar couplings · Alignment media · Folding/unfolding under pressure

Defining the physical–chemical determinants of protein folding and stability, under normal and pathological conditions has constituted a major subfield in biophysical chemistry for over 50 years (Dill and MacCallum 2012; Wolynes et al. 2012). Although a great deal of progress has been made in recent years towards this goal, a number of important questions remain. These include characterizing the structural, thermodynamic and dynamic properties of the barriers between conformational states on the protein energy landscape, understanding the sequence dependence of folding cooperativity, defining more clearly the role of solvation in controlling protein stability and dynamics, investigating the structural and dynamic properties of unfolded and partially folded ensembles and probing the high energy thermodynamic states in the native state basin and their role in misfolding and aggregation. Fundamental to the elucidation of these questions is a complete structural, dynamic and thermodynamic description of protein folding landscapes.

Since the first pressure–temperature phase diagrams of protein folding were published in the early 1970s (Brandts et al. 1970; Hawley 1971; Zipp and Kauzmann 1973), the community of protein physical chemists has succeeded in providing a rather complete molecular and thermodynamic understanding of the effect of temperature on protein stability and structure (D'Aquino et al. 1996; Fu and Freire 1992; Gomez et al. 1995; Hilser et al. 1997; Kauzmann 1959; Murphy et al. 1990; Privalov and Gill 1988). In contrast to this rather satisfying and highly useful physical understanding of temperature effects on protein structure, the physical basis for the effects of pressure on protein

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structure and stability has remained poorly understood. Recently, thanks to the analysis of the folding properties of model proteins [Nank (Rouget et al. 2011) and Staphyloccocal nuclease (SNase) (Kitahara et al. 2011; Roche et al. 2012a, b, c)], we have identified internal solvent excluded void volume as a major determining factor in the value of the decrease in protein molar volume upon unfolding, and hence of the magnitude of the pressure effect. In contrast to the large contribution of internal voids to pressure effects, differences in solvent density upon hydration of exposed protein surface were not found to make a significant contribution. In these recent studies of SNase, a residue-per-residue quantification of pressure effects was obtained by coupling 2D NMR spectroscopy with pressure perturbation. A drawback of this approach is the paucity of information gained concerning the structural properties of the unfolded state. Pressure unfolded states are of interest because, due to the specific effect of pressure on cavities, they are thought to be less disrupted than unfolded states obtained at high temperature or high concentrations of chemical denaturant. Hence, they may more closely resemble the unfolded state accessed by proteins under native conditions all information on the unfolded state of the protein can be obtained only indirectly.

Precise characterization of the unfolded state is necessary to fully describe the folding pathways and mechanisms. NMR spectroscopy is well adapted to describe this disordered state: chemical-shift and/or residual dipolar coupling (RDC) measurements have provided detailed descriptions of the structural and dynamic features of intrinsically disordered proteins (IDPs) (Dyson and Wright 2004). Investigation of conformational sampling using orientational restraints such as residual dipolar couplings (RDC) has been carried out under denaturing conditions at high urea concentrations (Meier et al. 2007). Nevertheless, urea is suspected to greatly disturb the hydrogen bond network within the denatured state of the protein, including H-bonds that involve water molecules. The use of high pressure to induce protein unfolding in non-denaturing buffer conditions will provide complementary information about the unfolded state manifold.

