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# Impact of Hydrostatic Pressure on an Intrinsically Disordered Protein: A High-Pressure NMR Study of α-Synuclein

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*This paper is dedicated to the memory of our friend and colleague Ivano Bertini*

The impact of pressure on the backbone $^{15}N$, $^{1}H$ and $^{13}C$ chemical shifts in N-terminally acetylated α-synuclein has been evaluated over a pressure range 1–2500 bar. Even while the chemical shifts fall very close to random coil values, as expected for an intrinsically disordered protein, substantial deviations in the pressure dependence of the chemical shifts are seen relative to those in short model peptides. In particular, the nonlinear pressure response of the $^{1}H^{N}$ chemical shifts, which commonly is associated with the presence of low-lying "excited states", is much larger in α-synuclein than in model peptides. The linear pressure response of $^{1}H^{N}$ chemical shift, commonly linked to H-bond length change, correlates well with those in short model peptides, and is found to be anticorrelated with its temperature dependence. The pressure dependence of $^{13}C$ chemical shifts shows remarkably large variations, even when accounting for residue type, and do not point to a clear shift in population between different regions of the Ramachandran map. However, a nearly universal decrease in $^{3}J_{HN-H\alpha}$ by $0.22\pm0.05$ Hz suggests a slight increase in population of the polyproline II region at 2500 bar. The first six residues of N-terminally acetylated synuclein show a transient of approximately 15% population of α-helix, which slightly diminishes at 2500 bar. The backbone dynamics of the protein is not visibly affected beyond the effect of slight increase in water viscosity at 2500 bar.

## Introduction

Intrinsically disordered proteins (IDPs) are abundant in eukaryotes where they are involved in many regulatory and signaling processes. Due to their inherent structural plasticity, IDPs are able to form specific interactions with a large variety of partners, playing a pivotal role in the cellular protein-protein network.[1,2] In-depth knowledge of the structural features of IDPs is crucial to understand their functions and their connection with a range of diseases. Multi-dimensional solution NMR spectroscopy is a method of choice for analyzing the transient backbone conformations populated by IDPs and disordered regions of otherwise ordered proteins. A growing body of evidence, emerging from the study of chemical shifts, *J*-couplings and residual dipolar couplings, shows that IDPs populate all the favored regions of the Ramachandran plot, including the alpha, the beta and the polyproline II (PPII) regions.[3–7]

The conformational propensities of short peptides have been extensively studied over the past years as a model for disordered regions of proteins.[8,9] Systematic measurements of $^{3}J_{HN-H\alpha}$ couplings revealed that the PPII conformation is dominant for most amino acids at position X in Ac-G-G-X-G-G-$NH_2$ host-guest peptides, with the highest PPII propensity measured for Ala while β-branched or aromatic residues (Val, Ile, Tyr, and Phe) have a larger β-propensity.[10] The intrinsic conformational propensities of amino acids are strongly influenced by the solvent conditions and a change in the solvent properties represents the most efficient strategy to explore the conformational free-energy landscape of peptides and disordered proteins. For example, the PPII propensity is known to decrease in low-polarity solvents,[11] while a chemical denaturant such as urea, which stabilizes extended backbone conformations, promotes the formation of PPII.[12,13] The PPII conformation is also strongly influenced by temperature, showing an increase in PPII propensity at low temperature, while the beta conformation is favored at high temperature.[13,14]

Despite the fact that pressure is a fundamental thermodynamic variable, as important as temperature, the effect of pressure on disordered proteins has not yet been explored. High pressure is known to unfold globular proteins due to the smaller volume occupied by the unfolded states of proteins compared to their native states.[15] High-pressure NMR has been used extensively over the last 20 years to study the response of native proteins to compression,[16–20] to characterize low-lying excited states,[21–24] and to monitor the complete unfolding of globular proteins.[19,25–27]

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In this study, we used high-pressure NMR to analyze the impact of hydrostatic pressure on human α-synuclein, a widely used model for the study of intrinsically disordered proteins. α-Synuclein is a 140-residue protein composed of three distinct regions: a positively charged N-terminal region (residues 1–60), a central hydrophobic segment (residues 61–95) often referred to as the non-amyloid-beta-component (NAC) region, and a highly negatively charged and proline-rich C-terminal region (residues 96–140). The chemical shifts of α-synuclein are exceptionally close to typical random coil values.[28,29] The α-helical propensity of the first six N-terminal residues is also substantially increased by N-terminal acetylation,[29] a post-translational modification in eukaryotes. Moreover, the N-terminal and hydrophobic regions of α-synuclein are known to adopt an α-helical conformation when bound to the surface of negatively charged vesicles or detergent micelles.[30–32]

To explore in detail the effect of high-pressure on the structural properties of α-synuclein, we monitored the pressure-induced changes of the $^{1}H^{N}$, $^{15}N$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$ chemical shifts and measured a nearly complete set of $^{3}J_{HN-H\alpha}$ couplings at 2500 bar. The change in the $^{15}N$ spin relaxation was also investigated through $R_{1\rho}$ measurements. Although the pressure-induced $^{1}H^{N}$ shifts are in close agreement with those predicted for model tetrapeptides[33] and can reasonably be explained in terms of hydrogen bond compression, additional long-range factors are likely affecting the observed $^{15}N$ pressure-induced shifts. Analysis of the $^{13}C$ chemical shifts and $^{3}J_{HN-H\alpha}$ couplings of α-synuclein suggests that pressure shifts the PPII-β equilibrium of the random coil towards increased PPII propensity.

