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Detergent isolation stabilizes and activates the *Shigella* type III secretion system translocator protein IpaC

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Abstract

Shigella rely on a type III secretion system (T3SS) as the primary virulence factor for invasion and colonization of human hosts. While there are an estimated 90 million *Shigella* infections, annually responsible for more than 100,000 deaths worldwide, challenges isolating and stabilizing many T3SS proteins have prevented a full understanding of the *Shigella* invasion mechanism and additionally slowed progress toward a much needed *Shigella* vaccine. Here, we show that the non-denaturing zwitterionic detergent LDAO and non-ionic detergent OPOE efficiently isolated the hydrophobic *Shigella* translocator protein IpaC from the co-purified IpaC/IpgC chaperone-bound complex. Both detergents resulted in monomeric IpaC that exhibits strong membrane binding and lysis characteristics while the chaperone-bound complex does not, suggesting that the stabilizing detergents provide a means of following IpaC “activation” *in vitro*. Additionally, biophysical characterization found that LDAO provides significant thermal and temporal stability to IpaC, protecting it for several days at room temperature and brief exposure to temperatures reaching 90°C. In summary, this work identified and characterized conditions that provide stable, membrane active IpaC, providing insight into key interactions with membranes and laying a strong foundation for future vaccine formulation studies taking advantage of the native immunogenicity of IpaC and the stability provided by LDAO.

Keywords

circular dichroism; physical stability; physical characterization; light scattering (dynamic); liposomes

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CONFLICT OF INTEREST

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INTRODUCTION

Shigella are Gram-negative facultative anaerobic bacteria that cause a severe form of bacillary dysentery (shigellosis) in humans.¹ Infections occur throughout the world with an estimated 90 million annual cases leading to greater than 100,000 deaths each year.² *Shigella* exposure generally occurs through the fecal oral route and/or contaminated drinking water with consumption of only 10–100 organisms leading to infection.³ As a result, most reported cases of shigellosis occur in developing regions where sanitation and access to clean drinking water are limited. There is currently no Federal Drug Administration (FDA) approved vaccine available for prevention of shigellosis, which likely contributes to the alarming increase in the number of infections reported in industrialized regions and the appearance of antibiotic-resistant strains throughout the world.⁴ These recent developments underscore the need to better understand the mechanism *Shigella* uses to infect human hosts and to formulate efficient countermeasures against it.

Following ingestion, acid-tolerant *Shigella* pass through the stomach and the small intestine to enter the colon where they rely on a complex type three secretion system (T3SS) to invade the cells of the colonic epithelium,^{5,6} evade host immune defenses,^{7,8} and spread throughout the infected tissues.^{1,9,10} The T3SS serves as the primary virulence factor for *Shigella* and encodes a hollow needle-like apparatus that provides a unidirectional conduit through which effector proteins are directly transported from the bacterial cytoplasm into the host cell.^{11,12} The type three secretion apparatus (T3SA) is highly conserved among related pathogens and resembles a nano-needle and syringe consisting of a cytoplasmic bulb, basal body that spans the inner and outer membranes of the bacterium, a hollow needle that extends beyond the thick lipopolysaccharide (LPS) layer, and an associated tip complex that interacts with the host cell membrane.^{11–14} The hydrophobic *Shigella* translocator proteins IpaB and IpaC are differentially recruited to the tip of the maturing apparatus by exposure to the bile salt deoxycholate (DOC)^{15,16} and specific membrane lipids,¹⁷ respectively. The orchestrated recruitment of IpaB and IpaC to the tip of the T3SA is essential to the formation of the translocon complex that penetrates the host cell membrane¹¹ and phenotype studies clearly demonstrate that both IpaB and IpaC are essential for the ability of *Shigella* to invade host cells.^{17–20} Despite their clear importance in supporting virulence, defining the roles of the *Shigella* translocators and the mechanisms that drive their observed membrane interactions have been challenging due to their hydrophobic nature and difficulties associated with producing recombinant protein.

We have previously used a detergent-based purification method to directly assess membrane interaction and disruption capabilities of identified IpaB oligomers, finding that the pre-formation of stable IpaB homotetramers was necessary for phospholipid membrane disruption but not for interaction.²¹ In this study, we expand on these findings to develop detergent-based purification strategies for IpaC using the non-ionic detergent *n*-octyl-oligo-oxyethylene (OPOE) and the zwitterionic detergent *N,N*-dimethyldodecylamine *N*-oxide (LDAO) to separate IpaC from the stable complex it forms with its cognate chaperone IpgC. Biophysical characterization of purified IpaC found that the detergent-based purification method significantly increases protein yield over traditional refolding techniques and provides improved protein stability even compared to the physiological IpaC/IpgC complex.

