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The use of high-sensitivity sapphire cells in high pressure NMR spectroscopy and its application to proteins

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Abstract. The application of high pressure in bioscience and biotechnology has become an intriguing field in un/refolding and misfolding processes of proteins. NMR spectroscopy is the only generally applicable method to monitor pressure-induced structural changes at the atomic level in solution. Up to now the application of most of the multidimensional NMR experiments is impossible due to the restricted volume of the high pressure glass cells which causes a poor signal-to-noise ratio. Here we present high strength single crystal sapphire cells which double the signal-to-noise ratio. This increased signal-to-noise ratio is necessary to perform, for example, phophorus NMR spectroscopy under variable pressures.

To understand the effect of pressure on proteins, we need to know the pressure dependence of ^{1}H chemical shifts in random coil model tetrapeptides. The results allow distinguishing structural changes from the pressure dependence of the chemical shifts. In addition, the influence of pressure on the buffer system was investigated.

Since high pressure was shown to populate intermediate amyloidogenic states of proteins the investigation of pressure effects on proteins involved in protein conformational disorders like Alzheimer's Disease (AD) and Transmissible Spongiform Encephalopathies (TSE) is of keen interest. ^{1}H-^{15}N-TROSY-spectra were acquired to study the effects of pressure and temperature on chemical shifts and signal volumes of the human prion protein. These measurements show identical pressure sensitivity of *hu*PrP(23–230) and *hu*PrP(121–230). First results suggest a folding intermediate for the human prion protein which can be populated by high hydrostatic pressure.

1. Introduction

High pressure NMR-spectroscopy can yield local information about mechanical and dynamical properties of proteins and can be used to stabilise folding and unfolding intermediates [1–3]. At pressures of 200 MPa the phase behaviour of water allows the observation of protein denaturation in aqueous solution at temperatures down to 255 K [4]. In addition, high pressure influences protein aggregation and association as well [5].

Currently, two conceptually different methods are applied in high pressure NMR experiments. The first method is known as the high pressure probe method and uses specifically designed non-magnetic metal autoclaves [6,7]. The second method has been called 'Yamada glass cell method' [8–10]. Generally pressurising the whole probe would allow obtaining very high pressures. Nevertheless, the design of special metallic high pressure probes leads to severe problems: (i) limitation of space in high resolution high field NMR spectrometers, (ii) perturbations of the magnetic field homogeneity and (iii) the difficulty

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to construct reliable low impedance radiofrequency feedthroughs through the thick metal parts of the autoclaves. The big advantage of the 'Yamada glass cell method' is its use in all commercially available probe heads. A modified version by Lang and Lüdemann [11] is used in our laboratory. Due to a rather small sample volume in the thick-walled sample tubes the glass cell method displays an inherent low sensitivity. Typically, borosilicate or quartz glass capillaries with an outer diameter of 5 mm and an inner diameter of 1.0 to 1.2 mm are required to withstand pressures up to 200 MPa [12,13].

2. Sapphire cells lead to higher sensitivity

In search for a better signal-to-noise ratio we devised a sapphire cell system (Fig. 1) with single crystalline sapphire capillaries having an inner diameter of 1.78 mm and an outer diameter of 3.14 mm, which are available from Saphikon (Milford, New Hampshire 03055, USA). ^{1}H-^{15}N-HSQC spectra were measured on a uniformly ^{15}N-enriched 0.5 mM sample of the cold shock protein (Csp) from *Thermotoga maritima* in a sapphire cell and under identical experimental conditions in a borosilicate glass cell with 5 mm outer diameter and 1.2 mm inner diameter [14]. A comparison of selected regions of the measured ^{1}H-^{15}N-HSQC spectra with the data plotted at the same contour level is shown in Fig. 2. The use of sapphire cells leads to much better signal-to-noise ratio as is expected from the approximately two-times larger active volume in the probe. 1D slices through the maximum of the H^N crosspeaks of K19 in the 2D HSQC spectra show an increase of the signal-to-noise ratio by a factor of 2 [14].