## Results

### Effects of pressure on the $^{15}N$ and $H^{N}$ amide chemical shifts

The pressure dependence of the $^{15}N$ and $H^{N}$ amide chemical shifts was monitored by recording a series of $^{15}N$ TROSY-HSQC spectra of N-acetylated α-synuclein at 288 K, pH 6.0, with pressure varying from 1 bar to 2500 bar. An overlay of the 2D $^{15}N$–$^{1}H$ spectra recorded at four different pressures, 1 bar, 500 bar, 1250 bar and 2500 bar, shows large downfield shifts for all the amide crosspeaks with the notable exception of Gly41, which shows an upfield $H^{N}$ shift with increasing pressure (Figure 1 A). Figure 1 B and C display the $^{15}N$ and $H^{N}$ chemical shift differences between 2500 and 1 bar ($\Delta\delta_{2500}$) as a function of residue number. With a Pearson 's correlation coefficient of $R^2 = 0.60$ (RMSD = 0.024 ppm), the $\Delta\delta_{2500}(^{1}H^{N})$ values correlate closely with the chemical shift differences predicted for the same amino acid sequence using the pressure coefficients reported by Koehler et al.[33] for model tetrapeptides (Figure 1 B). By contrast, the correlation observed for $\Delta\delta_{2500}(^{15}N)$ is much weaker ($R^2 = 0.18$, RMSD = 0.179 ppm; Figure 1 C). The difference between the observed and predicted $\Delta\delta_{2500}$ values shows no clear correlation with residue type: for both $^{15}N$ and $^{1}H^{N}$ chemical shifts, a considerable spread of $\Delta\delta_{2500}$ values is observed regardless of the nature of the amino acid (Figure S1 A and B in the Supporting Information). Such variations narrow down slightly when taking into account the nature of the preceding residue (Figure S1 C and D). Since the α-synuclein sequence contains seven imperfect repeats of the hexamer motif KTKEGV in the N-terminal and NAC regions, we also examined

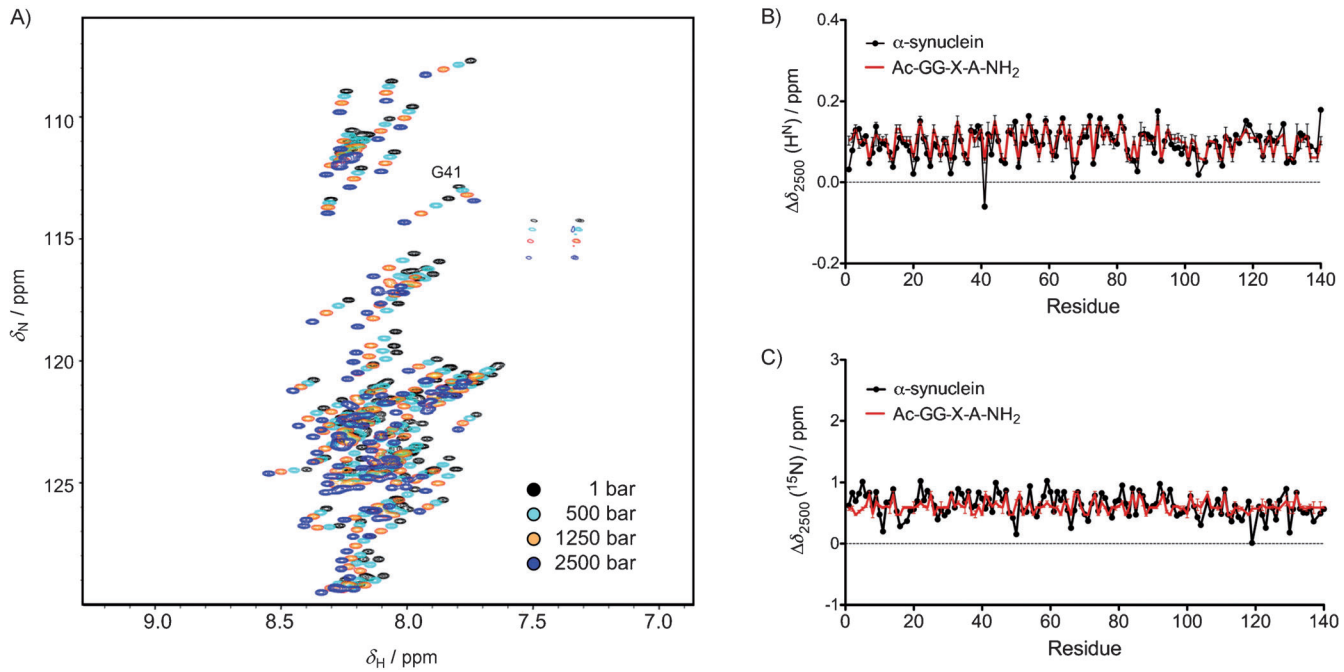


**Figure 1.** A) Overlay of the $^{15}N$ TROSY-HSQC spectra recorded at 500 MHz, between 1 bar and 2500 bar, at 288 K, recorded on a uniformly labeled $^{15}N/^{13}C$-enriched sample of N-acetylated α-synuclein (0.4 mM) in sodium phosphate buffer (20 mM, pH 6.0). The chemical shifts differences between 2500 bar and 1 bar ($\Delta\delta_{2500}$) are displayed in black as a function of the protein sequence for B) $^{1}H^{N}$ and C) $^{15}N$ and compared with the corresponding predicted chemical shift differences (red) for residues of the same type when embedded at position X in model tetrapeptide Ac-G-G-X-A-$NH_2$.[33]

