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Solution NMR Investigation of the Response of the Lactose Repressor Core Domain Dimer to Hydrostatic Pressure

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Abstract

Previous investigations of the sensitivity of the lac repressor to high-hydrostatic pressure have led to varying conclusions. Here high-pressure solution NMR spectroscopy is used to provide an atomic level view of the pressure induced structural transition of the lactose repressor regulatory domain (LacI* RD) bound to the ligand IPTG. As the pressure is raised from ambient to 3 kbar the native state of the protein is converted to a partially unfolded form. Estimates of rotational correlation times using transverse optimized relaxation indicates that a monomeric state is never reached and that the predominate form of the LacI* RD is dimeric throughout this pressure change. Spectral analysis suggests that the pressure-induced transition is localized and is associated with a volume change of approximately -115 ml/mol and an average pressure dependent change in compressibility of approximately $30 \text{ ml mol}^{-1} \text{ kbar}^{-1}$. In addition, a subset of resonances emerge at high-pressures indicating the presence of a non-native but folded alternate state.

Graphical abstract

Keywords

Lac repressor; high pressure NMR; conformational drift; local and subglobal unfolding

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1. Introduction

The Lac repressor (LacI) has long been a model system in the study of protein-DNA interactions in genetic regulation [1–3]. LacI is a tetrameric regulator of the bacterial *lac* operon, which contains a cluster of three genes involved in lactose regulation and binds with high affinity to an operator sequence that is upstream of these genes. The binding of allolactose, a lactose analog, induces release of LacI from the operator sequence and transcription of the operon gene cluster is subsequently initiated.

In addition to its storied role in the study of gene regulation, LacI has long been studied in the context of oligomeric dissociation. A pioneering study characterized the pressure sensitivity of LacI using tryptophan fluorescence [4]. This study was performed on full-length LacI and concluded that the tetramer transitioned to monomer under the application of hydrostatic pressures and that this transition is coupled to the dissociation of IPTG. A subsequent study using fluorescence polarization concluded that the LacI tetramer transitions to the dimeric state under application of high-hydrostatic pressure [5]. This is consistent with an earlier observation that hydrostatic pressure induces depression of the *lac* operon in living *E. coli* cells [6]. These studies fit into the view of dissociation of oligomers into lower order states upon application of relatively modest hydrostatic pressure [7]. However, these studies reached somewhat different conclusions, which may be potentially attributed to an assumption of the oligomeric state, with one assuming a monomeric end-point to the pressure transition [4] and the other observing a dimeric end-point [5]. Subsequent work characterizing the unfolding and oligomeric state of LacI upon addition of urea concluded that the transition of the tetramer to unfolded monomers goes through an intermediate state consisting of unfolded but still tetrameric LacI [8]. Enhanced detail of the folding mechanism was achieved with a monomeric construct using a combination of experiment and computation [9,10], which gave insight into intermediate states of the lac repressor in the folding process without the complication of coupled monomer-oligomer equilibria.

High-pressure NMR spectroscopy is well positioned to further investigate the effect of high-hydrostatic pressure on the lactose repressor. The emergence of a high active volume solution NMR cell [11] with robust physical characteristics [12] has enabled state-of-the-art NMR-based studies of pressure-dependent phenomena biological macromolecules to 3 kbar. NMR relaxation provides a convenient method to characterize molecule size and therefore oligomeric state [13,14], as well as providing an atomic resolution view of structural perturbations and unfolding transitions [15–20]. Here we focus on the regulatory domain of LacI (LacI RD), which is comprised of residues 60 – 331 of the full length construct. Recent advances in the production of recombinant LacI and its regulatory domain permits preparation of isotopically labeled protein appropriate for solution NMR spectroscopy [21]. LacI* RD is a dimer under native conditions and binds two IPTG molecules (Figure 1). It is a convenient form of LacI to study the pressure effects on without complications from the head-piece domain, which is not involved in dimerization [21].