3. Pressure shifts proline *cis-trans*-isomerization

A first result of the sapphire cells shows the pressure sensitivity of the *cis-trans*-isomerization of the proline peptide bond. In the NMR spectra, both isomers can be distinguished by the different chemical shift values of the prolyl signals. For our experiments we used a 5 mM solution of the random-coil peptide GGPA (glycyl-glycyl-prolyl-alanine). In a recent study we could not find a significant pressure dependence of the *cis-trans*-equilibrium using a glass cell [15] within the experimental error. The conformational equilibrium of the prolyl peptide bond was studied by integrating the H^{α}-signals of *cis*- and *trans*-isomer of proline which are well separated in the 1D-spectra (Fig. 3). Integration of the resonance lines gives the population of the corresponding isomer. As a result now a significant shift of the equilibrium constant $K = [trans]/[cis]$ can be observed when the pressure is varied. Increasing pressure leads to a higher population of the *cis*-isomer of the peptide bond. At 0.1 MPa and 305 K the value of K is 3.381 ± 0.008. Assuming a logarithmic pressure dependence the change of the equilibrium constant $\mathrm{d}\ln K/\mathrm{d}p$ with pressure can be calculated as -10^{-4} MPa^{-1} with a correlation coefficient of 0.94. The difference of the partial molar volume ΔV_0 is -0.25 ml mol^{-1} at 305 K [14]. A possible explanation for this effect is the break of two H-bonds which are forming γ-turns [16] in the short peptide GGPA between the carboxyl C and the amide N of the C-terminal alanine and the second glycine but may also represent differences of the partial charges of the peptide bond itself in the two isomers.

4. High pressure effects in model peptides

In high resolution solution NMR spectroscopy a wealth of information about the chemical shift in model random coil peptides is available [17]. For high pressure NMR spectroscopy we evaluated the