the variation of the $\Delta\delta_{2500}$ values among the six residues composing this motif (Figure S1 E and F). Interestingly, a clear pattern of $\Delta\delta_{2500}$ values appears for the $^1H^N$ chemical shifts, with the largest $\Delta\delta_{2500}(^1H^N)$ for the second residue of the motif and the smallest $\Delta\delta_{2500}(^1H^N)$ for the fifth residue of the motif (Figure S1 F). Residue Thr72, the fourth residue of the sixth repeat and the sole Thr at this position (in all other repeats the fourth residue is Glu) is a distinct outlier in this pattern.

### Nonlinear pressure dependence of the $^1H^N$ chemical shifts

The nonlinear pressure response of the $^1H^N$ chemical shifts has been extensively studied for globular proteins and has been attributed to the presence of low-lying "excited states".[21] Surprisingly, the $^1H^N$ chemical shifts reported by Koehler et al.[33] for model tetrapeptides also exhibit a significant nonlinear pressure dependence. To analyze this nonlinear pressure response in the case of the α-synuclein, we fitted the pressure dependence of the $^1H^N$ chemical shifts, measured at nine different pressures, ranging from 1 to 2500 bar, to a second order polynomial function (Figure 2 A). Comparison of the nonlinear coefficients of the $^1H^N$ pressure dependence with those reported by Koehler et al. (2012) shows that the values obtained for α-synuclein are, on average, about three times larger than the ones reported for model tetrapeptides $(-8.99\pm3.60)\times10^{-9}$ ppm bar$^{-12}$ compared to $(-3.10\pm2.19)\times10^{-9}$ ppm bar$^{-12}$, respectively; Figure 2 B). The largest differences between the measured and predicted coefficients are observed in the N-terminal region (residues 6–36) and in the central region (residues 50–92). Again, no significant correlation was observed between the nature of the amino acid and the magnitude of the nonlinear pressure response.

### Correlation between the $^1H^N$ pressure and temperature coefficients

It has been proposed that the upfield chemical shift observed for the amide protons with increasing temperature is caused by lengthening of the hydrogen bonds, which weakens the $H^N$ electron polarization.[37,38] Analogously, Akasaka and co-workers have interpreted the downfield shift of the amide protons with increasing pressure as resulting from a shortening of the H-bond length.[39] If the residue-by-residue variation in $\delta(^1H^N)$ temperature and pressure coefficients indeed reflects variations in the sensitivity of the corresponding H bonds to temperature and pressure, a correlation between these temperature and pressure coefficients is expected to be present. Although the degree of variation in $\delta(^1H^N)$ temperature and pressure coefficients is much smaller for an IDP than for a folded protein, we nevertheless examined the potential presence of such a correlation for acetylated α-synuclein. The pressure coefficients used here correspond to the first order coefficients extracted from the fit of the $^1H^N$ chemical shifts measured between 1 and 2500 bar, at 288 K, while the temperature coefficients were calculated by a linear least-square fit of the $H^N$ chemical shifts measured at 283 K, 288 K and 293 K, at atmospheric pressure. A modest correlation ($R^2=0.31$) with a negative slope is indeed observed between the temperature and pressure coefficients (Figure 3). Interestingly, the amide proton of Gly41 for which the sole upfield effect of pressure was measured with a pressure coefficient of $-2.8\times10^{-5}$ ppm bar$^{-1}$, exhibits by far the smallest temperature coefficients ($-1.2$ ppb K$^{-1}$). Recently, the Gly41 $^1H^N$ also was shown to have an exceptionally large isotope shift when comparing the protein where all nonexchangeable hydrogens had been deuterated with its fully protonated form.[40] However, no structural explanation for this outlier behavior of Gly41 $^1H^N$ has emerged so far.

### Effect of pressure on $^{15}N$ spin relaxation

The effect of pressure on the backbone dynamics has been described in a few cases, for globular proteins[41] and for an isolated α-helix,[42] leading to the general conclusion that rapid internal motions of the N–H vectors on the nanosecond or subnanosecond time scale are not affected by pressure. To extend

