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Structural Insights into the *Yersinia pestis* Outer Membrane Protein Ail in Lipid Bilayers

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Abstract

Yersinia pestis the causative agent of plague, is highly pathogenic and poses very high risk to public health. The outer membrane protein Ail (Adhesion invasion locus) is one of the most highly expressed proteins on the cell surface of *Y. pestis*, and a major target for the development of medical countermeasures. Ail is essential for microbial virulence and is critical for promoting the survival of *Y. pestis* in serum. Structures of Ail have been determined by X-ray diffraction and solution NMR spectroscopy, but the protein's activity is influenced by the detergents in these samples, underscoring the importance of the surrounding environment for structure–activity studies. Here we describe the backbone structure of Ail, determined in lipid bilayer nanodiscs, using solution NMR spectroscopy. We also present solid-state NMR data obtained for Ail in membranes containing lipopolysaccharide (LPS), a major component of the bacterial outer membranes. The protein in lipid bilayers, adopts the same eight-stranded β -barrel fold observed in the crystalline and micellar states. The membrane composition, however, appears to have a marked effect on protein dynamics, with LPS enhancing conformational order and slowing down the ^{15}N transverse relaxation rate. The results provide information about the way in which an outer membrane protein inserts and functions in the bacterial membrane.

Graphical Abstract

antibiotic resistance, all contribute to the classification of *Y. pestis* as a category A Select Agent, and underscore the need to develop medical countermeasures.^{8–11}

The membrane protein Ail (Adhesion invasion locus) is an attractive target for drug development.^{12,13} At 37 °C, the conditions of human infection, Ail is the most abundant protein on the bacterial cell surface, constituting 30% of the *Y. pestis* outer membrane proteome.^{14–17} Ail interacts with human host factors to promote pathogenesis and has established roles in promoting serum resistance, host cell adhesion/invasion, and biofilm formation.^{14,18–30} Despite its importance, very little molecular data exist to describe its interactions with human host partners.

Structures of Ail have been determined by X-ray diffraction for crystalline Ail²⁶ and by solution NMR for Ail in decylphosphocholine (DePC) micelles.³¹ Ail folds as an eight-stranded β -barrel, with three short intracellular turns (T1–T3) and four long extracellular loops (EL1–EL4) that are significantly more flexible than the barrel body. The extracellular loops of Ail are important for function.^{28,32,33} Moreover, positively charged Arg and Lys side chains on the extracellular surface are proposed to form two heparin binding sites, on the basis of cocrystallization of Ail with the small molecule sucrose octasulfate (SOS).²⁶ Other outer membrane proteins, utilize similarly positioned Arg and Lys to bind lipopolysaccharide (LPS) at the membrane surface,³⁴ and this may also be important for Ail. To date, the structural basis for the interaction of Ail with any of its ligands is unknown and its interaction with LPS has not been examined.

The ligand binding activity of Ail is compromised in detergents.²⁸ This complicates functional assays based on the purified protein and underscores the need to perform structure/function studies in detergent-free membranes. Here we report the backbone structure of Ail determined in phospholipid bilayer nanodiscs by solution NMR spectroscopy, and present solid-state NMR spectra that reflect the effects of LPS on Ail dynamics in phospholipid liposomes. These detergent-free membrane samples enable structure and dynamics to be probed in environments that more closely resemble the native membranes where the protein is active.

MATERIALS AND METHODS

Protein Preparation

Ail was cloned and expressed in *Escherichia coli* BL21(DE3) cells and purified as described previously.²⁸ Uniformly ¹⁵N and ¹³C labeled (u-¹⁵N, u-¹³C) protein was obtained by growing bacteria in M9 minimal medium containing (99% ¹⁵NH₄)₂SO₄ and (99% ¹³C)-D-glucose (Cambridge Isotope Laboratories) as the sole sources of nitrogen and carbon. For ²H labeling, the growth medium was further supplemented with (99.99% ²H)-water and (98% ²H, 99% ¹³C)-D-glucose for uniform deuteration (u-²H). The ²H atoms at exchangeable sites were replaced with ¹H atoms during protein purification under denaturing conditions.

Nanodisc and Proteoliposome Preparation

Ail nanodiscs were prepared as described previously,²⁸ using the membrane scaffold protein MSP1D1 h5,³⁵ and the phospholipids dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG), at a molar ratio 2:1:75:25 of MSP1D1 h5/Ail/DMPC/DMPG. The phospholipids were both ²H labeled at all 54 acyl chain sites (Avanti). Briefly, Ail was refolded in 170 mM *n*-decylphosphocholine (DePC; Avanti) and then mixed with MSP1D1 h5 and lipids in 20 mM sodium cholate. Detergent was removed by dialysis and treatment with Bio-Beads SM-2 (Bio-Rad). The nanodiscs were transferred to buffer A (25 mM sodium phosphate, pH 6.5, 5 mM NaCl, 1 mM EDTA), supplemented with 10% ²H₂O, for solution NMR experiments. The NMR sample contained 0.6 mM (u-¹⁵N, u-¹³C, u-²H) Ail in a 500 μ L volume of the 5 mm NMR tube.