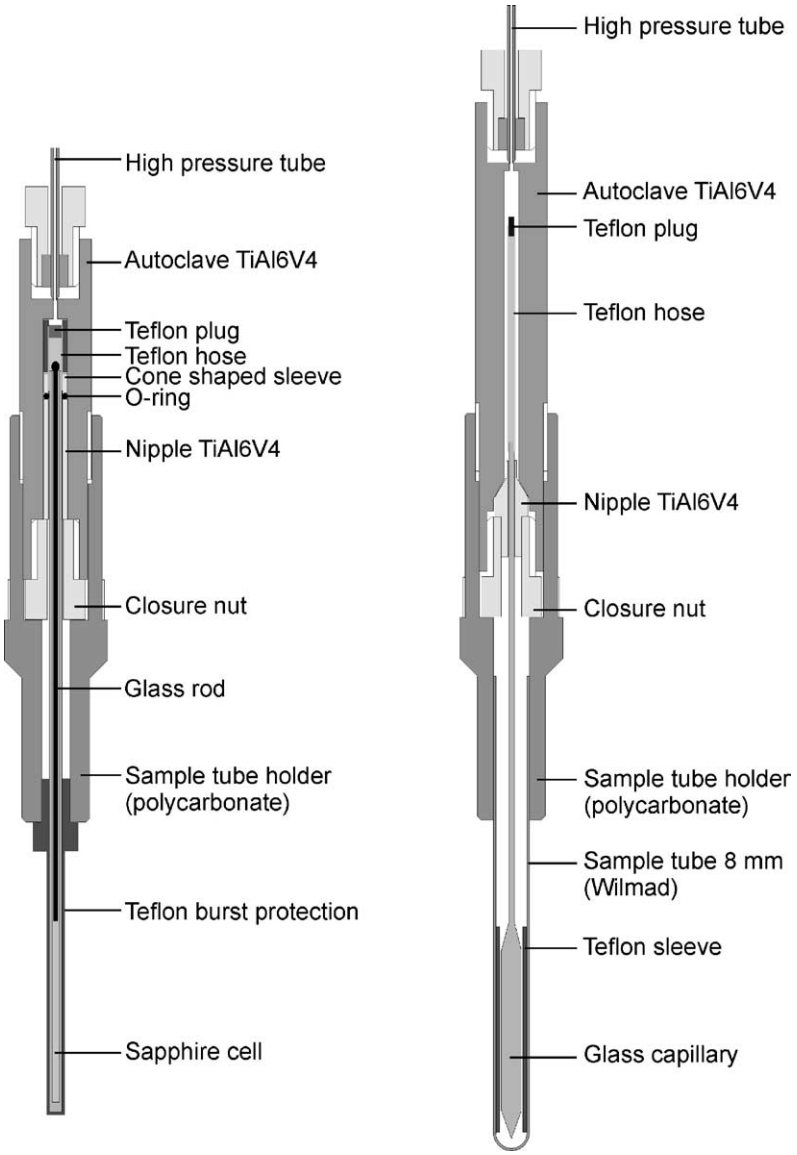

Fig. 1. *Left*: Sapphire cell system with O-ring gasket. The pressurising fluid and sample are separated by a Teflon shrink hose, which is closed by an Teflon plug. Outer diameter of the sapphire cell 3.18 mm, inner diameter 1.73 mm. As burst protection either a Teflon hose with 0.2 mm wall thickness or an especially manufactured closed Teflon tube (PTFE, outer diameter 4.8 mm, inner diameter 3.5 mm) was used. *Right*: Glass cell system with cone shaped metal sealing. The Duran 50 borosilicate glass capillary is glued into a cone shaped TiAl6V4 nipple. Outer diameter of the glass capillary 5.0 mm, inner diameter 1.2 mm.

influence of pressure on the chemical shift of all of these model peptides [15]. The pressure dependence of the ^{1}H-NMR chemical shift of the amino acids X in the random-coil model peptides Gly-Gly-X-Ala was studied for the 20 common amino acids at two pH values (pH 5.0 and 5.4 in phosphate buffer) at 305 K in the pressure range from 0.1 to 200 MPa and showed only two nonlinear behaving examples: the backbone amide proton resonance of glutamate and the side chain NH-resonance $H^{\varepsilon 1}$ of tryptophan [15]. The methylation of the C-terminal carboxyl group which has a pK_a-value of approximately 3.3 led to a