2. Methods

2.1 Protein production and sample preparation

The LacI* RD was prepared and purified as previously described [21] with some differences. The pASK derived plasmid [22] containing the LacI* RD gene was transformed into competent BLIM strain *E. coli* cells [23] and plated onto LB agar with ampicillin. In order to acclimate the cells to $^2\text{H}_2\text{O}$ M9 media, a 5 ml sample of LB/amp media was prepared, and a single colony of the freshly transformed LacI* RD was added to the media. When the OD_{600} reached 1.0, the cells in the LB media were centrifuged, were used to inoculate 75 ml $^2\text{H}_2\text{O}$ M9 solution to an OD_{600} of 0.05 and were left to grow overnight at 37°C with a final OD_{600} of 1.2. The starter growth was centrifuged and the pellet was used to inoculate a 0.5 L growth at a starting OD_{600} of 0.05 for ^2H - ^{15}N labeling. The labeling was achieved by growing in 91% $^2\text{H}_2\text{O}$ media with 1 g/L $^{15}\text{NH}_4\text{Cl}$ and 2 g/L glucose. This was induced at OD_{600} 0.5 using anhydrotetracycline [22], at which point the growth temperature was reduced to 25°C for overnight expression, with a final OD_{600} of 2.0. Harvested cells were suspended in 0.1 % Triton $\times 100$, 10 mM MgCl_2 , 5 mM CaCl_2 , 0.14 g/ml lysozyme, 0.1 g/ml DNase, 0.5 mM PMSF and a cOmpleteTM EDTA free protease inhibitor tablet (Sigma-Aldrich) and lysed using sonication.

Purification of the 6xHis tagged protein was achieved with a 5 ml His60 Ni Superflow column (Clontech Laboratories, Inc.). Buffer consisting of 50 mM NaPO_4 , 500 mM NaCl , pH 7.8 was used with 40 mM imidazole used to wash after loading the cell lysate, and 250 mM imidazole used to elute 6xHis LacI* RD. Proteolytic cleavage of the 6xHis tag was achieved with 6xHis-TEV protease and the reaction was dialyzed overnight against 4 L of 50 mM NaPO_4 , 500 mM NaCl , 3 mM β -mercaptoethanol at a pH of 7.8. The proteolytically-processed protein was flowed through a 5 ml His60 Ni Superflow column to capture the cleaved 6xHis tags and the 6xHis-TEV protease, with the flow-through containing the processed LacI* RD. Quantification of protein concentration was achieved using A_{280} with a calculated $\epsilon = 18450 \text{ M}^{-1}\text{cm}^{-1}$. 0.5 L of minimal media culture yielded ~15 mg of pure LacI* RD. Samples for NMR spectroscopy were prepared by first buffer exchanging pure fractions of LacI* RD into a buffer of 10 mM DTT, 20 mM Tris and 50 mM NaCl , pH = 7.4 with 10% $^2\text{H}_2\text{O}$ and concentrated to a final protein concentration of 400 μM with 10 mM IPTG added to the sample.

2.2 NMR spectroscopy

The LacI* RD sample was loaded into a 5 mm outer diameter/2.8 mm inner diameter ceramic NMR tube rated for 3 kbar and tethered to a computer controlled pressure generator (Daedalus Innovations). Experiments were performed at 298 K and collected on a 750 MHz Bruker Avance III spectrometer equipped with a triple-resonance cryo-probe. TROSY ^1H - ^{15}N HSQC [24] spectra were collected to monitor unfolding and structural perturbations and ^{15}N - ^1H TRACT [13] measurements were performed to monitor the rotational correlation time of the folded fraction of LacI* RD. The pressure points collected were 1, 500, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, and 3000 bar and the pressure series was brought back to 1 bar in a single step to characterize reversibility as monitored by TROSY ^1H - ^{15}N HSQC spectrum and ^{15}N - ^1H TRACT. The pressure series was collected

within 16 hours to mitigate potential aggregation effects. TROSY ^1H - ^{15}N HSQC spectra were collected with 1024×100 complex points and 16 scans, ^{15}N - ^1H TRACT experiments were collected in 1D with 16 scans and 2048 complex points using 30 relaxation delays and an additional 3 replicates for error analysis.

TROSY ^1H - ^{15}N HSQC spectra were processed in NMRpipe [25] and analyzed in SPARKY [26] with TRACT analysis performed with an in-house script interfacing with ALNMR [27]. TRACT spectra for each relaxation delay were integrated in the range of 8.7 to 9.6 ppm and the integrals were fit to a single exponential decay and τ_c calculations were performed as described previously [13,14].

3. Results and Discussion

3.1 LacI* RD responds reversibly to high-hydrostatic pressure

In our hands, apo-LacI* RD was not stable for a sufficient period to allow high-pressure NMR experiments to be carried out. Accordingly, all experiments reported here refer to the IPTG-bound state of LacI* RD. In order to understand the effect of pressure on LacI* RD, TROSY ^1H - ^{15}N HSQC [24] spectra were acquired at a series of pressures ranging up to 3000 bar using a sample that was 400 μM in monomer with an excess of ligand (10 mM IPTG). The TROSY ^1H - ^{15}N HSQC spectra reveal that application of high pressure causes manifestation of a variety of effects. Largely linear chemical shift perturbations of resonances were observed throughout the spectrum (Figure 2). Such changes correspond to local bonding effects [28–30] and minor changes in the protein structure [16] and changes the effect of solvent on protein backbone chemical shifts [31]. Some portion of the chemical shift changes could also arise from the small pressure sensitivity of the pH of the Tris buffer employed [32]. Due to the difficulty in interpretation of chemical shift perturbations of proteins under high-hydrostatic pressure [31], these were not analyzed further in quantitative detail.