Ail liposomes were prepared as described previously,³⁶ by mixing a solution of refolded Ail and 170 mM DePC in buffer A, with a solution containing phospholipids and 100 mM Nacholate in buffer A. Detergent was removed by dialysis against three 4 L volume changes of buffer B (5 mM sodium phosphate at pH 6.5 and 1 mM EDTA) over the course of 24 h, followed by incubation with Bio-Beads SM-2 (Biorad) overnight. The samples contain ~3 mg of Ail and have a protein/lipid molar ratio of 1:100, as evidenced by measuring the ¹H NMR signal intensity at 1.2 ppm from the lipid acyl CH₂ groups.³⁶ Proteoliposomes were harvested by ultracentrifugation at 168000g, 4 °C, for 16 h, and then packed in a 4 mm MAS rotor with a 50 μ L insert (Bruker). Two samples were prepared, one containing Ail, DMPC, and DMPG at a molar ratio of 1:74:25, and another containing Ail, DMPC, DMPG, and di(3-deoxy-D-manno-octulosonyl)-lipid A (Kdo₂-Lipid A or KLA; Avanti) at a molar ratio of 1:68:23:8.

Differential Scanning Calorimetry (DSC)

DSC experiments were performed using an N-DSC II instrument (Calorimetry Sciences Corporation). Pure phospholipid and proteoliposomes samples were suspended in buffer B at concentrations between 0.5 and 3.0 mg/mL and scanned at a rate of 1 K/min, under a constant pressure of 3 atm. Buffer B served as the reference. The DSC data were analyzed with NanoAnalyze software (TA Instruments).

Solution NMR Experiments

Solution NMR spectra were recorded for (u-¹⁵N, u-¹³C, u-²H) labeled Ail in nanodiscs prepared with ²H labeled lipids, at 45 °C, on Bruker AVANCE 600 MHz and Bruker AVANCE III 800 MHz spectrometers, each equipped with a ¹H/¹⁵N/¹³C triple-resonance cryoprobe. TROSY-based³⁷ 2D HN and 3D HNCA experiments, and 3D ¹⁵N-resolved 1H-¹H NOESY experiments were performed with ²H decoupling. The NOESY data were recorded with 100 ms mixing time and 25% of the initial number of indirect hypercomplex points in a random nonuniform sampling (NUS) manner; the NUS spectrum was reconstituted using compressed sensing.³⁸ Chemical shift assignments were obtained by analysis of these data sets and aided by comparison with the assigned spectra of Ail in micelles.³¹ Rotational diffusion correlation times were measured 1H-¹⁵N TRACT experiments.³⁹ Assigned chemical shifts have been deposited in the Biological Magnetic Resonance data Bank (BMRB 30284).

Solid-State NMR Experiments

Solid-state NMR spectra were recorded for (u- ^{15}N , u- ^{13}C) labeled Ail in liposomes, at 10 °C, on a 500 MHz Bruker AVANCE spectrometer equipped with a Bruker 4 mm E-free MAS probe, operating at a spinning rate of 12 kHz $\pm$ 5 Hz. Typical $\pi/2$ pulse lengths for ^1H , ^{13}C , and ^{15}N were 2.5, 2.5, and 5 μs . SPINAL64,⁴⁰ implemented with a 90 kHz RF field strength, was used for ^1H decoupling during data acquisition. The bulk, ^{15}N transverse relaxation (T_2) was measured by appending a spin echo sequence (τ - π - τ) after 1D CP. 2D heteronuclear ^{13}C - ^{15}N correlation spectra were acquired using SPECIFIC-CP⁴¹ for band selective polarization transfer, during which continuous wave (CW) ^1H decoupling (100 kHz RF field strength) was applied, and a tangent ramp was applied on the ^{15}N channel with contact times of 3 ms for N-CA transfer. Resonance assignments were described in a previous study;³⁶ they were obtained with 2D and 3D ^{13}C - ^{15}N correlation spectra and assisted by direct comparison with the solution NMR data obtained for Ail in nanodiscs.