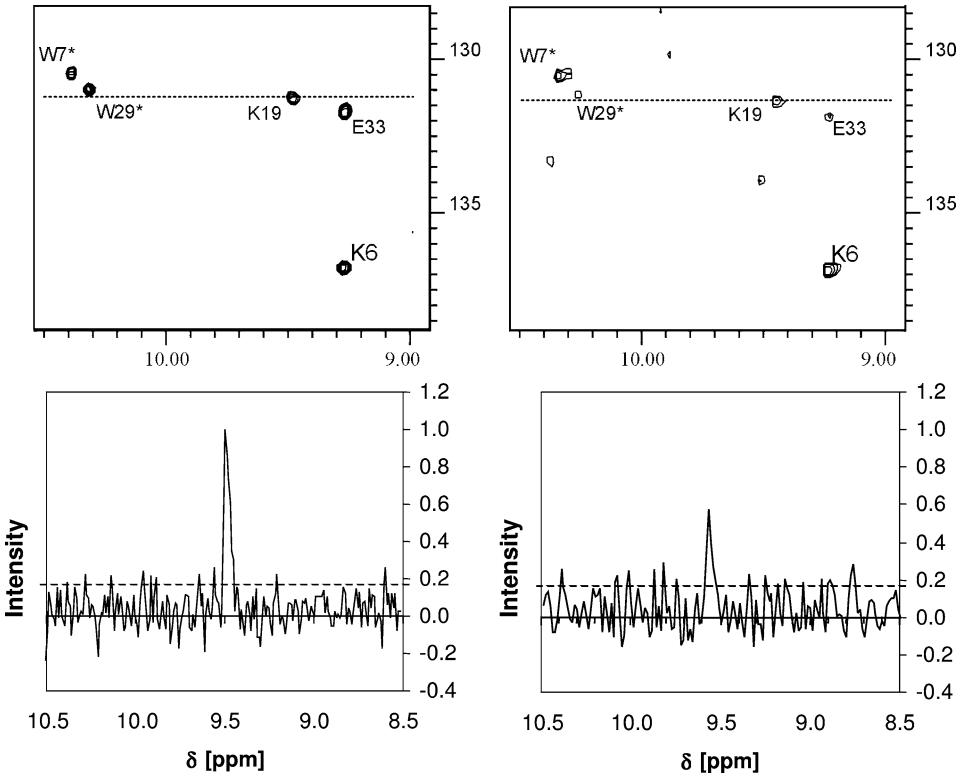

Fig. 2. *Top*: Selected regions of ^{1}H-^{15}N correlation spectra. The sample contained 0.5 mM uniformly ^{15}N-enriched cold shock protein from *Thermotoga maritima* (*Tm*Csp) in 50 mM phosphate buffer (pH 6.5), 20 mM NaCl, 0.2 mM Na-EDTA, 0.1 μM NaN_3, 10% D_2O and 90% $^{1}H_2O$. Gradient selected sensitivity enhanced ^{1}H-^{15}N-HSQC spectra were recorded under identical experimental conditions either in a sapphire cell (left) with an outer diameter 3.2 mm, inner diameter 1.7 mm or a borosilicate glass capillary (right) with an outer diameter of 5.0 mm and an inner diameter of 1.2 mm. Data were recorded with a 8 mm inverse triple resonance probe head at 600 MHz proton frequency. Total acquisition time approximately 2.5 h, resolution 2048 points in the direct dimension and 256 points in the indirect dimension. The temperature was adjusted to 303 K. The same contour levels for the two experiments were used. *Bottom*: 1D-slices through the maximum of the H^N-signal of K19. (Reprinted with permission.)

disappearance of the nonlinear pressure dependence indicating an interaction between the Glu $^{1}H^N$ and the C-terminal Ala in the non-methylated form (see Table 1).

5. High pressure NMR on the human prion protein

We investigated the effects of pressure and temperature on chemical shifts and signal volumes of two variants of the human prion protein, *hu*PrP(121–230) and *hu*PrP(23–230). 1D ^{1}H-NMR as well as ^{1}H-^{15}N-TROSY spectra of *hu*PrP^{c}(121–230) and *hu*PrP^{c}(23–230) at variable pressure and temperature show that the application of pressure is reversible and we see virtually no difference between *hu*PrP^{c}(121–230) and *hu*PrP^{c}(23–230) [18].

We observed 1D ^{1}H- and 2D ^{1}H-^{15}N-TROSY NMR spectra of ^{15}N enriched *hu*PrP(23–230) and *hu*PrP(121–230) at pH 4.5 (acetate buffer) at variant pressures and temperatures. At 20°C we applied hydrostatic pressures of 0.1, 50, 100, 125, 150, 175 and 200 MPa at both, *hu*PrP(23–230) and *hu*PrP(121–230). At ambient pressure and 200 MPa we studied the temperature dependence of *hu*PrP(23–230) and

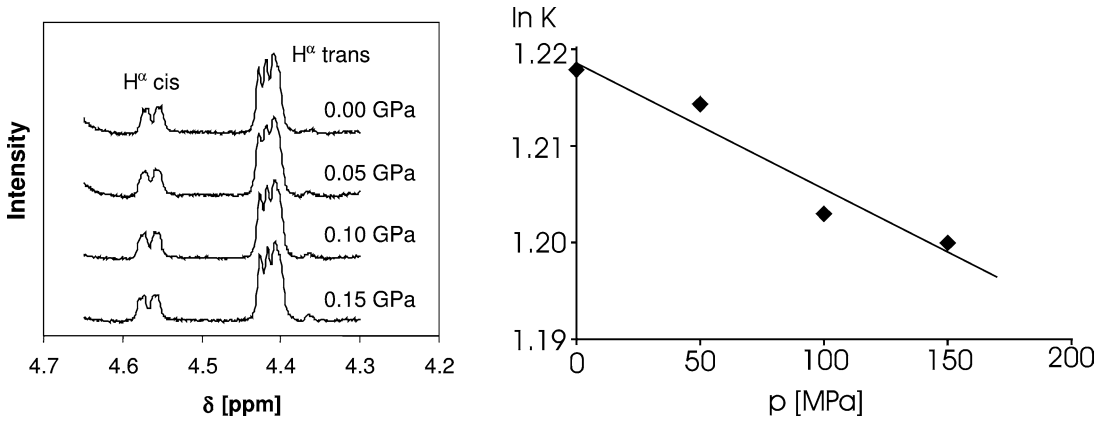

Fig. 3. *Left*: Part of 1D ^{1}H-NMR spectra at various pressures showing the H$^\alpha$-signal of proline in *cis*- and *trans*-conformation, respectively. The sample contained 5 mM GGPA in 50 mM Tris/HCl buffer (pH 7.0) and 0.1 mM DSS in 99% D_2O. The pressure was changed from 0.1 MPa to 150 MPa in steps of 50 MPa at a temperature of 305 K. *Right*: The ratio of the integrals of the signals of the *trans*- to the *cis*-conformer are plotted as function of pressure. (Reprinted with permission.)