Having access to the crucial structural information given by RDCs under extreme conditions, such as high pressure, is not a new idea. Attempts in that direction has been already made by Kalbitzer and col. (Brunner et al. 2001) who have characterized the pressure/temperature dependence of DMPC/DHPC bicelles [doped with hexadecyl(cetyl)trimethylammonium bromide (CTAB)]. They have shown an amazing strong stabilizing effect of temperature under pressure: although bicelles collapsed at pressure below 400 bar at ambient temperature, the oriented phase remained stable up to ca. 2,000 bar at high temperature (102 °C). The aim of the present study is to identify and characterize among the most common ordering media, used under standard conditions for protein study (pH, temperature…), those that are able to resist high pressures (up to 2,500 bar). We have tested the pressure effect on four dilute liquid crystal media: Pf1 filamentous phage (10 mg/mL) (Hansen et al. 1998), n-alkyl-poly(ethylene glycol)/n-alkyl alcohol mixture [the molar ratio r of $C_{12}E_5$ to n-hexanol was 0.85 and the $C_{12}E_5$/water ratio was 5 wt% (PEG/Hex)] (Ruckert and Otting 2000), phospholipid bicelles [5 % (w/v) total DLPC and CHAPSO in water in a ratio of 4.2] (Wang et al. 1998), and DNA nanotubes (DNA of 400-nm-long 6-helix bundle nanostructure (6HB), 18 mg/mL) (Bellot et al. 2011). All media were dissolved in a 10 mM BisTris buffer (H_2O:D_2O; 95:5) pH 6.5. To monitor the behavior of the commonly used alignment media under high pressure, we measured first the ^2H quadrupolar splitting of D_2O as a function of pressure in different liquid crystal media. ^2H quadrupolar splitting of D_2O were used as a sensor of the liquid crystalline state to check the constancy of the degree of alignment with increasing pressure. Quadrupolar splittings were measured at 25 °C and 600 or 700 MHz Bruker Avance III spectrometers equipped with either a conventional ambient-temperature or a cryogenic triple-resonance probe, respectively. A commercial high-pressure NMR ceramic cell and an automatic pump system (Daedalus Innovations, Philadelphia, PA) were used to vary the pressure in the 1–2,500 bar range.

Pressure had no effect on the ^2H quadrupolar splitting of D_2O measured in a $C_{12}E_5$/Hex mixture in the 1–2,500 bar range, whereas a transition was observed for Pf1 phage and 6HB nanotubes: the value of the quadrupolar splitting decreased by approximately 50 and 25 % for the two media, respectively, between 300 and 900 bar, then remained virtually constant up to 2,500 bar (Fig. 1). A similar transition was observed after a twofold dilution of the Pf1 sample: ^2H quadrupolar splitting decreased by approximately 30 %. In all cases, this transition was fully reversible, demonstrating that pressure does not alter irreversibly Pf1 phage or DNA nanotubes structure. In contrast, phospholipid bicelles did not tolerate high hydrostatic pressure: the effective alignment was abolished as the pressure was increased beyond 400 bar (Fig. S1), as shown by the disappearance of the quadrupolar splitting in the ^2H spectra and the concomitant appearance of a narrow singlet resonance indicating the onset of isotropic reorientation of the lipid molecules (Fig. S1). These results are in general agreement with those recently published (Fu and Wand 2013). However, neither the reversibility nor the apparent transition observed in Pf1 media were reported in that previous work. This is probably due to smaller pressure jump increments (of 200 bar instead of 500 bar) used in the present study. Similar to what was observed for DMPC/

DHPC bicelles (Brunner et al. 2001), the pressure stability of the DLPC/CHAPSO bicelles was found to increase with the temperature: at 45 °C, the oriented phase remains stable up to ca. 600 bar. But this is far beyond the stabilization observed for CTAB-doped DMPC/DHPC bicelles, where the oriented phase remained stable up to 2,000 bar for temperature above 62 °C. The addition of CTAB to the solutions has been suggested as the most likely reason for the extraordinarily high temperature stability of DMPC/DHPC bicelles.

Due to its robustness and its stability over a large pressure range, the neutral alcohol mixture, which forms an α lamellar crystalline phase, appeared to be the most promising medium for pressure dependent RDC measurements on proteins. The Pf1 phage and DNA nanotubes, which usually form a nematic crystalline phase under the standard conditions used for RDC measurements, underwent a reversible pressure-induced ^{2}H quadrupolar transition. This medium can be used nonetheless for studies under pressure with this qualification in mind. Given their high cost and a pressure response similar to that of the Pf1 phage, DNA nanotubes were not considered further.