Structure Calculations

Structure calculations were performed with Xplor-NIH^{42,43} using the EEFx energy function for implicit membrane solvation,^{44,45} as described previously.⁴⁶ Values of $T = 25 \text{ \AA}$ and $n = 10$ were used for the EEFx term, to set the hydrophobic membrane thickness (T) and the rate of transition (n) of the electrostatic and solvation parameters from the hydrophobic membrane core to bulk water. These parameters reflect the profile of the DMPC and DMPG phospholipids used in the experimental sample, and mimic the relatively thin bacterial outer membrane. The program TALOS +⁴⁷ was used to derive backbone dihedral angles from the assigned chemical shifts. The Xplor-NIH statistical torsion angle potential, torsionDB,⁴⁸ was used to further restrain other dihedral angles. Long-range amide HN-HN distances were derived from ^1H - ^1H NOE measurements. Long-range amide O-HN hydrogen bonds were derived from a $^1\text{H}/^{15}\text{N}$ NMR spectrum acquired in $^2\text{H}_2\text{O}$ to detect amide hydrogens that resist exchange with water. A total of 100 structures were refined, starting from the coordinates of the 10 lowest energy structures calculated for Ail in micelles (PDB: 2N2L).³¹ The 10 refined structures with lowest energy were selected for the final ensemble. The structural coordinates and experimental restraints have been deposited in the Protein Data Bank (PDB: 5VJ8). Structure statistics are reported in Table 1. Structures were rendered with PyMol.⁴⁹

RESULTS AND DISCUSSION

Correlation Time of Ail Nanodiscs and Self-Association of Ail

In a previous study,²⁸ we showed that homogeneously sized Ail nanodiscs can be prepared for solution NMR. Structural studies, described in this work, required additional sample optimization to achieve the highest sensitivity and narrowest possible lines for resonance assignment and the measurement of NOE distance restraints. The combined use of small nanodiscs, extensive ^2H -labeling of protein and lipids, TROSY-based sequences at high magnetic fields, and nonuniform sampling (NUS) experiments has been effective for solution NMR structural studies of two other eight-stranded β -barrel membrane proteins, OmpX and OmpA.^{35,50} Following this approach, we prepared nanodiscs with the short MSP1D1 h5 scaffold protein and ^2H -labeled phospholipids; we labeled Ail with

(~99%) ^{13}C , ^{15}N , and ^2H , with backbone ^1H back-exchanged during protein refolding in water; and we performed TROSY-based experiments with NUS at 800 MHz magnetic field.

Sample dilution, obtained by adding buffer to Ail nanodiscs while maintaining a constant Ail to lipid molar ratio of 1:100, has a dramatic effect on the solution NMR signal intensity (Figure 1A). Diluting the sample by 50%, from an initial concentration of 1.60 mM to 0.80 mM (determined on the basis of Ail absorbance at 280 nm), results in a 40% increase in signal intensity, and further dilution to 0.66 mM increases the signal intensity by 60%. Further decreases in sample concentration, to 0.30, 0.15, and 0.10 mM Ail, lead to progressively lower signal intensity, as expected for dilution of the number of spins, indicating that 0.66 mM is the optimal concentration for solution NMR studies of Ail nanodiscs.

Size exclusion chromatography indicates that Ail nanodiscs elute as a single homogeneous fraction, with an apparent size similar to that of empty nanodiscs, and no evidence of significant aggregation.²⁸ To explore the reason for the observed dilution-dependent sensitivity enhancement, we measured the correlation times of the Ail nanodisc samples at different concentrations, using NMR TRACT experiments. The TRACT data (Figure 1B) show that the signal enhancement coincides with slower relaxation and a reduction in the apparent correlation time (τ_c) upon sample dilution, from a value of $\tau_c = 43.7$ ns at 1.60 mM, to $\tau_c = 35.4$ ns at 0.66 mM. Additional sample dilution to 0.30 mM has no effect on the measured correlation time.

The slower relaxation observed at and below 0.66 mM Ail reflects enhanced dynamics and faster overall tumbling at lower sample concentration. The data suggest that Ail nanodiscs are prone to reversible self-association, a process that is expected to increase solution viscosity and reduce overall tumbling at higher protein concentration. Self-association could be promoted by the charge distribution of the extracellular loops of Ail and is consistent with Ail's role in promoting the autoaggregation phenotype that is associated with pellicle formation and flocculent bacterial growth,³⁰ features that are connected with virulence in a number of pathogens, including certain *Y. pestis* strains.

The shorter correlation time observed at 0.66 mM Ail is close to the value ($\tau_c = 34$ ns) reported for OmpX,³⁵ in nanodiscs prepared with the same short MSP1D1 h5 scaffold protein, with the minor difference (~1 ns) possibly attributed to experimental uncertainty and to the dynamic properties of the extracellular loops in the two proteins. OmpX (148 residues) and Ail (156 residues) are similar in size and share significant sequence identity in the transmembrane β -barrel but differ in the sequences and lengths of their extracellular loops, which are shorter, more ordered, and carry relatively low net charge in OmpX, and longer, much more dynamic, and more charged in Ail.

