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High-pressure EPR spectroscopy studies of the *E. coli* lipopolysaccharide transport proteins LptA and LptC

Kathryn M Schultz and Candice S Klug*

National Biomedical EPR Center, Department of Biophysics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226 USA

Abstract

The use of pressure is an advantageous approach to the study of protein structure and dynamics because it can shift the equilibrium populations of protein conformations toward higher energy states that are not of sufficient population to be observable at atmospheric pressure. Recently, the Hubbell group at the University of California, Los Angeles, reintroduced the application of high pressure to the study of proteins by electron paramagnetic resonance (EPR) spectroscopy. This methodology is possible using X-band EPR spectroscopy due to advances in pressure intensifiers, sample cells, and resonators. In addition to the commercial availability of the pressure generation and sample cells by Pressure Biosciences Inc., a five-loop–four-gap resonator required for the initial high pressure EPR spectroscopy experiments by the Hubbell group, and those reported here, was designed by James S. Hyde and built and modified at the National Biomedical EPR Center. With these technological advances, we determined the effect of pressure on the essential periplasmic lipopolysaccharide (LPS) transport protein from *Escherichia coli*, LptA, and one of its binding partners, LptC. LptA unfolds from the N-terminus to the C-terminus, binding of LPS does not appreciably stabilize the protein under pressure, and monomeric LptA unfolds somewhat more readily than oligomeric LptA upon pressurization to 2 kbar. LptC exhibits a fold and relative lack of stability upon LPS binding similar to LptA, yet adopts an altered, likely monomeric, folded conformation under pressure with only its C-terminus unraveling. The pressure-induced changes likely correlate with functional changes associated with binding and transport of LPS.

1. Introduction

The application of high hydrostatic pressure to a purified protein is often a reversible approach to the study of protein structure and dynamics because it can shift the conformational equilibrium populations of proteins to adopt higher energy conformational states that are not of sufficient population to be observable at atmospheric pressure (e.g., [1, 2]). Proteins placed under pressure must take on a structure that is of the smallest partial molar volume. Pressure can shift the conformational equilibria toward higher energy states, including pressure unfolded conformational states. This is a concept that has been extensively utilized in the nuclear magnetic resonance (NMR) field but with limited use by electron paramagnetic resonance (EPR) spectroscopists. Recently, the application of high pressure to the study of proteins by EPR spectroscopy (e.g., [3]) has been reintroduced by

*Corresponding author information: candice@mcw.edu, Phone: 1-414-955-4015, ORCID: 0000-0003-4547-320X.

the Hubbell group at the University of California, Los Angeles [4–9]. This methodology is possible using X-band EPR spectroscopy due to a number of advances, including pressure intensifiers operating up to 6.2 kbar, ceramic sample cells withstanding up to 2 kbar pressure, and EPR resonators that can accommodate and enhance the sensitivity of samples contained in pressurizable sample cells. The recent commercial availability of the HUB440 and HUB880 pressure generators (named for Wayne Hubbell, UCLA) and sample cells by Pressure BioSciences Inc. (South Easton, MA), and the five-loop–four-gap resonator used to accommodate the large ceramic sample cell, designed by James S. Hyde and built and modified at the National Biomedical EPR Center (Medical College of Wisconsin, Milwaukee, WI) [10], enabled the initial high pressure continuous wave (CW) EPR spectroscopy experiments by the Hubbell group [5–8] as well as those reported here.