Table 1

Chemical shifts and pressure coefficients of the amide protons of amino acid Glu in Gly-Gly-X-Ala and [b]Gly-Gly-X-Ala-methyl at 305 K in aqueous solution at pH 5.4[a]

X3	First order model			Second order model		
	δ_0^{HN} [ppm]	$\delta_{\Delta p}^{HN}$ [ppm GPa^{-1}]		δ_0^{HN} [ppm]	$\delta'^{HN}_{\Delta 2p}$ [ppm GPa^{-1}]	$\delta_{\Delta 2p}^{HN}$ [ppm GPa^{-2}]
Glu	8.522	−0.026	±0.004	8.525	−0.15	0.63
Glu[b]	8.709	0.017	±0.001	8.709	−0.98	0.53

[a]The chemical shift δ_0^{HN} at 0.1 MPa, the linear pressure coefficient $\delta_{\Delta p}^{HN}$, the first order and second order pressure coefficients $\delta'^{HN}_{\Delta 2p}$, $\delta_{\Delta 2p}^{HN}$ were obtained by fitting the data according to [15].

found that at 60°C the 1D ^{1}H-NMR spectra were characteristic of an unfolded protein. Here, the release of the pressure did not result in a refolded protein. Up to 50°C the pressure-induced unfolding was completely reversible. Figure 4 shows 2D ^{1}H-^{15}N-TROSY spectra of *hu*PrP(121–230) and *hu*PrP(23–230) at ambient pressure and 200 MPa. Increasing the pressure results in changes in the resonance frequency. In addition even in the TROSY spectra the increased pressure leads to more broadened signals, indicating a tentative increase in molecular mass or exchange (broadening) between the native and a pressure-stabilized conformer. Many signals broaden such that they disappear from the spectra. Between 175 and 200 MPa the amide protons of residues 128, 131, 134, 136, 139, 141–144, 150, 156, 160, 161, 163, 174, 178, 182, 199, 200, 202, 210, 214, 215, 217 and 221 are not observable in case of *hu*PrP(121–230). Especially, residue 131 disappears already at 125 MPa, while residues 139, 141, 160, 161, 163 and 178 are undetectable at 150 MPa. These residues mainly cluster to the loop between the strand β1 and helix α1, near helix α3 and close to the β-sheet (see Fig. 5). In case of *hu*PrP(23–230) due to severe signal overlap induced by the pressure-induced line broadening only the disappearance of residues 131, 139, 141, 156, 157 and 178 can be reliably confirmed. By releasing the pressure we observe the original spectra at ambient pressure again, thus the pressure-induced changes are completely reversible. Upfield shifted methyl groups of Ile139, Leu130 and Ile182 show a similar broadening (data not shown) indi-