Resonance Assignment of Ail Nanodiscs

The $^1\text{H}/^{15}\text{N}$ correlation TROSY spectra (Figure 2A) of Ail nanodiscs, acquired at 1.60 and 0.66 mM Ail, mirror the concentration-dependent signal intensity and correlation time data. The $^1\text{H}/^{15}\text{N}$ spectrum of 0.66 mM Ail contains a number of additional peaks, which are absent from the spectrum of 1.60 mM Ail. Resonance assignments indicate that these

include low intensity signals from residues at the intracellular and extracellular (e.g., D40, L140) water–membrane interfaces and in the extracellular loops (e.g., G60, G105).

At 0.66 mM Ail, the ^1H and ^{15}N line widths compare favorably with those reported for OmpX and OmpA nanodiscs.^{35,50} The narrowest and most intense lines (Figure 2B) are observed for β -barrel residues located in the hydrophobic core of the membrane, such as G76 (^1H = 38 Hz; ^{15}N = 40 Hz) and G89 (^1H = 37 Hz; ^{15}N = 36 Hz). By contrast, the lines from interfacial residues, such as D40 (^1H = 50 Hz; ^{15}N = 43 Hz) and L140 (^1H = 66 Hz; ^{15}N = 47 Hz), are significantly broader. Signals from G60 (^1H = 45 Hz; ^{15}N = 78 Hz) and G105 (^1H = 38 Hz; ^{15}N = 67 Hz), each located at the apices of extracellular loops EL2 and EL3, are significantly less intense and moderately broader than transmembrane peaks. These signals were assigned by comparison with the spectrum of Ail in detergent micelles. Their low intensity is likely due to both conformational exchange broadening as well as ^1H exchange with water at pH 6.5. A similar signal intensity profile was also observed in the NMR spectra of OmpA, in both nanodiscs and micelles, where residues at the membrane interface were “NMR-invisible”.⁵⁰

The NMR spectra of Ail in micelles²⁸ also exhibited line broadening, reduced intensity, and reduced heteronuclear ^1H – ^{15}N NOE intensities, for interfacial and extracellular loop residues. The line widths in micelles, however, are significantly narrower than nanodiscs, so that many more of these signals could be detected and assigned.

The optimized 0.66 mM sample allowed us to assign backbone resonances (HN, N, and CA) using three-dimensional HNCA and ^{15}N -resolved ^1H – ^1H NOESY experiments (Figure 2C,D). A total of 111 backbone resonances could be identified in the spectrum of Ail in nanodiscs, and 101 could be assigned. In addition, several long-range NOEs, between neighboring β -strands (Figure 2D,E), could be measured and assigned in the NOESY spectrum, and $^1\text{H}/^{15}\text{N}$ correlation spectra obtained in $^2\text{H}_2\text{O}$ allowed us to identify amide sites that form strong hydrogen bonds resistant to exchange with water (Figure 2E). Line broadening and low intensity remained limiting factors for NMR of Ail nanodiscs. Many signals from the extracellular loops could not be detected or assigned, and HNCACB data acquisition was not successful, likely because the fast transverse relaxation prevented coherence transfer. As a result, solution NMR structural analysis was limited to the Ail β -barrel backbone.

Conformation of Ail in Nanodiscs

The assigned chemical shifts provided a useful set of backbone dihedral angles, and the NOEs, together with hydrogen bonds, provided a good set of long-range distance restraints between neighboring β -strands, for structure calculation. The β -barrel of the resulting structure is well-defined by the NMR data (Table 1), with a backbone RMSD of 0.84 Å (residues: 5–14, 27–35, 42–54, 66–80, 86–97, 113–123, 130–140, 143–154) for the ten lowest energy models in the ensemble (Figure 3A). As expected, the lowest energy model is similar to the solution NMR structure in micelles (Figure 3B) and to the crystal structure, with respective backbone atom RMSDs of 1.67 and 1.89 Å for the β -barrel.

The extracellular loops are significantly disordered, due to sparse resonance assignments and the lack of restraints for this region of the protein. Loop disorder is also evident in the micelle and crystal structures, where it manifests as broadened resonance lines, reduced heteronuclear ^1H – ^{15}N NOE intensities, and sparse distance restraints²⁸ in one case and incomplete observation of electron density²⁶ in the other.

The implicit membrane model used in the calculations yields a membrane-embedded structure with the long barrel axis tilted $\sim 10^\circ$ relative to the lipid bilayer normal. The membrane-embedded position of the β -barrel agrees with the $^1\text{H}/^2\text{H}$ exchange data, placing the ring of positively charged Lys and Arg side chains at the extracellular membrane surface, and the two bands of aromatic residues near the membrane-water interfaces (Figure 3C).