A much easier approach is to follow the loss of cross peak intensity due to conversion to an alternate local structure. Many peaks were observed to undergo a reduction in intensities upon application of high-hydrostatic pressure with a major transition for this set of peaks observed above 1 kbar (Figure 2). At these pressures, broadened peaks emerge in the center of the spectrum, which is characteristic of unfolded disordered protein structure, and corresponds to a reduction in population of the native state of LacI* RD as it exists at ambient pressure. Of the resonances that respond to high-pressure by reduction in peak intensities, a subset of these show the emergence of nearby resonances in apparent slow exchange on the chemical shift timescale. These are attributed to the emergence of alternate but locally folded structure in the pressure-induced state of LacI* RD.

To characterize the reversibility of the observed pressure transitions, TRACT and TROSY spectra were collected at ambient pressure (1 bar) after a one-step drop after collection of the pressure series. Resonance peak positions returned to those of the native state with an average of 85% of their initial value indicating the pressure response is largely reversible with only minor conformational drift [33–35]. TRACT measurements confirmed the retention of the dimeric state.

3.2 The oligomeric state of LacI* RD under hydrostatic pressure

A series of ^{15}N - ^1H TRACT [13] experiments were collected at pressures ranging from ambient to 3000 bar to determine the oligomeric state of the remaining folded fraction of protein. TRACT provides a convenient estimation of rotational correlation times of large proteins while eliminating contaminating effects from relaxation arising from chemical exchange [13]. However, due to the rigid body approximation used in TRACT analysis a systematic underestimation of τ_c persists and can amount to about 2–5 ns [13,14]. The ^{15}N filtered 1D TRACT approach allowed data collection at elevated pressure to be kept short and avoid contaminating effects from protein aggregation and conformational drift phenomena. ^{15}N - ^1H TRACT experiments were performed with 30 relaxation delays for measurement of both $^{15}\text{N}_\beta^1\text{H}_\alpha$ and $^{15}\text{N}_\alpha^1\text{H}_\alpha$ spin states with three duplicate relaxation delays for error estimation. Relaxation rates of each spin state were analyzed by integration of the 1D spectra corresponding to each relaxation delay and fit to a single exponential decay [13]. Care must be taken in TRACT analysis to avoid contaminating effects from protein regions or fractions of the protein population containing a fast internal correlation time, i.e. highly dynamic regions, side-chains, or unfolded protein. In order to avoid these contaminating signal regions, the 1D spectra were integrated in the range from 8.7 to 9.6 ppm, a spectral region that contains only dispersed resonances arising from the folded fraction of protein throughout the pressure series (see Figure 2). The molecular reorientation correlation time of LacI* RD at ambient pressure was determined to be ~27 ns, which is consistent with the 58 kDa dimer [14]. Throughout the pressure series, the TRACT estimated molecular reorientation correlation time remained within ± 2.0 ns, indicating that the fraction of protein that remains folded remains predominately as a dimer.

3.3 Pressure induced transition of the native state

The disappearance and reduction of peak intensities of the ambient pressure state is observed to be in slow-exchange on the NMR chemical shift time scale, facilitating simple analysis via peak intensities by scaling the fraction in the folded state to the resonance peak intensity of the ambient pressure state as described previously [18,19]. Though the resonance assignments of LacI* RD have yet to be fully established and are on-going, analysis of individual residues provides insights into the effect of hydrostatic pressure on structure and stability. Resonance peaks of the folded state were followed from the ambient pressure state and intensities were measured throughout the pressure perturbation series. LacI* RD was assumed to be 100% folded at ambient pressure, so TROSY ^1H - ^{15}N HSQC peak intensities upon applied hydrostatic pressure were scaled to those at 1 bar in order to characterize a fraction of protein that remains folded at each pressure. Analysis was performed only on crosspeaks that were well resolved throughout the pressure series and have ample signal-to-noise. Many crosspeaks in the central portion of the spectrum were not analyzed as well due to the emergence of unfolded protein signal at high pressure points (Figure 2). In total, 118 resonances of a possible 262 observable residues were characterized using this method.