To further explore the utility of Pf1 phage and the PEG/Hex mixture as suitable weak alignment media for high pressure studies of proteins, we measured the pressure dependence of the $^{1}D_{N-H}$ RDCs for an ultra-stable variant of Staphylococcal Nuclease protein (SNase Δ+PHS) (Kitahara et al. 2011; Roche et al. 2012a, b, c), dissolved in these media. RDCs measured on the backbone nuclei allows to calculate an alignment tensor which depends on the alignment of the protein along the magnetic field direction (Tjandra and Bax 1997). Around neutral pH and in absence of denaturant SNase Δ+PHS neither unfolds nor experiences substantial conformational changes up to 2,500 bar (Kitahara et al. 2011) (Fig. S2). The lack of pressure effect is particularly apparent in the OB-fold sub-domain (SubD1, FigS2A) (Roche et al. 2012c) encompassing the β-barrel and helix 1. Focusing on the pressure dependence of the $^{1}D_{N-H}$ RDCs measured on this sub-domain, and more especially on the β-barrel, allowed characterization of the pressure dependence of the alignment frame without any interference from possible conformational rearrangements in the protein. All the experiments were performed at 20 °C with the same experimental set up as for quadrupolar splitting measurements. Uniformly ^{15}N labeled SNase

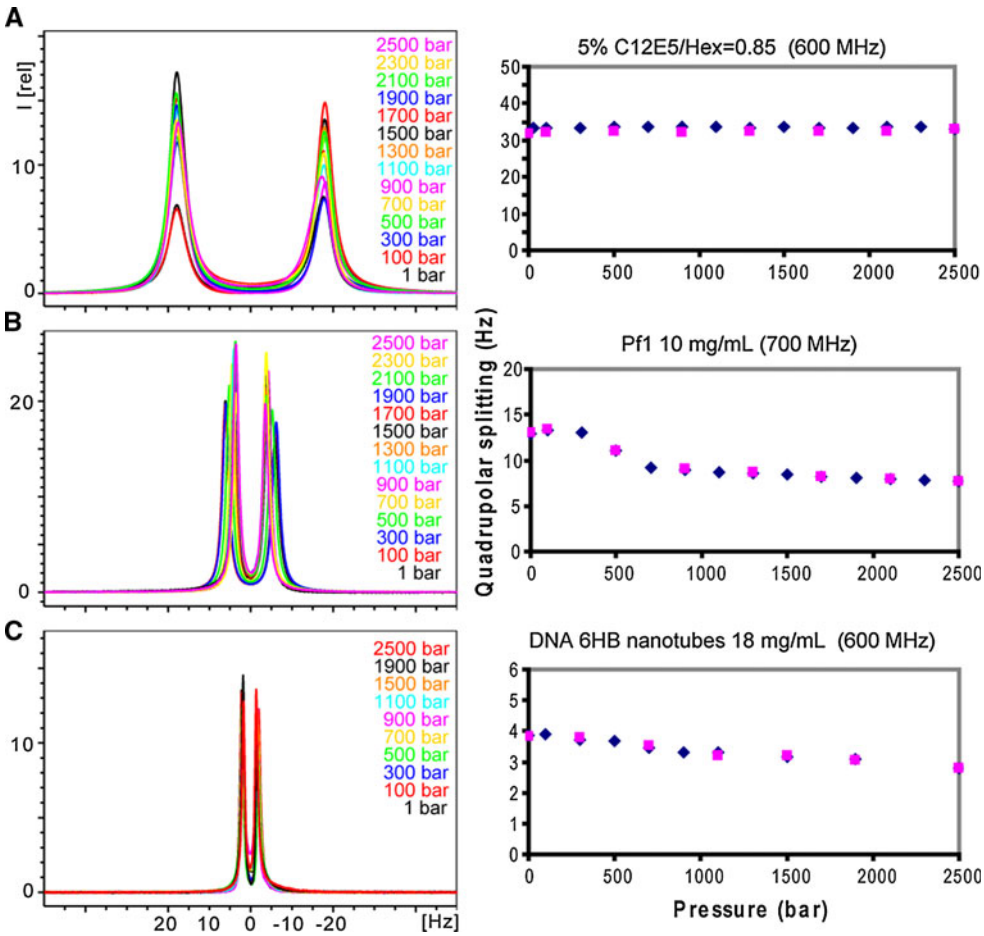

Fig. 1 ^{2}H quadrupolar splitting of D_2O (Hz) measured in **a** $C_{12}E_5$/*n*-hexanol system, *r* was 0.85 and the $C_{12}E_5$/water ratio was 5 wt%; **b** Pf1 filamentous phage (10 mg/mL); **c** DNA nanotubes of 400 nm 6-helix bundle (18 mg/mL); (*left*) spectra; (*right*) values as a function of pressure (*blue diamonds* positive P-jumps, from 1 to 2,500 bar; *pink squares* negative P-jumps, from 2,500 to 1 bar)