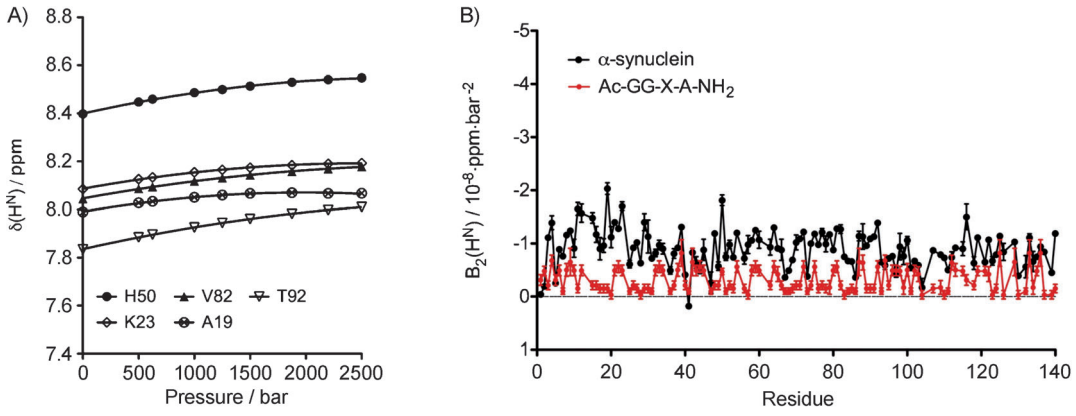


**Figure 2.** Effect of pressure on $^1H^N$ chemical shifts. A) Pressure dependence of the $^1H^N$ chemical shifts of N-acetylated α-synuclein measured through a series of $^{15}N$ TROSY-HSQC experiments, recorded at 500 MHz, 288 K, and pressures ranging from 1 to 2500 bar. The changes of the $^1H^N$ chemical shifts with pressure ($\delta_{HN}(p)$) are fitted to a quadratic function: $\delta_{HN}(p)=\delta_0(p_0)+B_1(p-p_0)+B_2(p-p_0)^2$. B) The second order coefficients ($B_2$) are displayed as a function of the protein sequence (black) and compared with those predicted for residues of the same type when embedded in the model tetrapeptide Ac-G-G-X-A-NH$_2$ (red).[33]

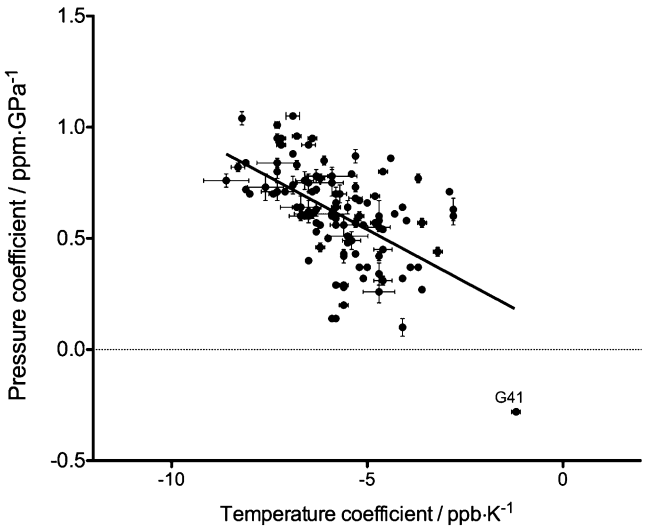


**Figure 3.** Correlation between the $^{1}H^{N}$ pressure and temperature linear coefficients, measured for a $^{15}N/^{13}C$-enriched sample of N-acetylated α-synuclein at pH 6.0. The pressure coefficients were extracted from the fit of the $^{1}H^{N}$ chemical shifts as a function of pressure, between 1 bar and 2500 bar, at 288 K, while the temperature coefficients were calculated from a linear least squares fit of the $^{1}H^{N}$ chemical shifts at 283, 288 and 293 K, under atmospheric pressure.

these observations to IDPs, we measured the $R_{1\rho}$ relaxation rates at 600 MHz on a nonacetylated α-synuclein, at 1 bar and 2500 bar (Figure 4). A modest and rather uniform increase of approximately 5% in the relaxation rates was observed at 2500 bar ($R_{1\rho}=3.28\pm0.55\ s^{-1}$), compared to 1 bar ($R_{1\rho}=3.13\pm0.54\ s^{-1}$), with a correlation coefficient, $R^2=0.89$ (RMSD = $0.24\ s^{-1}$) between the two data sets (Figure 4). Such a nonspecific, uniform effect of pressure on the transverse relaxation rates simply reflects the small increase in the solvent viscosity[43] and suggests that the backbone dynamics of α-synuclein is not affected by pressure. The rapid internal dynamics of α-synuclein has previously been highlighted by $R_1$ relaxation dispersion measurements.[44] Increased flexibility of the NAC

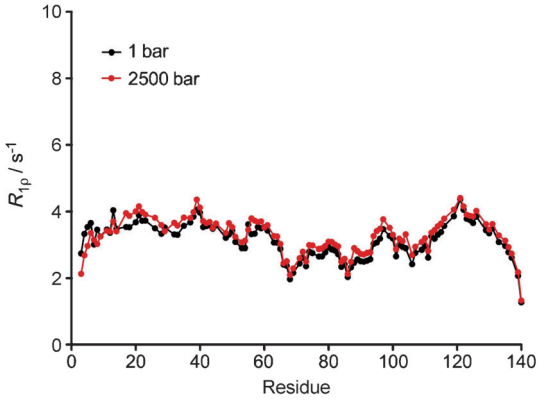


**Figure 4.** $^{15}N$ $R_{1\rho}$ relaxation rates measured using a 1.3 kHz RF field at 60.8 MHz $^{15}N$ frequency, at 1 bar (black) and 2500 bar (red), displayed as a function of the protein sequence. The sample conditions are: $^{15}N$-enriched nonacetylated α-synuclein (0.5 mM) in sodium phosphate buffer (20 mM, pH 6.0). All the experiments were performed at 288 K.

region relative to the remainder of the protein, as judged by decreased $^{15}N$ transverse relaxation rates, has been reported previously,[45] but is more evident in the current data due to increased sensitivity associated with the use of a cryogenic probe head used in our measurements. Our data clearly show that this increased flexibility of the NAC region is not impacted by pressure.