Viewed in the context of the phospholipid membrane structure,^{51–55} where the bilayer thickness is defined as the distance between the regions of 50% water volume probability at $\pm D_B/2$, and the hydrocarbon region is defined by the acyl chains, with thickness $2D_C$ (Figure 3), the intracellular aromatic belt occupies the water–membrane interfacial region centered around $-D_C$, while the extracellular aromatic belt is embedded within the hydrocarbon region of the membrane just below D_C of the opposite interface. The basic Arg and Lys side chains occupy the extracellular interfacial region between D_C and $D_B/2$, where the membrane polarity transitions from 0% to 50% water. The negatively charged phosphate groups of the lipids are known to be very near $D_B/2$ and, thus, would be able to interact with the basic side chains of Ail.

Interestingly, the aromatic belts are not symmetrically disposed relative to the membrane hydrophobic center. While the intracellular aromatic belt aligns with the water–membrane interface, the extracellular belt is more deeply embedded in the membrane, and it is the basic Arg and Lys side chains that occupy the water–membrane interface. This feature has also been noted for other transmembrane β -barrels. For example, MD simulations⁵⁶ showed that the OmpA β -barrel interacts with the phospholipid headgroups in two regions: one narrow band, situated at the intracellular surface of the barrel, is composed primarily of aromatic residues, while a second band, at the extracellular surface, is significantly broader and includes both the extracellular aromatic belt and residues in the extracellular loops of the protein. This is very similar to the NMR conformation of Ail calculated in the implicit membrane model (Figure 3C). MD simulations of Ail in explicit lipid bilayers, and experiments aimed at measuring lipid–protein interactions, will be needed to confirm this result.

Effect of the Membrane Environment

Comparison of the NMR spectra of Ail in micelles and nanodiscs shows that the assigned resonances have very similar chemical shifts in the two environments. Combined N, HN, and CA chemical shift differences greater than 0.2 ppm are observed for only 16 residues located near the membrane–water interfaces, and particularly near the extracellular membrane surface (Figure 3D). The solution NMR structure of OmpX in nanodiscs revealed an influence of the membrane environment on protein dynamics,³⁵ and the NMR spectra from OmpA in nanodiscs⁵⁰ had highly reduced intensity for signals from residues near the

extracellular membrane surface, suggesting that this region of the β -barrel is sensitive to changes in membrane environment. Ail is similar to OmpX and OmpA in this regard.

Recent MD simulations^{57,58} indicate that the structure and dynamics of β -barrel outer membrane proteins are influenced by membrane composition. Notably, the presence of LPS, the major component of the extracellular leaflet of the outer membrane of Gram negative bacteria, was found to significantly reduce the flexibility of the extracellular loops of β -barrels, with loop rigidity increasing as a function of increasing number of sugars in the LPS extracellular core.

Ail is expressed by all three pathogenic *Yersinia*^{14,23,29} but is most significant in *Y. pestis*, partly because *Y. pestis* does not express the other adhesion/invasion factors YadA and Inv,^{19,21,59–62} but also because the rough LPS on the surface of *Y. pestis* is much shorter than the smooth O-antigen LPS on the surface of *Y. enterocolitica* and *Y. pseudotuberculosis* and does not mask the extracellular structure of Ail.^{22,63,64} Moreover, the serum resistance function of Ail appears to be linked to the LPS core structure^{65,66} and Ail deletion mutants of *Y. pestis* appear to be less resistant to cell wall stress, suggesting that Ail plays a structural role in strengthening the outer membrane. To date, the interactions of Ail with LPS have not been examined.

To probe the effect of LPS on the structure and dynamics of Ail, we reconstituted the protein in liposomes prepared either with phospholipids only (DMPC and DMPG) or with phospholipids plus 8% mole fraction of Kdo₂-Lipid A (KLA; Figure 4A), the lipid moiety of LPS, and we analyzed the samples by DSC and solid-state NMR spectroscopy.

DSC analysis shows that the KLA-containing, KLA(+), Ail lipid bilayers undergo a main endothermic phase transition (T_m) at 24 °C (Figure 4B, black), very close to the transitions observed³⁶ for KLA(–) Ail liposomes at 24.7 °C (Figure 4B, red), and for pure lipids at 24.2 °C (Figure 4B, blue). The DSC endotherms reflect the cooperative transformation from the lamellar gel phase ($L\beta'$), where the lipid acyl chains are tilted and all-trans, to the lamellar liquid crystalline phase ($L\alpha$), where the lipid chains are disordered and fluid.^{67–69}

Pure DMPC:DMPG lipids undergo an additional pretransition (T_{pre}) at 14.2 °C, from $L\beta'$ to a rippled gel phase ($P\beta'$), where the lipid chain tilt is reduced. The pretransition is not observed for either bilayers containing Ail or Ail plus KLA. This is consistent with attenuation of the enthalpy of the pretransition in mixed membranes, where the intermolecular interactions with the lipid headgroups and acyl chains are chemically diverse.