Δ+PHS (1 mM) was dissolved in 10 mM BisTris buffer at pH 6.5. 5 % $C_{12}E_5$/Hex = 0.85 alcohol mixture or 10 mg/mL of Pf1 filamentous phage (plus 200 mM NaCl) were added for $^1D_{N-H}$ RDCs measurements. In the Pf1 phage sample, ^2H quadrupolar splitting measurements revealed a transition over the 300–900 bar pressure range, as already observed in absence of the protein (Fig. 1). The alignment strength is lower (8 Hz instead of 14 Hz, Fig. 1b), due to the high concentration of salt added to screen strong electrostatic interactions between the basic protein and the acidic phage and possibly due to some Pf1 aggregation during salt addition (Hansen et al. 1998). At each pressure, $^1D_{N-H}$ RDCs were extracted from the difference between the $^1J_{N-H}$ couplings measured with ^1H–^{15}N TROSY and semi ^1H–^{15}N TROSY experiments (Weigelt 1998) in the isotropic sample and the $^1J_{N-H} + {}^1D_{N-H}$ couplings measured with ^1H–^{15}N IPAP-HSQC experiments (Cordier et al. 1999) in the anisotropic samples.

A preamble to this study was to ensure that the protein behaved equivalently with pressure when dissolved in an isotropic medium or diluted in a slightly anisotropic medium. Pressure is known to perturb protein NMR spectra, even at non-denaturing values. For instance, amide chemical shifts are pressure sensitive, their variation has been mostly associated to protein compressibility (Li et al. 2000) (Fig. S2). A good sensor of this compressibility is the average difference in chemical shift between 2,500 and 1 bar, calculated for each amide group as $\Delta\delta^{(2,500-1)\text{bar}} = [\Delta\delta^2_{HN} + \Delta\delta^2_N/25]^{1/2}$ (Grzesiek et al. 1996).

The values of the amide resonance chemical shift variation $\Delta\delta^{(2,500-1)\text{bar}}$ measured for SNase Δ+PHS in each medium versus the protein sequence are reported on Fig. S3. Figure 2 highlights the chemical shift behavior of the amide group of residues belonging to the β-barrel of the protein. While Pf1 diluted phage have no effect on the protein compressibility, in particular in the β-barrel domain, significant variations are observed in the alcohol mixture. This later medium apparently destabilizes the protein, as already suggested by the poor quality of spectra recorded in this medium at atmospheric pressure (Fig. S4), and the protein is even further destabilized with pressure. This is probably due to a specific interaction between PEG and the protein, already detectable in the HSQC spectra of SNase Δ+PHS at $C_{12}E_5$ concentrations far below those used in the anisotropic medium composition. Note that addition of *n*-hexanol alone up to similar concentrations did not give rise to any spectral modification (data not shown). Thus, in the following, we focused on the $^1D_{N-H}$ extracted from the diluted Pf1 phage sample to follow the pressure dependence of the alignment tensor parameters.

To investigate the behavior of the phage alignment medium under pressure, we characterized the alignment tensor parameters (relative to an arbitrary molecular frame) of the protein dissolved in this anisotropic medium by following the pressure dependence of the axis orientation defined by the Euler angles (α, β, γ) and the eigenvalues of the tensor (axial and rhombic component, Aa and Ar, respectively, and R = Ar/Aa as the rhombicity). Depending on the pressure-affected parameter, these measurements should indicate whether the decrease of the ^2H quadrupolar splitting of D_2O was related to reversible changes at the surface of the phage Pf1, responsible for changes in the interaction between the protein and the particle, (R), or to a decrease in the order of magnitude of the alignment (Aa).