With the technology in place, we studied the effect of pressure on LptA, the essential periplasmic lipopolysaccharide (LPS) transport protein (Lpt) from *Escherichia coli*, and one of its binding partners, LptC, to investigate the functional characteristics of these proteins that enable LPS binding and release during transport across the periplasm. *E. coli*, and many other important pathogens classified as Gram-negative bacteria, have an inner membrane (IM) and an asymmetric outer membrane (OM) that protect the cytoplasm and act as highly selective permeability barriers [11]. The outer leaflet of the OM is mainly composed of LPS (endotoxin), a large lipid essential for the survival of nearly all Gram-negative bacteria and a major factor in the protection of bacteria from adverse environmental stresses such as toxins and the hostile environments found during infection or host colonization [11, 12]. The transport of LPS from inside the cell to the outer leaflet of the OM in *E. coli* occurs through the combined action of MsbA and LptFGB₂ in the IM, LptC and LptA in the periplasm, and LptDE in the OM [13–21]. Structures of all proteins involved in LPS transport have now been solved, yet many aspects of the functional dynamics and mechanism of action of these proteins are still unresolved. A major strength of the site-directed spin-labeling (SDSL) EPR spectroscopy technique is its ability to detect and follow changes in local structure due to intra- and intermolecular conformational changes or dynamic interactions with other proteins or substrates based on spin label mobility changes or distance changes between two spin labels. The spin label side chain (R1) is an excellent reporter of local packing, protein-ligand, and protein-protein interaction sites (e.g., [22–24]). With relatively short data collection times, small sample requirements, and no protein size restrictions, the EPR technique is ideally suited to take advantage of the benefits of high pressure to study Lpt protein folding and dynamics. The results presented here indicate that LptA unfolds from the N-terminus to the C-terminus, binding of LPS does not appreciably stabilize the protein under pressure, and monomeric LptA, as compared to oligomeric LptA, only somewhat more readily unfolds upon pressurization up to 2 kbar as detected by SDSL EPR spectroscopy. LptC unfolds at its C-terminal edge strand, yet the core and N-terminal edge of the protein adopt an alternate, likely monomeric, folded conformational state in the presence of pressure. These insights will aid in a better understanding of LPS transport across the periplasm of *E. coli*.

2. Materials and Methods

2.1. Protein expression, purification, and labeling

Unique cysteine mutations were introduced into the naturally cysteine-free soluble domain of LptC (amino acids 24–191) or mature LptA (amino acids 28–185) using High Fidelity PCR EcoDry Premix (Clontech, Mountain View, CA) and verified (Retrogen, San Diego, CA) as described previously [25, 26] or by gene synthesis (GenScript, Piscataway, NJ). The LptC protein was expressed from a pET28b (Novagen, EMD Millipore, Billerica, MA) vector with an N-terminal 6xHis tag in *E. coli* BL21(DE3) cells, and the LptA proteins were expressed from a pET21b vector with a C-terminal 6xHis tag in *E. coli* BL21(DE3)pLysS cells using cobalt affinity chromatography (Clontech, Mountain View, CA). Proteins were spin labeled at the unique cysteine residues using a 50-fold excess of the sulfhydryl-specific spin probe, 2,2,5,5-tetramethylpyrroline-3-yl-methanethiosulfonate spin label (Toronto Research Chemicals, New York, ON) to generate the R1 side chain as described [25, 26]. The purified, spin labeled proteins were concentrated using Microcon YM-10 centrifugal concentrators. Protein concentrations were determined using the Thermo Scientific Pierce BCA Protein Assay Kit (Rockford, IL) with lysozyme (14 kDa) as the protein standard. LPS from *E. coli* O111:B4 was purchased from List Biological Laboratories, Inc. (Campbell, CA) and solubilized to a concentration of 200 μ M in 50 mM NaPO₄, 300 mM NaCl, pH 7 buffer.

2.2. High pressure CW EPR spectroscopy

Pressure was generated using a Pressure Biosciences HUB440 pressure intensifier (Pressure Biosciences Inc., South Easton, MA) [4]. Samples were contained in a 3 mm OD $\times$ 0.78 mm ID yttrium-stabilized zirconia (ceramic) sample cell (Pressure Biosciences Inc.) inserted into a National Biomedical EPR Center (Milwaukee, WI) custom-built five-loop–four-gap silver plated MACOR resonator designed by James S. Hyde [10], built by Tadesz Oles and modified with a 2 mm brass rod introduced into an outer loop by Jason W. Sidabras to compensate for the resonant frequency shift induced by the ceramic cell. The X-band five-loop–four-gap resonator with the yttrium-stabilized zirconium sample cell has an eigenmode Q_0 -value (Q_0) of 700 and an efficiency parameter (Λ) of 2.7 G/W^{1/2} as calculated using Ansoft HFSS software. The dielectric constant of the cell was calculated to be 43 and the loss tangent to be 0.0055 using experimental bench data combined with Ansoft HFSS analysis and is consistent with the literature [27]. For comparison, this five-loop–four-gap resonator with a 2 mm OD $\times$ 1 mm ID TPX sample tube with a dielectric constant of 2.1 and a loss tangent of 0.0008 has a calculated Q_0 of 907 and a Λ of 2.2 G/W^{1/2}.

