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Influence of the lipid membrane environment on structure and activity of the outer membrane protein Ail from *Yersinia pestis*

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ABSTRACT

The surrounding environment has significant consequences for the structural and functional properties of membrane proteins. While native structure and function can be reconstituted in lipid bilayer membranes, the detergents used for protein solubilization are not always compatible with biological activity and, hence, not always appropriate for direct detection of ligand binding by NMR spectroscopy. Here we describe how the sample environment affects the activity of the outer membrane protein Ail (attachment invasion locus) from *Yersinia pestis*. Although Ail adopts the correct β-barrel fold in micelles, the high detergent concentrations required for NMR structural studies are not compatible with the ligand binding functionality of the protein. We also describe preparations of Ail embedded in phospholipid bilayer nanodiscs, optimized for NMR studies and ligand binding activity assays. Ail in nanodiscs is capable of binding its human ligand fibronectin and also yields high quality NMR spectra that reflect the proper fold. Binding activity assays, developed to be performed directly with the NMR samples, show that ligand binding involves the extracellular loops of Ail. The data show that even when detergent micelles support the protein fold, detergents can interfere with activity in subtle ways.

1. Introduction

The biological functions and molecular structures of proteins are highly dependent on the physical and chemical properties of the surrounding environment [1]. Just as water is essential for supporting the native states of soluble proteins, the lipid bilayer is critical for preserving the functional and structural integrity of membrane proteins. By contrast, the detergents used to solubilize membrane proteins for structural studies by NMR and crystallography can interfere with biological activity in multiple ways [2].

Among the methods for three-dimensional molecular structure determination, the principal advantage of NMR spectroscopy is its ability to examine proteins in samples that are very close to their native environments. This enables structure and biological function to be characterized in the same sample and useful structure–activity correlations to be established by direct spectroscopic detection of ligand binding or conformational changes [3]. Various NMR experimental approaches and sample types have been developed for membrane protein structural studies in detergent-free lipid samples. Solid-state NMR methods can be used for proteins in a variety of lipid bilayer assemblies, including planar supported lipid bilayers, lipid bilayer macrodiscs and liposomes [4–10]. More recently, lipid bilayer nanodiscs [11,12] have been used effectively for solution NMR studies of membrane proteins [13–24].

Here we describe the influence of the sample environment on the activity of the outer membrane protein Ail (attachment invasion locus) from *Yersinia pestis*, an extremely pathogenic organism with a long history of precipitating massive human pandemics [25–27]. We show that Ail in detergent micelles adopts an eight-stranded β-barrel conformation, with three intracellular loops (IL1–3) and four extracellular loops (EL1–4), similar to that of its crystal structure [28]. However, we find that the micellar detergent concentrations required for high resolution NMR spectroscopy are not compatible with functional assays of ligand binding. By contrast, optimized preparations of Ail in phospholipid bilayer nanodiscs support both function and structure, and can be used for parallel activity and NMR studies on exactly the same samples.

The pathogenicity of *Y. pestis* is associated with its exceptional abilities to proliferate in diverse environments and overcome the defenses of the human host. *Y. pestis* Ail is an essential factor contributing to these properties by mediating cell adhesion [29,30], promoting bacterial cell auto-aggregation [29] and conferring serum resistance [29,31]. Ail-mediated cell adhesion is essential for delivering the *Yersinia* outer protein (Yop) effectors that protect the bacterial cell from phagocytosis and

Abbreviations: DDM, n-dodecyl-β-D-maltopyranoside; DePC, n-decyl-phosphocholine; DHPC, 1,2-dihexyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphatidylglycerol; DPC, n-dodecyl-phosphocholine; ELISA, enzyme linked immunosorbent assay; IPTG, isopropyl 1-thio-β-D-galactopyranoside; LDAO, n-dodecyl-N,N-dimethylamine-N-oxide; LPPG, 16,0-lysophosphatidylglycerol; MSP, membrane scaffold protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate

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interfere with the host's inflammatory response, enabling *Y. pestis* to survive and multiply extracellularly [30,32]. The interactions of *Y. pestis* Ail with the extracellular matrix proteins fibronectin and laminin have been shown to be important for both cell adhesion and Yop delivery [28,33,34], and amino acid residues in Ail's extracellular loops have been shown to play important roles in the adhesion of *Y. enterocolitica* and *Y. pestis* [35,36] as well as in the invasion and serum resistance of *Y. enterocolitica* [35]. The ability to perform parallel NMR and functional activity assays on samples of Ail in lipid bilayers, free of interference from detergent molecules, paves the way for structure–activity NMR studies and the development of Ail-targeted molecular intervention.