Addition of 8% KLA induces broadening and asymmetry in the DSC main transition endotherm, features that reflect a degree of departure from ideal mixing of the bilayer components. Quantitative thermodynamic studies will be needed to examine whether this broadening reflects a disruption, by KLA, of the lipid headgroup and acyl chain interactions that stabilize the gel phase of the phospholipids, or a phase separation of Ail with KLA, mediated by interactions of Ail side chains with LPS sugars at the extracellular membrane surface, as depicted in Figure 4A.

Interestingly, the ^{13}C solid-state NMR spectra of Ail indicate that inclusion of KLA in the liposome samples results in $\sim 28\%$ more efficient $1\text{H}-^{13}\text{C}$ CP, and $\sim 40\%$ more efficient $1\text{H}-^{15}\text{N}-^{13}\text{C}$ double CP (Figure 4C). Signal enhancement is also apparent in the 2D solid-state NMR NCA spectrum of KLA(+) Ail liposomes, where there are some additional peaks, in addition to some peak shifts, compared to the spectrum of the KLA(-) sample (Figure 4D).

We considered the possibility that signal enhancement in the presence of KLA might be simply due to the presence of more Ail, but a quantitative difference of 28% more protein in one sample seems unlikely in the absence of gross experimental error. Both KLA(-) and KLA(+) preparations were initiated with the same amount of Ail, verified by measuring UV absorbance at 280 nm. Both preparations proceeded smoothly, with no evidence of either precipitation or the presence of soluble Ail in the liposome supernatant. Moreover, the sample preparation has been optimized³⁶ to minimize lipid loss during reconstitution, as verified by ^1H NMR analysis of the lipids, and sample transfer from the centrifuge tube to the MAS rotor was quantitative in both cases.

It is more likely, therefore, that the observed increases in CP efficiency and signal intensity reflect a reduction in protein conformational dynamics and the presence of a stronger network of dipolar couplings in the KLA(+) sample. As the DSC data show that both KLA(-) and KLA(+) membranes undergo a gel to liquid crystalline phase transition at the same temperature, differential fluidity of the membranes, with greater rigidity and lower lateral diffusion in the KLA(+) samples, is not likely to be a dominant factor.

The DSC endotherms reflect the presence of hydrated lipid bilayers in all compositions examined in this study, it is possible, however, that the KLA(+) membranes retain less water. Though it is unlikely that this effect could account for 28% more protein in the sample, this raises the interesting possibility that the KLA sugar headgroup acts as an anhydro and cryo protectant, in a manner analogous to trehalose, which is thought to form a network of hydrogen bonds with protein sites, and appears to form a cage-like structure of water molecules with very slow dynamics, around proteins.^{70,71}

To further examine the effect of KLA on the NMR spectra of Ail, we analyzed the dynamic properties of the protein by measuring its apparent bulk ^{15}N transverse relaxation time (T'_2). The data (Figure 4E) show that the ^{15}N T'_2 of Ail increases significantly in the presence of KLA, from a value of 7.4 ms for KLA(-), to 13.5 ms for KLA(+) liposomes.

Taken together, these observations point to an effect of KLA on the slow conformational motions of Ail and suggest that slower relaxation in the transverse (R'_2) and, potentially, rotating ($R_{1\rho}$) frames may be responsible for the signal enhancement. Both R'_2 and $R_{1\rho}$ relaxation rates are sensitive to slow conformational motions in the millisecond time range, and site-resolved measurements of $R_{1\rho}$ can provide information about slow protein dynamics.⁷² The significantly slower transverse relaxation measured for the KLA(+) sample is consistent with a higher degree of conformational order in the presence of KLA. In addition to their dependence on slow conformational exchange, R'_2 and $R_{1\rho}$ relaxation processes also depend on coherent dipolar dephasing, and on the MAS rate. Thus, we expect

that further studies with deuterated samples and higher MAS rates will provide site-specific information about the stabilizing influence of LPS on Ail and membrane dynamics.

CONCLUSIONS

The backbone structure of Ail described in this study illustrates the effects of the lipid environment on protein dynamics. Although the eight-stranded β -barrel is maintained in the crystalline, micellar, and membrane-embedded forms of the protein, the conformations and dynamics of residues at the membrane–water interfaces and in the extracellular loops are sensitive to their environment, and to their distance from the lipid bilayer hydrophobic center.