CW EPR spectroscopy was carried out on a Bruker E500 or a Varian E-102 Century Series X-band spectrometer. Spectra were background corrected by subtracting out the EPR signal produced by the ceramic cell and analyzed using software written in LabView by Christian Altenbach (UCLA). Quantitation was carried out through spectral subtraction [26, 28] of spectra recorded at each pressure. The composite spectra were deconvoluted to obtain each pressure-populated spectrum through manual spectral subtraction of the corresponding apo spectrum. The double integration values of the resulting spectra (which correspond to the number of spins in the pressure-induced population) were compared to the double

integration value of each composite spectrum (i.e., the number of total spins in each sample) to obtain the percent of pressure-populated proteins at each pressure.

Double electron electron resonance (DEER) spectroscopy, also referred to as pulsed electron electron double resonance (PELDOR) spectroscopy, was carried out at 80K on a Q-band Bruker E580 equipped with an overcoupled Bruker EN5107D2 resonator. Samples contained 20% deuterated glycerol as a cryoprotectant and were held in 1.1 mm × 1.6 mm quartz capillaries. The 2 kbar sample was pressurized using the HUB440 and a custom-built pressure bomb as described by Lerch et al. [4, 5] and flash frozen at 200K while under pressure, a methodology proven to retain the pressure-populated conformational states of the protein [5]. The dipolar evolution data were phased and 3D-background corrected using the LongDistances software program [29]. The distance distributions were determined by fitting the phased and 3D-background corrected dipolar evolution data using the model-free algorithms included in the analysis program.

3. Results

High hydrostatic pressure can shift the conformational equilibria of proteins to populate higher energy conformational states that are not of sufficient population to be observable at atmospheric pressure, and it is often a reversible phenomenon (e.g., [1, 4]). Also, EPR spectroscopy is ideally suited to compare protein unfolding and dynamics between low and high energy conformational states. Therefore, high pressure EPR spectroscopy was utilized to study various populations of the pressure-populated conformational states of LptA and LptC.

3.1. High pressure EPR spectroscopy on LptA

LptA is a soluble protein that forms concentration-dependent end-to-end oligomers [25] and transports LPS across the periplasm, presumably receiving LPS from LptC at the inner membrane, protecting the hydrophobic acyl chains through the periplasm and delivering LPS to LptDE at the outer membrane. The N-terminal β -strand of LptA forms one end of the protein oligomerization interface as well as the interface for its interaction with LptC [25, 26, 30, 31]. The N-terminal helix and edge strand also were shown previously to become disordered upon LPS binding [28]. At 100 μ M, LptA is an average of a 25-mer and is therefore highly oligomerized.

To determine the effect of pressure on LptA, high pressure EPR spectroscopy was first carried out on I36R1, which is located on the N-terminal β -strand (Figure 1a). Under atmospheric pressure at 100 μ M concentration, the mobility of I36R1 LptA is slow. Despite its edge location, which would generally result in relatively unrestricted motion, the motion is slow because it is located on the oligomeric interface of the protein (Figure 2a; [25, 28]); tertiary contacts with another LptA protomer slow down the motion of the spin label reporter group. Upon application of pressure in the absence of LPS, the N-terminal β -strand containing residue 36 appears to completely unfold by 2 kbar of pressure, represented by fast motion of R1 (Figure 2a). Fast motion of R1 is represented by sharp, narrow spectral lines and results from largely unrestricted motion of R1, indicative of an R1 attached to an unfolded or highly flexible region of the protein. The spectra were also recorded at several

pressures up to 2 kbar and deconvoluted to determine the amount of folded protein in the absence and presence of LPS (Figure 2b). This pressure-induced unfolding is reversible, with 88% of the protein returning to the folded state upon release of pressure. In the presence of equimolar LPS and upon application of pressure, R1 at position 36 follows a similar trend (Figure 2b, open circles), with a somewhat increased population of unfolded protein as pressure is increased compared to apo LptA. Interestingly, the spectra for the LPS-induced state in the absence of external pressure [28] and the apo 2 kbar-induced state for I36R1 overlay (Figure 2c), as both indicate R1 is highly mobile on this edge strand. The pressure data recorded in the presence of LPS show an increase in the highly mobile (unfolded) population of LptA I36R1, representing additional LPS-induced unfolding of this strand.