2. Materials and methods

2.1. Expression and purification of Ail

Wild-type Ail and C-terminal His-tagged Ail (Ail–His) were prepared by cloning the gene corresponding to mature *ail* from *Y. pestis* KIM 10 (gene *y1324* without signal sequence) in the *Escherichia coli* pET-30b plasmid vector (EMD). For wild-type Ail, the gene was cloned between the *Nde*I and *Xho*I restriction sites of the plasmid. For His-tagged Ail, the gene was cloned between *Nde*I and *Kpn*I sites, to express Ail plus the His tag encoded after the *Kpn*I site of pET-30b. The expressed amino acid sequences of Ail and Ail–His begin with an extra N-terminal methionine before residue Glu1 of the native sequence (Fig. S1). The sequence of Ail terminates with the native residue Phe156, while Ail–His includes 33 additional C-terminal residues from the His tag of the pET-30b vector. The longer linker sequence preceding the His tag was essential for folding Ail–His. Protein folding was abolished when short His tags were directly appended to the C-terminus of Ail.

The *ail*-encoded plasmids were transformed in *E. coli* BL21 (DE3) cells and positive clones were grown at 37 °C, in minimal M9 medium [37], supplemented with 1 mM thiamine, and 35 μg/mL kanamycin to maintain plasmid selectivity. Cells were grown to a cell density of $OD_{600} = 0.6$, before induction with 1 mM IPTG (isopropyl 1-thio-β-D-galactopyranoside) for 3 h, then harvested by centrifugation (7200 ×*g*, 4 °C, 15 min), and stored at −80 °C overnight. For ^{15}N, ^{13}C and ^{2}H labeling of Ail, bacteria were grown in M9 medium prepared in 99% D_2O and containing 1 g/L of U-99%$^{15}NH_4Cl$ plus 2 g/L of U-99%^{13}C-glucose as the sole sources of N and C. All isotopes were from Cambridge Isotope Laboratories. Bacteria were adapted to culture in $^{2}H_2O$ by adding 1 mL of $^{2}H_2O$ M9 media to a 1 mL H_2O M9 starter culture every 2 h, until the volume reached 5 mL, and then growing overnight. After transferring this overnight culture into 20 mL of fresh $^{2}H_2O$ M9 media, growth was continued for 4 h at 37 °C to a cell density of $OD_{600} = 1.0$, then the entire volume was placed into 475 mL of fresh $^{2}H_2O$ M9 and cell growth and induction were carried out as described above.

Cells from 1 L of culture were suspended in 30 mL of buffer A (20 mM Tris–Cl, pH 8.0) and lysed by two passes through a French Press. After removing the soluble cell fraction by centrifugation (48,000 ×*g*, 4 °C, 30 min) the insoluble pellet, enriched in inclusion bodies, was suspended in 30 mL of buffer A, supplemented with 2% Triton-X, for 1 h, at 37 °C. The soluble fraction was removed by a second centrifugation step (48,000 ×*g*, 4 °C, 30 min) and the remaining pellet was first washed by suspension and centrifugation in 30 mL of water to remove residual detergent, and then dissolved in 30 mL of buffer B (20 mM Na-acetate, pH 5.0, 8 M urea) for Ail, or buffer A for Ail–His. Any insoluble material remaining after incubation at 37 °C for 1 h, was removed by centrifugation (48,000 ×*g*, 4 °C, 30 min). Ail and Ail–His were purified by cation exchange chromatography (HiTrap SP/HP 5 mL column, GE Healthcare) in buffer B with a NaCl gradient (Ail), or Ni affinity chromatography (HisTrap FF 5 mL column, GE Healthcare) in buffer A plus 8 M urea and 500 mM NaCl (Ail–His). Both Ail and Ail–His were further purified by size exclusion chromatography (Sephacryl S-200 HR HiPrep 16/60 column, GE Healthcare) in buffer B supplemented with 150 mM NaCl. Purified Ail was precipitated by dialysis (10 kDa molecular weight cutoff) against water, lyophilized, and stored at −20 °C.