Additionally, chemical crosslinking analysis identified the recombinant IpaC as monomeric under the tested detergent conditions while liposome flotation and liposome dye release assays showed that IpaC maintains its ability to interact with and disrupt phospholipid membranes, respectively. The development and assessment of detergent-based purification strategies for IpaC together with the “membrane activity” assays described here help to answer key questions concerning membrane recognition and disruption by IpaC and lay a strong foundation for additional studies designed to uncover mechanistic details of host cell recognition and *Shigella* translocon formation.

In addition to better understanding the specific roles and modes of action of T3SS components such as IpaC, there is a desperate need for the development of effective vaccines against *Shigella*. While there is currently no FDA approved *Shigella* vaccine available, several groups have made great progress in developing and testing *Shigella* vaccine candidates with many focusing on the highly immunogenic T3SS tip complex proteins.^{22–27} The Picking lab at the University of Kansas has recently characterized both IpaD and IpaB as pan-*Shigella* vaccine candidates finding that each provided significant protection when tested using a murine pulmonary infection model,²³ leading to the development of a novel IpaB-IpaD fusion complex that resulted in similar levels of protection and enhanced cytokine responses.^{22,28} While the tip proteins IpaD and IpaB are proving to be valuable vaccine components, pioneering *Shigella* immunogenicity studies showed that IpaC generates a strong immunogenic response during *Shigella* infections,⁸ driving the development of promising multicomponent *Shigella* vaccines containing IpaC^{26,27,29} as well as preliminary studies using urea-isolated IpaC.²⁵ Despite these advances, however, challenges obtaining and stabilizing recombinant IpaC have thus far prevented necessary vaccine formulation and full characterization of its efficacy. Our results in this study show that detergent-based purification of IpaC overcomes these challenges as low concentrations of the GRAS (generally regarded as safe) detergent LDAO provide significant thermal and temporal stabilization of IpaC when compared to traditional urea denaturation methods and even the native co-purified IpaC/IpgC complex, while maintaining what appears to be proper IpaC fold and function. These findings provide a strong foundation for IpaC-based *Shigella* vaccine formulation studies that will take advantage of the favorable effects of LDAO on IpaC as well as support studies testing the mechanistic role(s) of IpaC as a part of the *Shigella* T3SA and translocon complex.

MATERIALS AND METHODS

n-octyl-oligo-oxyethylene (OPOE) was purchased from Enzo Life Sciences (Farmingdale, NY). lauryldimethylamine N-oxide (LDAO), sulforhodamine B (SRB), dithiobis (succinimidyl propionate) (DSP) and asolectin were from Sigma Chemical (ST. Louis, MO). Urea and Triton X-100 were from Amresco (Solon, OH). *Escherichia coli* strains, protein expression plasmids, and Clonables 2X Ligation Premix were from Novagen (Madison, WI). Ampicillin, chloramphenicol, and IPTG were from Gold biotechnology, Inc. (Olivette, MO). Oriole UV-Fluorescent stain was obtained from Bio-Rad (Philadelphia, PA). Nickel chelating resin was from G biosciences (St. Louis, MO). All other solutions and chemicals were of reagent grade.

Expression and purification of IpaC

IpaC was cloned into pET15b and *ipgC* into pACYC prior to co-transformation into Tuner (DE3) *E. coli* cells. The cells were grown at 37°C in Terrific Broth to an OD₆₀₀ of 1.0 in the presence of 1 mg/mL ampicillin and 0.34 mg/mL chloramphenicol to ensure maintenance of the pET15b and pACYC plasmids, respectively. Protein expression was then induced with 1 mM IPTG for 18 hours at 17°C, resulting in complex formation between tagless IpgC and IpaC containing an N-terminal 6X Histidine tag. The soluble IpaC/IpgC complex was purified from the supernatant of the expression cells using Ni²⁺ chelation chromatography as described previously.³⁰ IpaC/IpgC was further purified using a Hiload 16/600 Superdex 200 GE size exclusion column to remove contaminants and buffer exchange the complex into phosphate buffered saline (PBS). The IpaC/IpgC complex was either maintained at 4°C and used within one week or the chaperone IpgC removed by re-binding the purified complex to a Ni²⁺ chelating column followed by washing with binding buffer containing 6 M urea, 1.0% OPOE, or 0.1% LDAO. During the wash steps, the histidine tagged IpaC remains bound while the tagless chaperone is cleared from the column. The isolated IpaC is eluted with buffer containing 400 mM imidazole and 6 M urea, 0.5% OPOE, or 0.05% LDAO to maintain stability. The isolated IpaC was dialyzed into PBS containing 2 M urea, 0.5% OPOE or 0.05% LDAO and evaluated by SDS-PAGE prior to use in subsequent experiments.