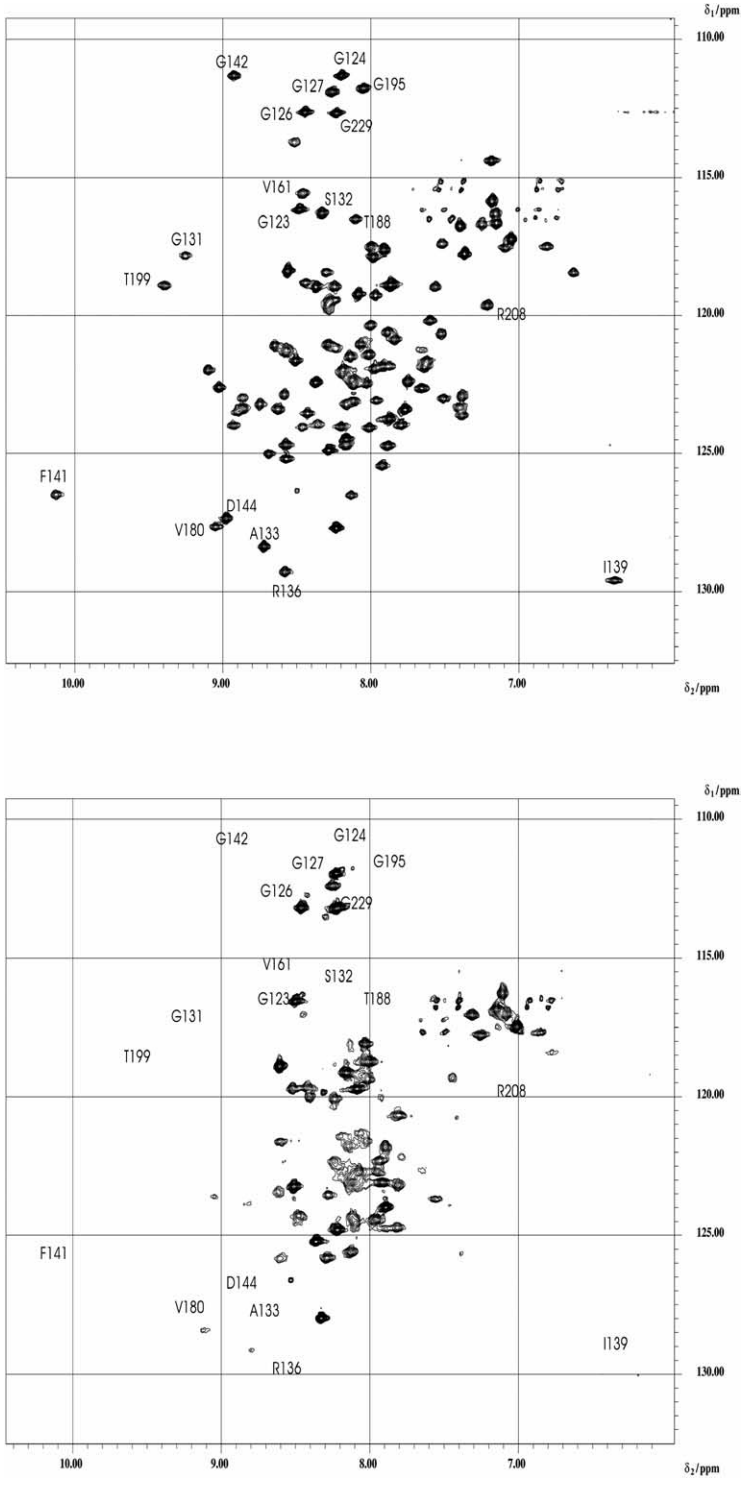

Fig. 4. 2D ^{1}H-^{15}N-TROSY spectra of *hu*PrP(121–230) at ambient pressure (*top*) and 200 MPa (*bottom*).

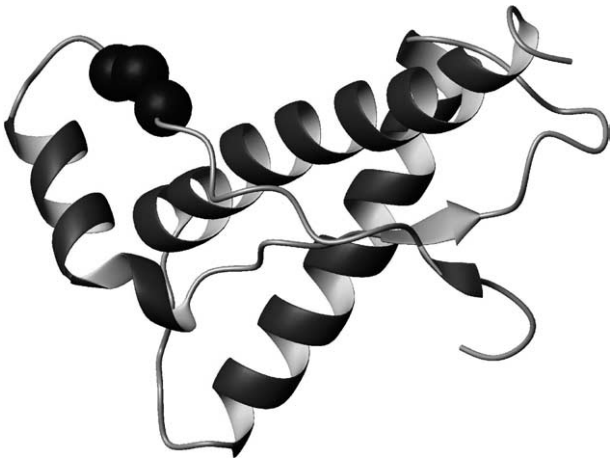

Fig. 5. Most pressure-sensitive region mapped on the tertiary structure of *hu*PrP (PDB-ID: 1QM2).

cating primarily an underlying structural conformational change rather than chemical exchange of the amide protons as origin for the line broadening [18].

6. Summary

In summary, we can state that the combination of high hydrostatic pressure and solution NMR spectroscopy allows studying local dynamics of proteins which might be important for function apart from the dynamic information gained from relaxation measurements. The examples described above are important for regulatory processes such as signal transduction. The aggregation of proteins into fibrils as seen in many of the protein conformational disorders might involve specific interaction sites of target proteins which can be characterized under steady state conditions in high pressure high field NMR spectroscopy. Especially the reversibility of these interaction modes and thus their population can be fine tuned by optimising the three parameters pH, temperature and pressure.

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