To discern whether the application of pressure results in an LPS-induced state of the entire protein or an unfolded state of LptA, two additional sites in the β -sheet domain of LptA were spin labeled and studied under pressure. S110R1 faces the outside of the protein and is located on the fold in β -strand 5, which is in the very center of the protein, and V165R1 is located on the C-terminal edge strand facing into the putative LPS binding pocket (Figure 1a). These positions on LptA do not unfold; rather, they become more immobilized in the presence of LPS under atmospheric pressure [28]. Upon application of hydrostatic pressure, large populations of S110R1 and V165R1 become highly mobile (Figure 3), suggesting that the entire protein unfolds in response to pressure. Quantitation of the spectral data indicates that 64% and 31% of the protein at positions S110R1 and V165R1, respectively, are unfolded under 2 kbar of pressure (Figure 4). Notably, LptA refolds upon return to atmospheric pressure. Additionally, the last residue of LptA, N185R1, is located in the unstructured tail region of the protein and remains unstructured upon application of pressure (Figures 3 and 4).

LptA forms concentration-dependent oligomers and thus is highly oligomerized at the concentrations examined using pressure EPR spectroscopy [25]. Therefore, we next tested the stability of the monomeric mutant, LptA I36R1/Q148A/K149A [28], under pressure. The largely monomeric protein shows a similar unfolding trend as oligomeric LptA, with a slightly shifted unfolding pattern (Figure 2b, open circles) indicating the monomer unfolds somewhat more readily than the oligomer under pressure. Additionally, LPS does not appreciably stabilize the monomeric protein during pressure-induced unfolding. Similarly, the presence of equimolar LPS does not affect the extent of pressure-induced unfolding of the core of the oligomeric protein, as evidenced by the mobility of S110R1 (Figures 4 and 5).

3.2. High pressure EPR spectroscopy on LptC

The soluble domain of LptC (amino acids 24–191) has a very similar fold to LptA (Figure 1b), and both proteins bind and release LPS to transport the lipid across the periplasm of Gram-negative bacteria; thus, it is expected that the two proteins may have other very similar structural and functional properties. However, LptC forms concentration-independent terminal dimers [20], which is in contrast to the concentration-dependent end-to-end high order oligomerization of LptA [25]. To study the pressure-induced changes in LptC, several

spin-labeled proteins were generated and examined by EPR spectroscopy, similar to the LptA studies described above.

Analogous to LptA, the C-terminal residue on LptC, P191R1, is located in an unstructured tail region and shows no changes in mobility upon application of up to 2 kbar of pressure (Figure 6). In addition, the spectra of A172R1, which is located on the C-terminal edge strand, indicate that 49% of this edge strand of LptC unravels in the presence of 2 kbar of pressure (Figures 4 and 6). This is also similar to the center and C-terminal edge strands of LptA that partially unfolded in the presence of pressure. Interestingly, T47R1, in the unstructured N-terminal tail region; Q65R1, located on the N-terminal edge strand; and S95R1, located on the exterior in a β -strand, each adopt a distinct, yet folded, conformation upon increase in pressure. The spectra of the center and N-terminal sites studied on LptC do not indicate that the protein is unfolding under pressure. Although the pressure-induced changes in structure are reversible according to the T47R1 and A172R1 reporters, Q65R1 and S95R1 data reveal irreversible changes in the core β -sheet structure and the N-terminal edge (Figure 4). LPS somewhat modulates the effects of pressure for T47R1 (Figures 5 and 6).