Characterization of IpaC by size exclusion chromatography

Isolated IpaC purified in the presence of urea, OPOE, or LDAO was analyzed on a Superdex 200 10/300 GE column connected to an AKTA purifier equipped with UV detection. Elution profiles and retention times of each condition were recorded and compared to one another as well as to those of protein calibration standards.

Chemical crosslinking

The oligomeric state of IpaC in OPOE and LDAO was investigated using chemical crosslinking. Protein concentrations of IpaC in PBS containing either 0.5% OPOE or 0.05% LDAO were adjusted to 10 µM and the protein covalently crosslinked using 0.25 mM DSP (dithiobis (succinimidyl propionate)). DSP was first prepared as a 10 mg/mL stock solution in DMF (dimethylformamide) and was made fresh for each reaction. The DSP-protein solution incubated for 45 min at room temperature to allow covalent crosslinking of available primary amines on the protein. Crosslinking was terminated by boiling the sample in SDS sample buffer containing 210 mM TRIS. Crosslinked samples were separated on 12% polyacrylamide SDS-PAGE gels and visualized using Oriole UV fluorescent protein stain. DSP includes an internal disulfide linkage that is cleaved in the presence of a reducing agent. The addition of 50 mM dithiothreitol (DTT) prior to boiling and analysis by SDS-PAGE cleaved the DSP and served as a control ensuring that DSP crosslinking was responsible for the shifts in migration patterns observed by SDS-PAGE. Chemical crosslinking could not be performed in the presence of urea as urea itself contains two primary amines.

Far-UV Circular Dichroism

Far-UV Circular Dichroism (CD) spectra and thermal stability profiles were obtained for IpaC in the tested purification conditions using a JASCO model J-1500 spectropolarimeter equipped with a 6 sample turret and a Peltier temperature controller (Jasco, Easton, MD). Spectral scans were obtained from 200 nm to 260 nm at 10°C, 0.5 nm spectral resolution, 50 nm/min scan rate, and a 1 sec data integration time in quartz cuvettes with 0.1 cm path length. Thermal stability profiles were generated by monitoring CD signal at 208 nm while the temperature was increased from 10°C to 90°C at 0.3°C/min. All measurements were at an IpaC concentration of 0.5 mg/mL with appropriate detergent concentrations as outlined for each experiment. To mimic experimental conditions, CD data for IpaC in urea was obtained by rapidly diluting concentrated IpaC in 2 M urea to 0.5 mg/mL IpaC and 200 mM urea. Lower urea concentrations were not possible for these measurements due to limitations of IpaC concentration that can be achieved in 2 M urea. All CD signals were converted and reported as mean residue molar ellipticity and the Dichroweb software package K2D was used to analyze secondary structure content.^{31,32} Thermal transition values were determined by plotting the derivative of each thermal unfolding curve and identifying the derivative maxima corresponding to the transition inflection.

Dynamic light scattering

The hydrodynamic radius (R_{hyd}) of IpaC in urea, OPOE, and LDAO was measured at 25°C using a DynaPro NanoStar (Wyatt Technology Corporation, Santa Barbara, CA) equipped with a 658 nm laser. The IpaC concentration for all measurements was between 0.05 – 0.3 mg/mL after passing through a 0.22 μ m filter. For urea-stabilized IpaC, measurements were taken in 2 M urea which mimics storage conditions as well as following rapid dilution with PBS to achieve 0.05 mg/mL IpaC and 64 mM urea concentrations, directly probing the conditions used in the activity assays described below.