### Effect of pressure on the backbone $^{13}C$ chemical shifts

The pressure dependence of the $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$ chemical shifts of N-acetylated α-synuclein was analyzed by recording HNCACB and HNCO experiments at 1 bar and 2500 bar (Figure 5A). A general upfield shift of the $^{13}C^{\alpha}$ chemical shifts was observed, with an average difference between 2500 and 1 bar of $\Delta\delta_{2500}(^{13}C^{\alpha})=-0.122\pm0.091$ ppm. Gly $^{13}C^{\alpha}$ resonances are an exception and show the opposite sign for the pressure dependence: $\Delta\delta_{2500}(^{13}C^{\alpha})=+0.124\pm0.038$ ppm (Figure 5B). A general upfield change was also observed for the $^{13}C^{\beta}$ chemical shifts ($\Delta\delta_{2500}(^{13}C^{\beta})=(-0.186\pm0.081)$ ppm), with the exception of Ala, which show a downfield shift: $\Delta\delta_{2500}(^{13}C^{\beta})=+0.288\pm0.069$ ppm (Figure 5C). The magnitude of the pressure induced shifts observed here for N-acetylated α-synuclein are comparable to those reported by Williamson and co-workers for the aliphatic carbons in two well-folded proteins, barnase and protein G, where an average $^{13}C$ shift difference between 2000 bar and 1 bar of $+0.24\pm0.18$ ppm for $CH_3$, $-0.09\pm0.17$ ppm for $CH_2$ and $-0.17\pm0.15$ ppm for the CH carbon atoms was found.[46] Surprisingly, even for α-synuclein which shows all the hallmarks of an IDP, remarkably large variations in $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ pressure dependence remain when comparing the effects seen for residues of a given type at different locations in the protein (Figure S2). This observation indicates that the variation in $\Delta\delta_{2500}(^{13}C^{\alpha},^{13}C^{\beta})$ values is not simply dominated by the amino acid type and instead must reflect a differential local effect in how the disordered chain reorganizes itself upon increasing pressure. Only in the case of $^{13}C^{\alpha}$ atoms did we notice a slight reduction in the $\Delta\delta_{2500}$ variations when the nature of the preceding residue was considered (Figure S2). For the $^{13}C'$ nuclei, a small but nearly universal downfield change in chemical shift was observed upon increasing the pressure to 2500 bar, with an average $\Delta\delta_{2500}(^{13}C')=+0.079\pm0.076$ ppm (Figure 5D). The opposite direction of the pressure-induced $^{13}C^{\alpha}$ and $^{13}C'$ changes in chemical shift suggests that these changes do not simply reflect a change in α-helical propensity of the N-acetylated α-synuclein, as the secondary $^{13}C^{\alpha}$ and $^{13}C'$ shifts associated with α-helix are both pronouncedly positive.

### Effect of pressure on $^{3}J_{HN-H\alpha}$

In addition to chemical shifts, $^{3}J_{HN-H\alpha}$ couplings are widely used as reporters for the backbone torsion angle, $\phi$. We recently demonstrated that $^{3}J_{HN-H\alpha}$ values in α-synuclein can be measured at very high precision (< 0.05 Hz).[29] Moreover, a study of the protein GB3 showed that the $^{3}J_{HN-H\alpha}$ values are quite insensitive to residue type, H-bonding effects, or structural variables other than $\phi$, as evidenced by an RMSD of 0.4 Hz between ex-