These studies also illustrate the challenges associated with solution NMR structure determination of membrane proteins in lipid bilayer nanodiscs. The detergent-free membrane environment of the nanodisc is ideally suited for supporting the function, structure, and dynamics of membrane proteins, without complications from detergents. It is not, however, surprising that most studies have focused on β -barrel membrane proteins, where three-dimensional folds can be determined with relatively sparse backbone restraints, without the need to assign and measure side chain resonances. In the case of Ail, we find that the long correlation times of the Ailnanodiscs, and slow conformational exchange in the extracellular residues, limit solution NMR experiments, despite the use of small nanodiscs prepared with short molecular scaffold protein, protein and lipid deuteration, and high magnetic field. Nevertheless, the solution NMR data were sufficient for backbone structure determination of the membrane-embedded portion of the β -barrel.

The solid-state NMR structural data obtained for *Y. pestis* Ail in detergent-free lipid bilayers represent a step toward analysis of the conformation and dynamics of the protein as it might be present in the native bacterial outer membrane, which is enriched in LPS. The emerging data suggest that protein dynamics may be modulated by LPS. This is intriguing, especially in light of the functional importance of the extracellular loops of Ail and the fact that *Y. pestis* alters the molecular composition of its outer membrane depending on physiological conditions. *Y. pestis* produces rough-type LPS with a hexasaccharide carbohydrate core, and no O-antigen polysaccharide repeating units. The major form of LPS produced by *Y. pestis* at 20–28 °C has six acyl chains whereas the form produced at 37 °C has only four acyl chains.⁷³ KLA, therefore, resembles the lipid A component of the low temperature LPS form of *Y. pestis*. LPS is present on the bacterial outer membrane surface at much greater levels than the 8% used in this study and is distributed asymmetrically, in the outer, and not inner, leaflet of the outer membrane. Additional studies with Ail in membranes containing native concentrations of *Y. pestis* LPS will be needed to characterize the conformations and dynamics of the functional extracellular loops.

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Figure 1.

Effect of dilution on the NMR signal intensity and correlation time of Ail nanodiscs. (A) 1D ^1H spectra of Ail nanodiscs acquired at sample concentrations of 1.60 mM (red), 0.80 mM (green), 0.66 mM (black), 0.30 mM (blue), 0.15 mM (orange), and 0.10 mM (cyan). Sample dilution was obtained by adding buffer to Ail nanodiscs, while maintaining a constant Ail to lipid molar ratio. Values of concentration reflect Ail UV absorbance at 280 nm (B) T₂ relaxation curves measured with the ^1H - ^{15}N TRACT experiment for Ail nanodiscs at 1.60, 0.66, and 0.30 mM. Exponential fits of the data yield rates for the amide TROSY (R_α) and anti-TROSY (R_β) relaxation components and derivation of the correlation times, as described.³⁹ The apparent correlation times were $\tau_c = 43.7$ ns (1.60 mM), $\tau_c = 35.4$ ns (0.66 mM), and $\tau_c = 35.9$ ns (0.30 mM). Slower relaxation rates at 0.66 and 0.30 mM reflect faster overall tumbling and enhanced dynamics on dilution.

Figure 2.

Examples of NMR data and summary of NMR restraints measured for Ail in nanodiscs. (A) 2D $^1\text{H}/^{15}\text{N}$ TROSY spectra of ($^2\text{H}, ^{15}\text{N}$)-Ail in nanodiscs obtained at 1.60 mM (red) and 0.66 mM (black) sample concentrations. Examples of new peaks appearing at 0.66 mM Ail are circled in red. (B) 1D ^1H chemical shift slices taken from the 2D $^1\text{H}/^{15}\text{N}$ TROSY spectrum of 0.66 mM Ail. Slices are representative of signals from Ail residues in the intracellular membrane–water interface (D40), the transmembrane β -barrel (G76, G89), the extracellular membrane–water interface (L140), and the extracellular loops (G60, G105). The ^1H and ^{15}N line widths for these peaks are 50 and 43 Hz (D40); 38 and 40 Hz (G76); 37 and 36 Hz (G89); 66 and 47 Hz (L140); 45 and 78 Hz (G60); 38 and 67 Hz (G105). (C, D) 2D

sequential strips taken from 3D HNCA (A) and 3D ^{15}N -edited NOESY (B) spectra, showing assignments and HN–HN NOEs connecting strands $\beta 5$ and $\beta 6$. (E) Schematic showing NOEs (black lines) and hydrogen bonds (dashed blue lines) connecting the eight strands of the Ail β -barrel. Assigned N, HN, and CA sites are labeled black. Unassigned sites are labeled red. Boxes indicate residues with experimental chemical shifts and/or NOEs indicative of β -strand conformation. Yellow boxes indicate residues exposed to the β -barrel exterior. Horizontal lines depict the implicit lipid bilayer membrane with thickness $T = 25$ Å.

Figure 3.