Temporal stability of IpaC

IpaC/IpgC in PBS and IpaC in 0.05% LDAO, 0.5% OPOE or 2 M urea were diluted to 10 μ M protein concentration to probe temporal stability. The IpaC solutions were stored at room temperature (21°C) for six days with daily supernatant samples taken from each following centrifugation to remove precipitated protein. The daily samples were boiled in SDS sample buffer and stored at –20°C until the final sample was collected. The samples were analyzed by SDS-PAGE for relative protein concentrations and the presence of IpaC degradation products.

Preparation of phospholipid vesicles

Asolectin, a natural soybean phospholipid mixture, was made to 8.7 mg/mL in PBS or PBS containing 100 mM of the fluorophore SRB. The mixture was briefly sonicated using a probe sonicator to hydrate the lipids and prepare them for extrusion according to manufacture protocol. Briefly, the mixture was brought to 42°C and was passed through a 100 nm pore sized membrane eleven times using an Avanti polar lipids extruder. Liposomes in PBS were used for the liposome flotation assay without further purification. Unincorporated dye was removed from the SRB containing liposomes using a Sephadex

G-25 size exclusion column prior to use in the liposome disruption assay. All purified liposomes were protected from light, stored at 4°C, and used within 1 week.

Liposome flotation assay

IpaC was incubated with asolectin liposomes for 30 min at room temperature in PBS at 3 μ M and 4.4 mg/mL, respectively. Following incubation, the mixture was brought up to 30% sucrose with the addition of a concentrated sucrose stock solution. 500 μ L of this solution was transferred to an 11 $\times$ 60 mm ultracentrifugation tube and overlaid with 3 mL of 22.5% sucrose in PBS followed by 150 μ L of PBS to form a discontinuous sucrose gradient with protein and liposomes at the bottom of the gradient. The samples were centrifuged in a Beckman SW-60 rotor using a Beckman XL-90 ultra-centrifuge for 1 hr 45 min at 310,000 $\times$ g and 4°C. 100 μ L aliquots were taken from the top, middle and bottom regions of the centrifuged solution and were analyzed for protein content using SDS-PAGE followed by protein detection with Oriole UV-fluorescent stain and a BioRad ChemiDoc MP imager. Biorad Image Lab software was used to perform densitometry analysis and quantitatively compare the protein levels detected in each fraction of the gradient to determine the extent of interaction between liposomes and IpaC.

IpaC induced fluorophore release from lipid vesicles

Fluorescence from liposome encapsulated SRB was monitored using an ISIS PC1 photon counting spectrofluorometer. The excitation wavelength was 565 nm with a 1 mm physical excitation slit and the emission was monitored at 586 nm with a 1 mm physical emission slit. A baseline fluorescence signal from 0.3 mg/mL SRB liposomes in PBS was collected for 30 sec followed by the addition of IpaC in the tested conditions to a final concentration of 10 nM protein and a 200-fold dilution of urea and detergents in the reaction mixture. Fluorophore release was detected as a function of time by monitoring SRB fluorescence intensity followed by the addition of Triton X-100 to a final concentration of 0.1% at T = 180 seconds. The addition of Triton X-100 completely lyses the liposomes and results in maximal fluorescence intensity, representing the 100% SRB release intensity value for each experiment. Protein free controls were performed with PBS or PBS containing appropriate concentrations of LDAO, OPOE, or urea. IpaC/IpgC in PBS and IpgC in each condition were used as additional negative controls to ensure that the observed IpaC induced lysis was specific.

RESULTS

Isolation of IpaC from the stably expressed IpaC/IpgC chaperone complex

Previous studies have shown that IpaC interacts with the *Shigella* T3SS chaperone protein IpgC, presumably stabilizing IpaC and preventing premature “activation” and interaction with the bacterial membrane.^{30,33–36} Upon secretion induction, IpgC is removed and IpaC is secreted through the T3SA needle where it can stably interact at the tip of the apparatus and complete the formation of the translocon pore in the targeted host cell membrane.¹⁷ Additionally, IpaC is secreted directly through the completed translocon into the host cell cytoplasm where it acts as a potent effector polymerizing host cell actin filaments at the site of interaction.^{37–39} This polymerization drives the macropinocytotic uptake of the *Shigella*