The correlation between back calculated and experimental RDCs and the alignment tensor parameters were determined with the Module software (Dosset et al. 2001), using the SNase Δ+PHS X-ray structure (3BDC PDB code) and the experimental $^1D_{N-H}$ data measured on the SNase Δ+PHS upon pressure. Hydrogens were added to the X-ray structure using the program CNS and a distance of 1.04 Å has been used for the internuclear distance of the N–H vectors. Aa is referred to as the magnitude of the residual dipolar coupling tensor, and Ar to as the rhombic component, both normalized to the N–H dipolar interaction (Dosset et al. 2001). Thus, Aa and Ar, described in (Dosset et al. 2001) are the unit-less axial and rhombic components of the molecular alignment tensor A, and are on the order of 10^{-4}.

The global fitting of all the $^1D_{N-H}$ RDCs measured on the SNase Δ+PHS X-ray structure (3BDC PDB code) is shown on Fig. S5. The relatively low agreement between back calculated and measured $^1D_{N-H}$ RDCs on the entire protein could come from a slightly different relative orientation of the different sub-domains in the X-Ray structure when compared to the protein in the NMR sample. In addition, the agreement slightly decreases when the pressure increases ($R^2 = 0.90$ at 1 bar and 0.87 at 2,500 bar), suggesting subtle reorganization of the different subdomains of the protein under pressure. This low agreement between back calculated and measured RDCs on the entire protein could prevent precise estimation of the global alignment tensor and add noise to the extracted tensor parameters. So we decided to focus only on the β-barrel of this protein, part of subD1 domain (Table S6), in order to have a more accurate determination of the alignment tensor. Alignment tensor parameters were determined at each pressure (1–2,500 bar, with 200 and 400 bar step below and above 900 bar, respectively) using 20 $^1D_{N-H}$ values (Fig. 3, Fig. S5). The pressure dependence of the Euler angles and eigenvalues of alignment tensors revealed an increase in the rhombicity (R = Ar/Aa) (Fig. 3b), but there was no change in the eigenvectors defined by the Euler angles (Fig. 3a), and the eigenvalue Aa revealed that the alignment strength remained virtually constant (Fig. 3c).