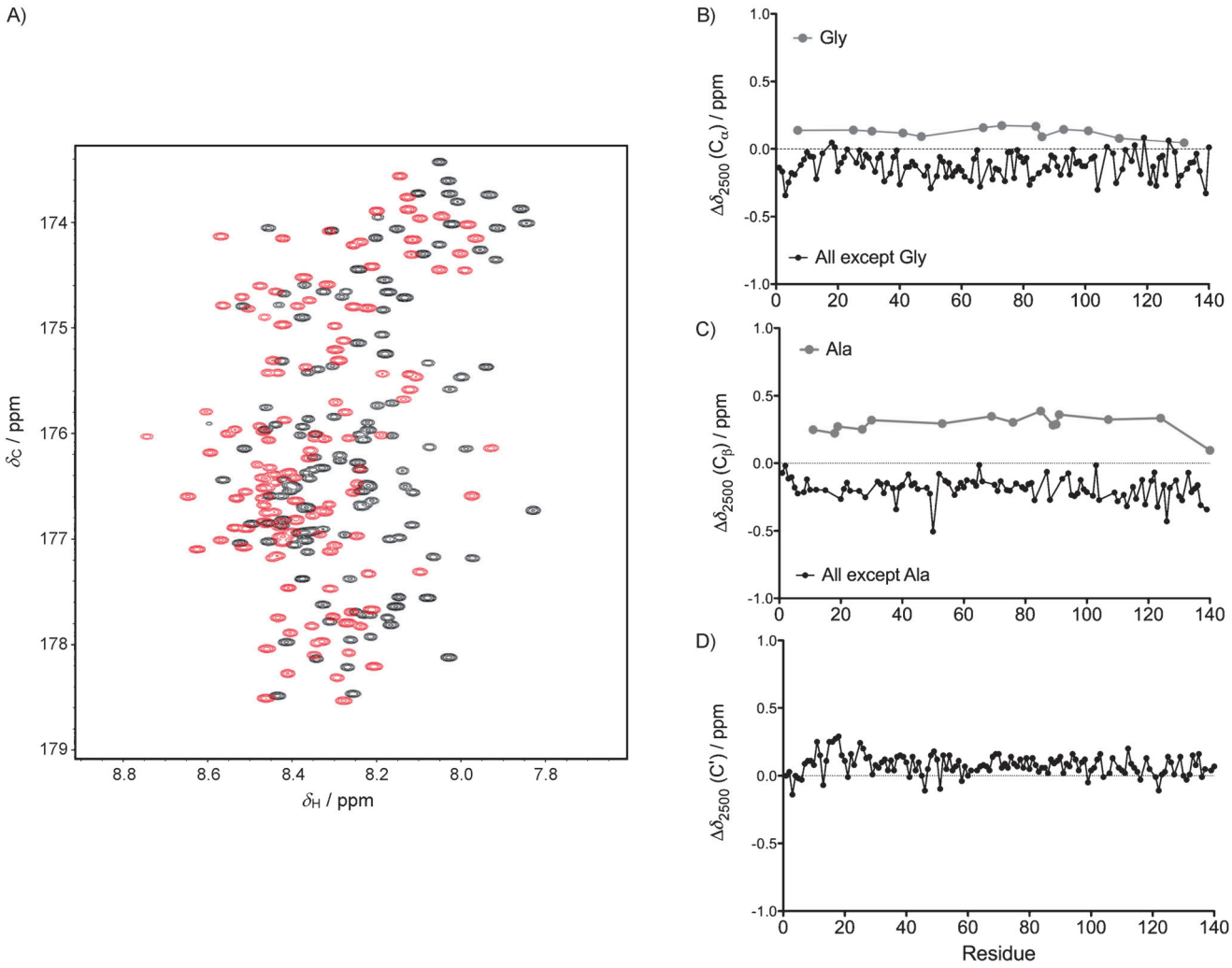


**Figure 5.** Effect of pressure on chemical shifts in α-synuclein. A) Superposition of the 2D projections of the TROSY-HNCO spectra recorded at 1 bar (black) and 2500 bar (red) on the $^{13}C/^{1}H$ plane. The chemical shifts differences between 2500 bar and 1 bar ($\Delta\delta_{2500}$) measured for the B) $^{13}C^{\alpha}$, C) $^{13}C^{\beta}$ and D) $^{13}C'$ at 288 K, are displayed as a function of residue number in the α-synuclein sequence. The $^{13}C$ chemical shifts were extracted from HNCACB and HNCO spectra collected at 500 MHz, using a uniformly $^{15}N/^{13}C$-enriched sample of N-acetylated α-synuclein in sodium phosphate buffer (20 mM, pH 6.0).

perimental values and those predicted by a Karplus curve when using an RDC-refined high resolution X-ray structure.[47] Here, we measured $^{3}J_{HN-H\alpha}$ couplings at 2500 bar and compared the values with our previously published set of $^{3}J_{HN-H\alpha}$ couplings measured for the same protein at atmospheric pressure.[29] This is to our knowledge the first report of the effect of pressure on $^{3}J_{HN-H\alpha}$ couplings. Although the effects are generally small, they are almost universally negative ($\Delta^{3}J_{HN-H\alpha} = -0.20 \pm 0.12$ Hz) and in many cases exceed the uncertainty in the measurement by five standard deviations or more (Figure 6). Again, little dependence on amino acid type is observed, and the range of $\Delta^{3}J_{HN-H\alpha}$ for a given type of residue, for example, Val or Lys, spans nearly the entire range of observed values, from 0 to −0.6 Hz. Perhaps surprisingly, even when selecting pairs of amino acids that are of the same type, considerable variation remains (Figure S3 B), indicating that the effect of pressure extends beyond impacting the interaction between side chains on adjacent amino acids.

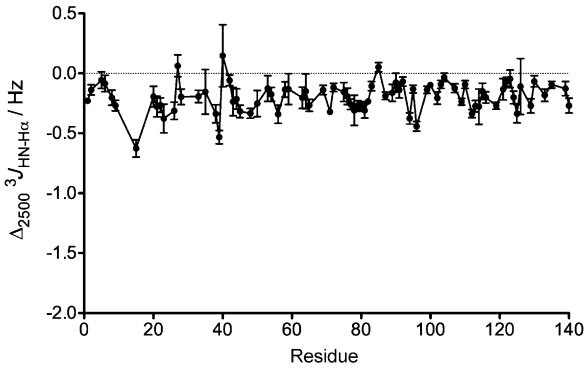


**Figure 6.** Differences in the $^{3}J_{HN-H\alpha}$ couplings measured at 800 MHz between 2500 and 1 bar, at 288 K, displayed as a function of the protein sequence. The sample conditions are: $^{15}N$-enriched N-acetylated α-synuclein (0.3 mM) in sodium phosphate buffer (20 mM, pH 6.0).