Given the irreversible motional changes associated with Q65R1 on the N-terminal dimer interface and the proximity of S95 to the N-terminus, coupled with the reversibility of the sites in the unstructured N-terminal tail and C-terminal edge strand that are not involved in the dimer interface, we hypothesize that LptC dimerization is affected by pressure. To examine this hypothesis, high pressure DEER spectroscopy was carried out using S95R1 to report on the extent of dimerization remaining following application of pressure. DEER spectroscopy is well suited to quantitate the number of interacting protomers within an oligomeric structure [32–34] and is used here to monitor differences in the number of interacting spins based on modulation depth differences. The experimental distance distribution is largely unchanged when comparing the 0 kbar and 2 kbar data for LptC S95R1 (Figure 7b), indicating that the average distance between intact dimers remains similar between the two samples; that is, the population of dimers remaining is not structurally affected by pressure. However, the modulation depths of the dipolar evolution data indicate that there is some loss of dimerization at 2 kbar of pressure (Figure 7a). The number of coupled spins can be linearly approximated to correspond to the modulation depth of the background-corrected dipolar evolution signal when the experimental conditions are similar between samples and the fraction of inverted spins is $\ll 1$ [32], as is the case for the data presented here. The modulation depth for the pressurized sample is 46% of that of the unpressurized sample, suggesting that approximately half as many spins are still interacting as an LptC dimer structure. In comparison, the CW data indicate that approximately 87% of the proteins are in a new conformational state upon application of 2 kbar pressure. In summary, a large population of the LptC protein as a whole appears to adopt a new conformation that is likely largely monomeric upon pressurization.

4. Discussion

Collectively, the data presented here indicate that pressure unfolds LptA from the N-terminus to the C-terminus and that LPS does not appreciably stabilize the protein in the

presence of pressure at the equimolar concentrations tested. It is possible that ratios of LPS:LptA greater than 1 would induce a more stabilizing effect. LptC unfolds at its C-terminal edge strand, yet the core and N-terminal edge of the soluble fraction of the protein adopt an alternate, likely monomeric, conformational state upon application of high hydrostatic pressure.

The N-terminus of LptA is completely unfolded by 2 kbar of pressure, whereas the center of the protein and the C-terminus are more stable and require more than 2 kbar of pressure to completely populate the unfolded state of the protein. At 1.5 kbar, the β -strand containing I36R1 begins to unfold, as indicated by the highly mobile nature of the spectrum. At 2 kbar, this edge strand is completely unfolded, likely due to the short N-terminal helix (residues 28–34; Figure 1a) swinging away from the protein and unraveling the adjacent β -strand containing I36R1. The fast motion of R1 corresponds to relatively unrestricted motion at I36, indicating this region of the protein is unstructured and unlikely to be able to form oligomers. Very fast spectral motion can indicate that the spin label has detached from the protein, which results in three very sharp, narrow spectral lines of equal intensity. The motion observed upon application of pressure or addition of LPS in these spectra is slower than the completely unrestricted motion of unattached spin label.

Surprisingly, the LptA I36R1 2 kbar spectrum overlays exactly with the 100-fold excess LPS-bound spectrum at atmospheric pressure (Figure 2c). These data suggest that this region of the protein may adopt a ligand-induced conformation upon application of pressure. It is also possible that the protein simply unfolds upon application of pressure. In either case, LPS and pressure populate the same conformational states for the N-terminal edge of LptA.

The mobility of S110R1 in the LPS-populated state at atmospheric pressure is distinct from the mobility of apo S110R1 in the pressure-populated state, and thus the pressure-induced state does not represent the LPS-induced state of the entire protein. LptA I36R1 and V165R1 have different, and much faster, motion under pressure than the motion observed in a monomeric protein (see [28]), so pressure is not solely shifting the transition from oligomer to monomer, it is also inducing additional motional changes. Plus, S110R1 becomes mobile upon the application of external pressure, but its motion is not significantly affected by concentration like 36 and 165 (see [28]), which are each located on an oligomeric contact site, because it is located in the center of LptA and not on an oligomeric interface.

Interestingly, the N-terminus and core of LptC adopt an alternate conformation upon application of pressure, with the changes unexpectedly irreversible. This is unlike the global unfolding observed for LptA. Rather, the LptC data suggest that the dimer may be disrupted by forming a conformationally distinct structure that is then unable to reform the dimer upon return to atmospheric pressure. This, too, may indicate a protein conformational change occurs during the binding or release of LPS in vivo, or that the protein dimer observed in solution is not representative of the oligomeric state in vivo. It is possible that LptC is monomeric in vivo with contacts at either the small jellyroll on the periplasmic tips of LptF or LptG [35] on its N-terminal edge and with LptA on its C-terminal edge [21, 26]. The distances between the LptC protomers within the dimer are most readily observed and

analyzed by DEER spectroscopy. While the spin-spin interaction observed in the CW spectra of LptC Q65R1 qualitatively indicate there may be some loss of the dimer up to 2 kbar of pressure, the largest change is in the motion of the R1 side chain; thus, quantitative evaluation of the DEER data for S95R1 was carried out. The distance distribution is largely unchanged when comparing the 0 kbar and 2 kbar data for LptC S95R1, yet the modulation depths of the dipolar evolution data indicate that approximately half of the dimers may be monomeric at 2 kbar of pressure.