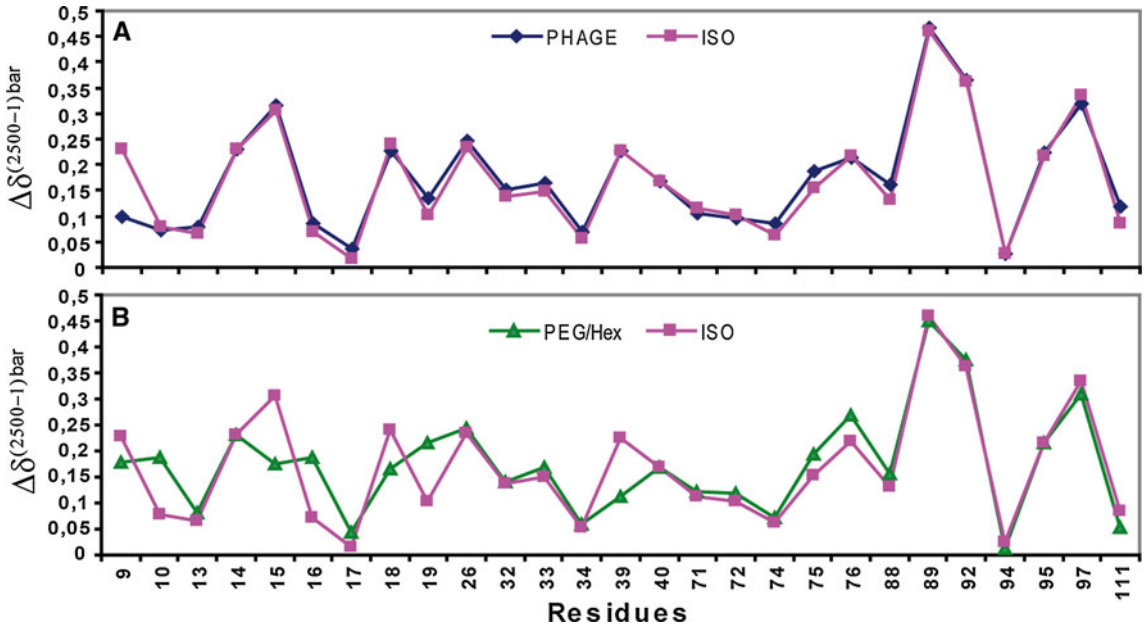

Fig. 2 Pressure effects sensed by the amide chemical shift variations $\Delta\delta^{(2,500-1)bar}$ measured for residues belonging to the β-barrel (Sub-Domain 1, SubD1) of SNase Δ+PHS. In both panels, the *pink curve* corresponds to $\Delta\delta^{(2,500-1)bar}$ values measured in an isotropic medium and has to be compared to values measured **a** in Pf1 phages (10 mg/mL) (*blue curve*) and **b** in 5 % $C_{12}E_5$/hexanol = 0.85 alcohol mixture (*green curve*)

The absence of variation upon pressurization of the axial component (Aa) is confirmed by the overall fitting of $^1D_{N-H}$ to the X-Ray structure at 1 bar ($Aa = 10.3 \times 10^{-4}$, $Ar = 1.7 \times 10^{-4}$, $R = Ar/Aa = 0.16$) and 2,500 bar ($Aa = 10.5 \times 10^{-4}$, $Ar = 2.8 \times 10^{-4}$, $R = Ar/Aa = 0.26$) (Fig. S5).

Highly charged liquid-crystalline media, such as the popular Pf1 phage particles, form nematic phases. However, just below the nematic phase boundary the behavior of the system is paranematic (Zweckstetter and Bax 2001). Given the low phage concentrations and high salt concentration used in the present work, the phase of the alignment medium should be paranematic.

According to Le Chatelier's Principle, pressure will shift equilibrium between multiple states towards those states that occupy the smallest molar volume. Thus, the transition observed on the pressure dependence of the quadrupolar splitting could possibly arise from a "pseudo"-phase transition shifting the initial ordering of the Pf1 at ambient pressure to a more compact one at high-pressure. If this is the case, the alignment strength of the protein should scale proportionately to that of the deuterium quadrupole splitting. Since in our work the value of the axial component (Aa) remains constant with pressure, the change observed in the 300–900 bar range in the ^2H quadrupolar splitting does not appear to be associated with a loss of alignment degree in the sample.

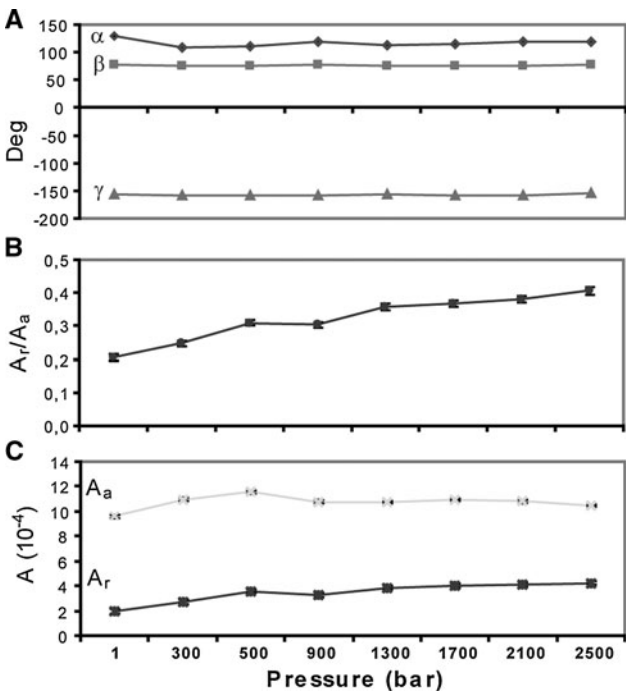

Fig. 3 Alignment tensor parameters extracted from 20 residues belonging to the SNase Δ+PHS β-barrel: **a** euler angles (α, β, γ); **b** rhombicity: R = Ar/Aa **c** axial (Aa) and rhombic (Ar) component. Errors in these alignment tensors (±0.2 for Aa and Ar) are mainly influenced by uncertainties in the reference structure and were estimated using the Monte Carlo method included in the Module software