## Discussion

### Effect of pressure on the amide chemical shifts and the hydrogen bonds length

The downfield shift observed at high pressure for the $H^N$ amide protons is commonly interpreted as resulting from the compression of the N–H···O=C hydrogen bond. Indeed, a close correlation between the strength of the hydrogen bond and the $^1H^N$ chemical shift has long been recognized.[39,48] The pressure induced changes in the through-hydrogen-bond $^{3h}J_{NC'}$ scalar couplings measured in streptococcal protein G[49] and ubiquitin[50] provide another piece of direct evidence that the electronic orbital overlap associated with H bonds is indeed affected by pressure. Based on an empirical relationship between the H-bond length and the $^1H^N$ chemical shift.[48] Akasaka and co-workers estimated that the N–H···O=C hydrogen bond distance is reduced by approximately 1% at 2 kbar in globular proteins.[39] The magnitude of the pressure-induced $^1H^N$ shifts measured here for α-synuclein, on average, is very similar to that of the solvent exposed amide protons in bovine pancreatic trypsin inhibitor (BPTI), for which an average of −0.129 ppm/2 kbar was reported.[39] Moreover, the close agreement observed between the linear component of the pressure-induced $^1H^N$ chemical shift changes in α-synuclein and those predicted on the basis of model tetrapeptides[33] (Figure 1B), suggests that the $\Delta\delta H^N$ shifts of α-synuclein are dominated by very local effects of pressure. On the other hand, Gly41 is clearly an outlier to this rule, as it shows an upfield change in chemical shift with pressure ($\Delta\delta H^N = -2.8 \times 10^{-5}$ ppm bar$^{-1}$). It is conceivable that this residue is transiently involved in intramolecular H-bonding interactions, the population of which may be sensitive to pressure. The correlation observed between the pressure and temperature coefficients of the $^1H^N$ chemical shifts in α-synuclein (Figure 3) agrees well the idea that both temperature and pressure affect the hydrogen-bond length, either by compressing (with increasing pressure) or expanding (with increasing temperature) the length of the N–H···O bond.

Interestingly, while the magnitude of the overall pressure-induced $^1H^N$ chemical shift changes in α-synuclein is similar to that of a small and rigid protein such as BPTI, the nonlinear component of the pressure dependence of the $^1H^N$ chemical shift are considerably larger than those in the model peptides (Figure 2B), and comparable to those reported for the dihydrofolate reductase or the Ras binding domain of Ral-GDS.[21] Akasaka and Li have correlated the magnitude of the nonlinear pressure response in globular proteins to the volume of internal cavities, suggesting that proteins with large internal void volumes are more likely to populate low-lying alternate conformers under the high-pressure condition.[21] Clearly, this explanation does not apply in the case of an IDP such as α-synuclein. The large nonlinear $H^N$ coefficients measured here for α-synuclein most likely arise from a shift in populations sampled by the peptide chain, as reflected by the decrease of the $^3J_{HN-H\alpha}$ couplings. However, we cannot exclude a contribution arising from a perturbation of the hydration shell at high pressure.[51,52]

The pressure-induced changes of the $^{15}N$ amide chemical shifts have been described in a few cases and remain poorly understood. Akasaka and co-workers suggested that the $^{15}N$ pressure coefficients are affected by site-specific variations of the backbone angles in addition to the decrease in the H-bonding distances.[16] A weak correlation was indeed found for BPTI between the magnitude of the $^{15}N$ shift and the $\psi_{i-1}$ angle in β-sheet but not in α-helices.[16] The absence of a correlation between the pressure-induced $^{15}N$ shifts measured here for α-synuclein and those predicted from model tetrapeptides, even when the nature of the preceding amino acid is considered (Figure 1C and Figure S1D), points to the influence of additional factors that extend beyond the amino acids sharing the peptide bond. It therefore seems clear that the $\Delta\delta_{2500}(^{15}N)$ values of α-synuclein cannot be explained solely by the compression of H-bond length to solvent, or to a strictly local interaction between the two sequential side chains, and instead must involve the differential sampling by the polypeptide backbone of the complex conformational landscape with increasing pressure.

### Effect of pressure on the conformational equilibrium of α-synuclein

The difference in protein volume associated with the unfolding of an α-helix is close to zero, such that α-helices in proteins are usually preserved under high-pressure conditions.[15] For example, a substantial amount of residual helical content was observed in the pressure-denatured state of staphylococcal nuclease (SNase).[53] Imamura and Kato[54] suggested that the volume change upon unfolding ($\Delta V_u$) could even be positive (i.e., pressure promoting the formation of helical content) in the case of a small helical peptide.

The N-terminal acetylation of α-synuclein resulted in a significant, approximately 15% transient population of α-helix by its six N-terminal residues, extending as far as residue 12 but at much decreased population.[29] We chose to study this N-acetylated form of the protein as it would provide direct data on the impact of pressure on the transient population of α-helix. The analysis of the $^{13}C$ chemical shift changes ($^{13}C^\alpha$, $^{13}C^\beta$, and $^{13}C'$, Figure 5) between 1 and 2500 bar shows that the N-terminal six residues exhibit, on average, chemical shift differences of $-0.211 \pm 0.075$ ppm ($^{13}C^\alpha$) and $-0.028 \pm 0.059$ ppm ($^{13}C'$), compared to $-0.118 \pm 0.034$ ppm ($^{13}C^\alpha$) and $+0.083 \pm 0.038$ ppm ($^{13}C'$) for all sliding windows of six amino acids between residues 10–140. The $^{13}C^\beta$ shows a smaller average change between 1 bar and 2500 bar for the first six residues ($-0.118 \pm 0.074$ ppm) compared to residues 10–140 ($-0.189 \pm 0.029$ ppm). Similarly, the average decrease in $^3J_{HN-H\alpha}$ with pressure for the first six residues ($\Delta^3J_{HN-H\alpha} = -0.127 \pm 0.076$ Hz) is slightly smaller than for the remainder of the chain $\Delta^3J_{HN-H\alpha} = -0.221 \pm 0.041$ Hz (Figure 6), suggesting that relative to the fully disordered region, population of α-helix is slightly decreased at 2500 bar and therefore that the $\Delta V_u$ for α-helix is negative.