Structure of Ail in nanodiscs. The implicit membrane is depicted as horizontal lines separated by the membrane hydrocarbon thickness ($T = 25 \text{ \AA}$), with $D_B = 36.3 \text{ \AA}$, and $2D_C = 25 \text{ \AA}$, as expected for DMPC and DMPG.⁵¹ (A) Ensemble of 10 lowest energy structures calculated for Ail in nanodiscs (PDB: 5VJ8). The ensemble is aligned to the lowest energy model. Arg and Lys side chains at the extracellular membrane surface are shown as yellow sticks. (B) Structure of Ail in nanodiscs (cyan) superimposed on the structure in micelles (orange; PDB: 2N2L). The lowest energy models of each ensemble are shown. (C) Lowest energy model in the Ail ensemble showing the bands of aromatic side chains near the membrane–water interfaces. (D) Lowest energy model in the Ail ensemble showing amide N atoms (blue spheres) with total chemical shift difference ($\Delta\delta$) > 0.2 ppm relative to the NMR data in micelles. (E) Chemical shift differences ($\Delta\delta$) between assigned backbone HN, N, and CA solution NMR signals of Ail in micelles versus nanodiscs. Values of $\Delta\delta$ were calculated by adding the changes in ^1H (ΔH), ^{15}N (ΔN), and ^{13}C (ΔC) chemical shifts as $\Delta\delta = [(\Delta H)^2 + (\Delta N/5)^2 + (\Delta C/3)^2]^{1/2}$, where the ^{15}N and ^{13}C chemical shifts are scaled by 1/5 and 1/3 to account for the 5-fold and 3-fold differences chemical shift dispersions relative to ^1H . The β -strands ($\beta 1$ – $\beta 8$), extracellular loops (EL1–EL4), and intracellular turns (T1–T3) are outlined above the graph.

Figure 4.

Effect of KDO2-lipid A (KLA) on solid-state NMR spectra of Ail in liposomes. (A) Structures of Ail and KLA positioned in a membrane (horizontal gray lines) with hydrocarbon thickness $T = 25 \text{ \AA}$ and bilayer thickness $D_B = 36.3 \text{ \AA}$. Basic Arg and Lys side chains of Ail (yellow sticks) are at the extracellular membrane surface. The KLA polar headgroup is colored by atom type: phosphorus (orange), nitrogen (blue), oxygen (red), polar hydrogen (white). The KLA acyl chains are shown as sticks. (B) DSC endotherms measured for a binary 75:25 molar mixture of DMPC:DMPG (blue), a ternary 1:74:25 molar mixture of Ail:DMPC:DMPG (red), and a quaternary 1:68:23:8 molar mixture of Ail:DMPC:DMPG:KLA (black). Note the endotherms are scaled to similar intensities for illustration. The main transition (T_m) from $L\beta'$ to $L\alpha$ and the pretransition (T_{pre}) from $L\beta'$ to $P\beta'$, are marked. (C) One-dimensional ^{13}C solid-state NMR spectra obtained for KLA(-) (red) or KLA(+) (black) liposomes, with ^1H - ^{13}C CP or ^1H - ^{15}N - ^{13}C double CP. The % signal enhancement is indicated in each case. (D) 2D solid-state NMR NCA spectra of Ail in

liposomes prepared without (red) or with (black) KLA. (E) Bulk ^{15}N T2 relaxation curves obtained by measuring ^{15}N signal intensity as a function of the relaxation delay τ in the spin echo sequence ($\tau-\pi-\tau$) applied after $^1\text{H}-^{15}\text{N}$ CP. The apparent T2 relaxation times were 7.4 ms in the absence of KLA (red) and 13.5 ms with KLA (black).

Table 1Structure and NMR Restraints Statistics^a

no. of experiment restraints	
dihedral angles	204
NOE distances ($i - j > 5$)	37
H-bonds	67
RMSD agreement with experimental restraints	
dihedral angle (deg)	0.686 ± 0.081
NOE (Å)	0.002 ± 0.003
H-bond (Å)	0.026 ± 0.007
RMSD agreement with ideal covalent geometry	
bonds (Å)	0.005 ± 0.000
angles (deg)	0.638 ± 0.010
impropers (deg)	0.320 ± 0.015
coordinate precision as average RMSD from mean (Å) ^b	
backbone CA, C, N atoms	0.839
all heavy atoms	1.591
MolProbity Statistics	
Ramachandran favored	93.77%
Ramachandran outliers	1.24%
favored side chain rotamers	95.83%
poor side chain rotamers	2.28%
clashscore	1.94
MolProbity score	1.59

^a Calculated for 10 structures, out of 100 refined structures, selected for lowest total energy.

^b Calculated for the β -barrel (residues: 5–14, 27–35, 42–54, 66–80, 86–97, 113–123, 130–140, 143–154).