The pressure studies uniquely indicate that LptA may unfold from the N-terminus toward the C-terminus, rather than from both ends simultaneously (Figure 4). And, LptA does not globally unfold in the presence of LPS; instead, the N-terminus appears to be the only region to unfold upon the binding of ligand [28]. This likely has mechanistic effects on the functional characteristics of LptA that enable LPS binding and release during transport across the periplasm. How LPS affects the global and directional unfolding of LptA and how these insights can aid in the understanding of LPS transport across an LptA bridge will be addressed in future studies.

The recent high pressure EPR studies were enabled by important technological developments. Opportunities for continuing advancement of the methodology still exist. For example, (1) upgraded resonators that recover the EPR signal intensity lost from the effect of a reduced filling factor due to the use of thick-walled sample cells necessary for pressure measurements, and (2) resonators that will accommodate very small diameter sample tubes that do not require thick walls to withstand high pressure without loss of signal intensity are under development to enable future studies by high pressure EPR spectroscopy.

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Fig. 1. Structures with studied positions highlighted for monomeric a) LptA (pdb: 2R1A) and b) LptC (pdb: 3MY2). The dashed lines represent terminal regions unresolved in the crystal structures.

Fig. 2.

Effect of pressure on EPR spectra of spin-labeled LptA. a) X-band EPR spectra recorded over 100 G for LptA I36R1 in the absence of LPS at the pressures indicated. As pressure increases, the β -strand containing I36R1 unfolds, as indicated by the increase in a fast motion component. The percent of unfolded protein is indicated. b) Plot of pressure vs. the percent of folded LptA I36R1 in the absence (closed symbols) and presence (open symbols) of LPS. Diamonds represent the percent of protein folded upon returning to atmospheric pressure after being subjected to high pressure. c) CW X-band EPR spectra of I36R1 LptA at 100 μ M under 2 kbar of pressure (black) and of the LPS-induced population at 2 μ M in the presence of 198 μ M LPS under atmospheric pressure (gray). Spectral width is 100 G.

Fig. 3. X-band EPR ceramic background-subtracted spectra recorded over 100 G for S110R1, V165R1, N185R1 LptA in the absence (black), and for S110R1 in the presence (gray), of LPS at the pressures indicated. Spectral width is 100 G.

Fig. 4.

Plots of pressure vs. the percent of folded protein for spin labeled LptA (left) and LptC (right). Closed symbols represent the percent of folded or new conformation as reported by sites on the N-terminal edge strand (black circles), the center (orange triangles), the C-terminal edge strand (aqua squares), and C-terminal tail residue (crosses) as a function of increasing pressure. Open symbols represent the percent of folded protein upon release of pressure. Quantitation was carried out through spectral subtraction of the spectra recorded at each pressure.

Fig. 5. Plot of pressure vs. the percent of folded LptA S110R1 and LptC T47R1 in the absence (closed symbols) and presence (open symbols) of LPS as quantitated by EPR spectroscopy. Diamonds represent the percent of protein folded upon returning to atmospheric pressure after being subjected to high pressure.

Fig. 6. X-band EPR ceramic background-corrected spectra recorded for LptC at the pressures and positions indicated. T47R1 spectra in the presence of equimolar LPS are shown in gray. Spectral width is 100 G for all spectra except the Q65R1 spectra, which are 200 G wide.

Fig. 7.

Q-band DEER spectroscopy data. a) Background-corrected dipolar evolutions (gray dots) and fits (solid lines) to the data. b) Distance distribution plot for LptC S95R1 in the absence (black) and presence (gray) of 2 kbar pressure. The y-axis represents the distance probability.