2.2. Expression and purification of membrane scaffold protein

Two variants of membrane scaffold protein (MSP) were expressed and purified as described previously: MSP1D1 [12] and MSP1D1Δh5 [18] lacking the fifth helical segment of MSP1D1. The C-terminal His tags were removed by proteolysis with tobacco etch virus and the MSPs purified by Ni-affinity chromatography. The pET-28a-MSP1D1 plasmid developed by Sligar and coworkers [38] was obtained from Addgene (Addgene plasmid 20061). A nucleotide encoding MSP1D1Δh5 was obtained from GenScript and cloned into the NcoI and HindIII restriction sites of pET-28a (EMD) by restriction and digestion with Gibson Assembly Master Mix (New England Biolabs).

2.3. SDS PAGE and Western blot analysis

Proteins were analyzed by 4–12% Bis–Tris SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis (PAGE) and visualized by staining with Coomassie brilliant blue, or transferred to nitrocellulose for Western immuno-blotting and visualized using antibody-conjugated alkaline phosphatase (Biorad) with 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt substrate and nitro-blue tetrazolium chloride developer. Western dot blots were performed on nitrocellulose in a similar manner, without prior separation on SDS-PAGE. Ail–His was probed with mouse anti-His monoclonal antibody (Qiagen; 1/5000 dilution). MSP was probed with goat anti-ApoA1 polyclonal antibody (Millipore; 1/1000 dilution). Ail was probed with rabbit anti-Ail-EL2 antibody (1/1000 dilution) specific for the second extracellular loop (EL2) of Ail. Anti-Ail-EL2 antibody was raised in rabbits against Keyhole limpet hemocyanin (KLH)-peptide (NH_2-CTRRGFEESVDGFKLIDGDF-COOH) conjugates, and purified via peptide affinity chromatography (Proteintech, Chicago, IL).

2.4. Protein refolding in detergent

Purified, lyophilized protein (1 mg of Ail or Ail–His) was dissolved in 100 μL of 6 M urea and added dropwise to 600 μL of refolding buffer (20 mM glycine, pH 10.2, 5 mM EDTA, 600 mM arginine, 300 mM KCl) containing 47 mM n-decyl-phosphocholine (DePC; Anatrace). The solution was gently stirred overnight at room temperature to achieve complete refolding, and then concentrated to 50 μL using a Vivaspin 500 centrifugal concentrator with 10 kDa cutoff (VivaProducts). The buffer was exchanged by consecutive addition of 150 μL of buffer C (5 mM Na-PO_4, pH 6.8, 5 mM NaCl, 19 mM DePC) and concentration to 50 μL, repeated four times to obtain a solution containing 1 mM Ail and 130–180 mM DePC.

The protein concentration was determined by measuring the UV absorbance at 280 nm, with a molar extinction coefficient of 28,880 M^{-1} cm^{-1}, whose value was confirmed by an independent Bradford assay. The DePC concentration was estimated by monitoring the intensity of the ^{1}H NMR peak from the trimethylamino protons at 3.15 ppm.

Protein folding was assessed by monitoring the shift in SDS-PAGE apparent molecular weight that correlates with the transition from unfolded to folded states of transmembrane β-barrels [39], and by solution NMR spectroscopy, where folded Ail yields high quality $^{1}H/^{15}N$ correlation spectra with ^{1}H chemical shifts dispersed over more than 3 ppm, and unfolded Ail yields spectra with all ^{1}H resonances collapsed within 0.5 ppm [40], as expected for random coil.