Having ruled out an increased α-helical propensity at high pressure, the small decrease of the $^3J_{HN-H\alpha}$ couplings measured here for nearly all residues of α-synuclein at 2500 bar (Figure 6) can be interpreted as arising from a pressure-induced decrease of the β conformation and a corresponding increase in the conformational populations with a less extended backbone geometry, namely PPII, $\alpha_L$ or β-turn conformations, all of which have smaller $^3J_{HN-H\alpha}$ couplings than the β conformation. Many studies have pointed out the crucial role of hydration in stabilizing PPII conformations relative to α or β conformations in peptides and proteins.[11–13,55] Importantly, the PPII-β equilibrium in peptides is highly temperature dependent, with the amount of PPII conformations decreasing with increasing temperature.[13,14] An increase in pressure will likely have an opposite effect to an increase in temperature, by shifting the PPII-β equilibrium toward more PPII content at high pressure. However, considering that $^3J_{HN-H\alpha}$ couplings do not uniquely report on secondary structure, further studies are needed to analyze the specific effect of pressure on PPII conformations relative to $\alpha_L$ or β-turn conformations.

## Conclusions

The NMR spectrum of α-synuclein is impacted by hydrostatic pressure to a degree that is comparable to that seen for folded proteins. While the changes in $^1H^N$ chemical shifts correlate well with values predicted from a series of short model peptides, large deviations from such model peptide behavior is seen for $^{15}N$, indicating that the pressure dependence of the α-synuclein spectrum is modulated by interactions that extend beyond the dipeptide unit for which chemical shift changes are observed. The nature of these remote interactions remains to be determined. The transient α-helical population observed for the first six N-terminal residues in the N-acetylated α-synuclein is only weakly diminished by pressure whereas the general decrease of the $^3J_{HN-H\alpha}$ couplings observed at high-pressure is suggestive of a shift in the PPII-β equilibrium towards PPII for most of the protein. Further computational studies are especially needed to explore in detail the opposite effects of heat and pressure on the PPII propensity of peptides and disordered fragments of proteins, and to explore in atomic detail which structural and hydration changes are compatible with the impact of pressure on the NMR parameters observed in our study.

## Experimental Section

N-acetylated α-synuclein was generated using a recently developed recombinant expression system that includes a plasmid for overexpression of the requisite acetylation enzyme NatB,[34] which permits bacterial expression of N-terminally acetylated and uniformly $^{13}C/^{15}N$-enriched α-synuclein, which was subsequently purified as previously described (Maltsev et al.[29]).

All NMR spectra were recorded at 288 K. The $^{15}N$ TROSY-HSQC spectra were recorded at a $^1H$ frequency of 500 MHz. A total of 200*×560* complex points were collected, for acquisition times of 164 and 80 ms in the $^{15}N$ and $^1H$ dimensions, respectively, using an interscan delay of 1.4 s.

3D TROSY-HNCO and TROSY-HNCACB spectra were recorded at 500 MHz with two scans per free induction delay. The HNCO spectra comprised 110*×160*×750* complex points, for acquisition times of 150.5, 144 and 107 ms in the $^{13}C$, $^{15}N$ and $^1H$ dimensions, respectively. For the HNCACB spectra, the final time-domain matrices consisted of 173*×160*×750* complex points, corresponding to acquisition times of 144 ($^{15}N$), 26 ($^{13}C$) and 107 ms ($^1H$). The $^{13}C$ chemical shift was recorded using constant-time evolution. Both HNCO and HNCACB experiments were collected using a 40% sparse sampling scheme, with the sampling schedule in the two indirect dimensions generated randomly (i.e., only 40% of the $(t_1,t_2)$ pairs were actually collected). The final spectra were reconstructed as previously described[35] using the algorithm developed by Hyberts et al.[36]

The $R_{1\rho}$ experiments were recorded at 600 MHz on a nonacetylated α-synuclein sample. Data matrices of 320*×1024* complex points were collected for acquisition time of 176 ms and 122 ms in the $^{15}N$ and $^1H$ dimensions, respectively. The strength of the radiofrequency spin-lock field was set to 1.3 kHz and the relaxation delays were sampled for eight different durations: 1, 20, 70, 110, 180, 230, 300 and 350 ms.

The $^3J_{HN-H\alpha}$ couplings were recorded at 800 MHz by monitoring the modulation of the cross-peak intensity from a series of constant-time $^1H,^{15}N$ HMQC spectra, as previously described.[35] Eight constant time delays were used, with durations of 50, 60, 75, 95, 140, 180, 210 and 240 ms. The data matrix sizes ranged from 82*×1024* points for the shortest constant-time duration to 402*×1024* points for the longest duration.

For all the experiments described above, a commercial ceramic high-pressure NMR cell and an automatic pump system (Daedalus Innovations, Philadelphia, PA) were used to vary the pressure in the 1 to 2.5 kbar range.

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