Alternatively, although protein alignment results from a coarser steric and electrostatic combination of forces, ^{2}H quadrupolar splitting results from D_2O molecule alignment, and water alignment results from the hydration distribution of the molecules on the atomic surface of the Pf1. Increasing hydrostatic pressure should provoke only subtle and reversible rearrangement of the Pf1 phage surface proteins, yielding concomitantly a decrease of ^{2}H quadrupolar splitting of D_2O, and an increase of the rhombicity of the alignment tensor of the co-dissolved protein. In our study, the change observed in the 300–900 bar range in the ^{2}H quadrupolar splitting must reflect modification of the Pf1 particle hydration that could induce a slight change in the interaction between the protein and the Pf1's surface, which, in turn, is reflected in a constant variation of the rhombicity ($R = Ar/Aa$) of the alignment tensor. Thus, in this case, the ^{2}H quadrupolar splitting only senses the hydration of the proteins at the surface of the Pf1. Such an interpretation is analogous to that of Ottiger and Bax (1998) for DHPC/DMPC bicelles. In that system, the ^{2}H quadrupolar splitting was strongly temperature dependent, but the protein alignment was not, and the temperature dependence of ^{2}H quadrupolar splitting simply reflects a change in hydration characteristics of the phospholipids.

NMR is an exquisite experimental approach to detect and characterize folding intermediates at the individual residue level. For instance, hydrogen/deuterium exchange experiments, relaxation dispersion have already been used to identify intermediate states during unfolding process, both at atmospheric (Boehr et al. 2006; Englander 2000) or at high hydrostatic pressure (Korzhnev et al. 2006; Nisius and Grzesiek 2012; Roche et al. 2012c; Fuentes and Wand 1998). However, the use of long-range structural restraints to characterize pressure-induced folding intermediates or denatured states were still missing. Here we demonstrate the feasibility of high pressure RDCs measurements on proteins in several alignment media. Indeed, we have identified and characterized three pressure stable and resistant alignment media, that can orient the co-dissolved bio-molecules via steric (alcohol mixture) and electrostatic (filamentous bacteriophage and DNA nanotubes) interactions. Fu and Wand have also observed the stability of alcohol mixture and Pf1 phage in slightly different conditions (Fu and Wand 2013). In their study, they have shown that RDCs measured at 2,500 bar exhibited significant deviations from those back calculated on the ubiquitin structure at atmospheric pressure, especially in regions where pressure-induced structural changes were previously highlighted in a solution structure determined using classical NMR restraints at 3,000 bar (Kitahara et al. 2005). We have chosen to focus on a different model: the ultra-stable Δ+PHS SNase, and more precisely on its β-barrel sub-domain that do not experience structural changes within the pressure range used for this study. This allowed us to focus only on alignment media properties, discarding any contribution from protein conformational changes. Thus, in the case of Pf1 alignment medium, we were able to safely conclude that the sigmoidal pattern exhibited by the variation of ^{2}H quadrupolar splitting with pressure can be solely related to alignment media perturbations: probably to reversible changes at the phage surface, affecting their hydration properties.

In addition, Fu and Wand found another stable medium composed of dinucleotides. Thus, four different alignment media appear suitable for high pressure studies. Moreover, our study has shown the pressure reversibility of three of these commonly used media making them useful for folding/unfolding studies. Thus, depending on the pI of the bio-molecule under study and its compatibility with the diluted liquid crystal, alternative alignment media can be chosen for RDC measurements under high hydrostatic pressure. In addition, the accessibility to several liquid crystal media with different alignment properties will allow the detailed investigation of the protein structure and dynamics. For instance, it will allow the refinement of structure of intermediate states during folding (Blackledge 2005), the accessibility to the dynamics over a broad timescale (Bouvignies et al. 2005), the study of domain rearrangement along the folding pathways of multi-domain proteins. Thus, measurement of RDCs under pressure will complete the toolbox for the characterization of these intermediate states, but can also bring unique information on the unfolded state. Indeed, RDCs contain both local and long range structural information that allow to establish the existence of transient and/or stable α helices, β extended or PPII (PolyProline II helix) elements of folded and unfolded states and thus to calculate an ensemble of relevant conformations. Consequently, the broad range of structural information afforded by measurement of RDCs (Bax and Grishaev 2005; Tjandra and Bax 1997; Tolman et al. 1995) can be now extended to the analysis of protein folding under pressure.

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