Of the characterized crosspeaks, sixty exhibited a clear uniform reduction in intensity upon application of hydrostatic pressure (Figure 3). Peak integrals rather than simple maxima were used where possible to minimize effects from line-broadening through the transition. These crosspeak intensities were averaged to obtain a global view of the pressure

susceptibility of LacI* RD (Figure 3). Only 8% of detectable intensity from these crosspeaks remains at the highest pressure point (3000 bar). On average, the application of 3 kbar of pressure makes the native state dimer 1.4 kcal/mol less favorable.

3.4 Volumetric analysis of the pressure transition

The intensities of individual crosspeaks of the native state spectrum of LacI* RD provide direct access to the thermodynamics of the response of the protein to hydrostatic pressure.

$$\Delta G(p) = \Delta G^\circ(p_0) + \frac{\partial G}{\partial p}(p - p_0) + \frac{1}{2} \frac{\partial^2 G}{\partial p^2}(p - p_0)^2 + \dots = \Delta G^\circ(p_0) + \Delta V^\circ(p - p_0) + \frac{1}{2} \Delta \beta'(p - p_0)^2 + \dots$$

(1)

with G° being the free energy difference between the native and pressure-induced states at 1 bar, V° and β' the molar volume change and change in compressibility respectively. Higher order terms that are sometimes necessary to describe the pressure dependence protein phenomena [15,36] were found to be unnecessary as the data obtained here are well described by truncation of Equation 1 at the quadratic term. The native state fractional population among all resonances showing a clear pressure transition (Figure 3) was averaged to obtain a global value for the native population remaining among all residues undergoing the pressure transition (Figure 3, red line). This was used to calculate the free energy between the native state and the pressure-induced state using Equation 1 (Figure 4).

The free energy difference at 1 bar between the native state and the pressure-induced state (G°) of the LacI* RD dimer was found to be 3.1 ± 0.1 kcal mol⁻¹. The molar volume difference between the states (V°) is found to be -115 ± 2 ml mol⁻¹, approximately half of the pressure induced volume change of 217 ml mol⁻¹ for the tetrameric construct of LacI [4]. It is noted that the latter study was performed with 1 μ M LacI and 1.8 mM IPTG. The change in compressibility (β'), which accounts for the non-linearity of the pressure dependence of $G(P)$ at high pressure was found to be 34 ± 2 ml mol⁻¹ kbar⁻¹. The volume normalized compressibility (V°/β') of 0.3 is comparable to that found previously for other proteins (e.g. HPr [29]).

The pressure induced transitions of individual cross peaks were also analyzed to understand if fitting equation 1 to the population average is an accurate approximation (Supplementary Figure 1). All resonances were fit to the linear and quadratic truncation of equation 1, and all but one were better described by inclusion of the quadratic term according to reduced χ^2 . Though the individual fits indicate that there is a range of local and sub-global stabilities, the free energy, volume differences and pressure dependent compressibility average to nearly the same as those of the population average fittings: $G^\circ = 3.6 \pm 2.0$ kcal/mol; $V^\circ = -122 \pm 75$ ml mol⁻¹; and $\beta' = 29 \pm 41$ ml mol⁻¹ kbar⁻¹. Gaussian fitting of a distribution of V° values among all resonances also recapitulates the average V° value with the distribution centered at -111 ± 7 ml mol⁻¹ (Supplementary Figure 2). This is a wider range of V° than seen in a previous study employing this method to extract V° values [19], though a

similarly wide range has been observed in studies where pressure denaturation is observed without the use of a chemical denaturant such as guanidinium chloride [18,37].

3.5 The emergence of an alternate state at high-pressure

In addition to the transition of many crosspeaks to spectral positions consistent with locally unfolded structure, many displayed an interconversion in slow-exchange ($< 10^{-1}$ s) with an alternate folded state. The alternate state is characterized by well-defined peaks emerging at a chemical shifts very similar to those of the native state (Figure 5). Thirty-three of these emerging crosspeaks with a clearly separated, slow-exchange peak indicate that the alternate state is stabilized by an average of ~ 0.5 kcal mol $^{-1}$ as compared to the native state at 3 kbar. Though volumetric analysis of the emergence of the alternate state is complicated by low signal-to-noise, resonance overlap and emergence of the resonances late in the pressure series, five resonance peaks could be analyzed at pressures above 1250 bar. Fitting to the first order truncation of equation 1 shows that these residues are in equilibrium with a high-pressure alternate state that has a molar volume difference from the native state (ΔV°) of -24 ± 6 ml mol $^{-1}$. Finally, the resonance chemical shifts were compared to those of apo LacI* RD to understand if high-hydrostatic pressure induces the dissociation of IPTG as previously concluded from observations of the tetrameric form of the protein under high-hydrostatic pressure [4]. No clear correspondence was observed between the high-pressure alternate state and the ambient pressure state of apo LacI* RD [21]. Details of the structural transition will await completion of the resonance assignments, which are on-going.

4. Conclusions

High-resolution NMR spectroscopy in conjunction with the application of high-hydrostatic pressure allows for an unprecedented view of the pressure response of the lactose repressor. Past studies have characterized the pressure response of LacI, but with varying conclusions as to the nature of the high-pressure state [4,5]. The current study takes advantage of recent technical developments in high-pressure NMR spectroscopy and efficient production of isotopically labeled protein. This has provided a highly site-resolved view of the effect of high-hydrostatic pressure on the oligomeric state and structure of LacI* RD. TRACT based estimations of the rotational correlation time of LacI* RD under pressured up to 3 kbar demonstrate that the predominate folded fraction remains dimeric throughout the entire pressure series, consistent with the dimerization constant of 77 nM reported for dimeric lac repressor [38]. This is in contrast with a previous study at similar temperature, pH, and large excess IPTG excess but at a much lower protein monomer concentration of 2 μ M [5]. Our results also contrast to previous observations that *tetrameric* lac repressor constructs dissociate into lower order quaternary structure elements over a relatively modest pressure range [4,5]. Here we find a large fraction of the protein is in slow exchange with a locally unfolded folded state that retains the dimer contacts, which is somewhat similar to a thermal transition seen for the dimeric Arc repressor above 100 μ M [39] and the tetrameric unfolded state of LacI proposed by Barry et al[8].

The thermodynamic parameters of the partial pressure denaturation elucidated in this study further illuminate the nature of the transition. The difference in volume between the folded

and pressure-denatured state (ΔV°) in the dimeric form of the lac repressor is found to be $\sim -115 \text{ ml mol}^{-1}$, which is consistent with the $\sim -220 \text{ ml mol}^{-1}$ reported for the assumed transition of the IPTG bound tetrameric construct to monomer under high hydrostatic pressure, for a value of $\sim 55 \text{ ml mol}^{-1}$ per protomer. The similarity of these values implies that early models of the pressure response may have omitted the effect of pressure on the fold stability of the protein decoupled from the transition into monomers [4,5], as was later included with chemical denaturation [8].

A preliminary structural analysis based on site-specific resonance assignments (to be reported elsewhere) suggests that the portion of the IPTG binding pocket that is furthest away from the dimerization surface is most susceptible to pressure induced denaturation and the emergence of this state. This may be explained by the incomplete filling of the binding pocket by the IPTG monosaccharide as opposed to the natural allolactose disaccharide ligand. Filling of the resulting hydrophobic cavity with water would be strongly disfavored [18]. Thus pressure driven solvation of this cavity could promote extensive unfolding of the protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The regulatory domain of the Lac repressor is probed using high pressure NMR
- The dimeric state of the regulatory domain is predominant at 3 kbar
- Application of pressure results in several structural transitions
- A volume change of -115 mL mol^{-1} is associated with a major subglobal unfolding

Figure 1. Structural representation of dimeric LacI* RD from PDBID 2P9H with one unit of the dimer represented as a surface (blue), the other represented by a cartoon (orange), and IPTG is represented as sticks (magenta). The N-terminus is oriented upwards.

Figure 2.

Overlay of TROSY ^1H - ^{15}N HSQCs of 400 μM LacI* RD with 10 mM IPTG at ambient pressure (black), 500 (purple), 1000 (blue), 1500 (green), 2000 (yellow), 2500 (orange) and 3000 bar (red). Contour levels are matched. Experiments were collected at 750 MHz (^1H) and 25° C.

Figure 3.

Pressure sensitivity of the native state of LacI* RD. Fractional population was determined by measuring resonance peak intensity of the TROSY ^1H - ^{15}N HSQC crosspeaks at each pressure point and normalizing to the intensity of the resonance at 1 bar. The red line represents the average pressure response to this subset of resonances with error bars representing the standard deviation among the set of peaks.

Figure 4. Free energy of the transition away from the native state upon application of hydrostatic pressure. Red dots are calculated from the global averages of the experimental data from figure 3 according to equation 1. Black line was fitted according to the quadratic of the pressure response presented in equation 2. See text for fit parameter details.

Figure 5. Pressure induced structural reorganization of LacI* RD. Selected expansions of TROSY ^1H - ^{15}N HSQC spectra overlays through the pressure series illustrating the emergence of the alternate state. Color scheme of the spectra match that of Figure 2. These are representative of 33 crosspeaks that exhibited this behavior.