which are ultimately released into the host cell cytoplasm where they multiply rapidly and avoid host immune detection.^{37,39,40} Much of the current understanding of IpaC's role in *Shigella* virulence has come from work with isolated protein that was either collected directly from the supernatant of *Shigella* cultures⁴¹ or was expressed recombinantly in *E. coli* and collected from inclusion bodies by denaturing with high concentrations of urea.^{38,42–44} Challenges posed by these purification techniques have limited the availability of pure recombinant IpaC in non-denaturing conditions. We have overcome this challenge by co-expressing and co-purifying the IpaC/IpgC complex in *E. coli* followed by removal of the chaperone and isolation of IpaC with mild detergents, resulting in high levels of stable, membrane active IpaC. Figure 1 shows the highly pure IpaC/IpgC complex that results from initial nickel affinity purification targeting the 6X Histidine tag expressed on the N-terminus of IpaC followed by size exclusion chromatography of the IpaC/IpgC hetero-complex. The figure also shows isolated IpaC that results from the on column removal of IpgC using denaturing (6 M urea) and non-denaturing conditions including the nonionic detergent OPOE or the zwitterionic detergent LDAO, followed by elution in the presence of the separating agents to maintain stability of IpaC. It is worth noting that we found it necessary to use concentrations of urea near 6 M and concentrations of the non-denaturing detergents 4 times the detergent critical micelle concentration (CMC) (OPOE CMC = 0.23%, LDAO CMC = 0.023%) to efficiently separate the IpgC from IpaC, though elution and storage in concentrations as low as 2 M urea and 2 times the detergent CMC were adequate for stabilizing IpaC and maintaining it in solution. Additionally, we found that isolation of IpaC from the co-purified IpaC/IpgC complex resulted in significantly higher protein yields than the traditional method of purifying IpaC from *E. coli* inclusion bodies using urea. Specifically, purification from inclusion bodies yielded 125 ± 21 mg IpaC/liter culture while purification using OPOE and LDAO resulted in yields of 320 ± 40 mg/liter and 460 ± 56 mg/liter, respectively.

IpaC remains monomeric following removal of its chaperone IpgC

Several pioneering studies were key to identifying virulence-associated roles of IpaC,^{38,41–45} however, the influence of contaminating secreted *Shigella* proteins and/or the effects of protein denaturation by urea during the purification process have thus far prevented a full characterization of IpaC. Biophysical characterization of IpaC obtained by urea denaturation followed by refolding and IpaC obtained by the detergent-based purification technique described in this paper provide insight into the effects of denaturant versus detergent on IpaC and provide the first comparative study involving detergent isolated and stabilized IpaC. As a part of the purification process, IpaC isolated using urea, OPOE, and LDAO were analyzed on a superdex 200 10/300 size exclusion column (Fig. 2A). The results show that IpaC in 0.5% OPOE and 0.05% LDAO both elute at approximately 12 mL (~1/2 column volume) while IpaC that was isolated in 6 M urea and dialyzed down to 2 M elutes just after 10 mL. When compared to retention volumes of known molecular weight standards, this suggests that IpaC in OPOE and LDAO has a complex molecular weight of 270 kDa and IpaC in 2 M urea has a molecular weight of 430 kDa, consistent with a homo-hexamer and homo-decamer, respectively. The prospect of isolating discrete IpaC homo-oligomers as was done for the *Shigella* translocator IpaB²¹ was exciting, however, the strong effect of protein shape

on SEC (size exclusion chromatography) retention times warranted additional characterization.

IpaC isolated and maintained in OPOE or LDAO were subjected to chemical crosslinking by the homo-bifunctional primary amine reactive crosslinking agent DSP. Though SEC suggested that IpaC was forming large homo-oligomers, chemical crosslinking followed by SDS-PAGE analysis clearly shows that the proteins exist almost exclusively as monomers in solution (Fig. 2B). The broadened crosslinked protein band is distributed around the same position in the SDS-PAGE gel as reduced and denatured IpaC, finding that IpaC remains monomeric in the tested detergent conditions though the monomers maintain a degree of tertiary structure as a result of intramolecular crosslinking. Addition of the reducing agent DTT cleaves the internal disulfide linkage in DSP and results in a tight IpaC band in the third lane of the gel that runs slightly heavier than native IpaC due to the additional mass of the cleaved crosslinking agent. Attempts to crosslink IpaC in urea failed due to the presence of two primary amines within urea itself, resulting in uncontrollable crosslinking of urea to IpaC and the formation of polymeric IpaC/urea structures.

Dynamic light scattering measurements of IpaC in OPOE and LDAO determined the hydrodynamic radii to be 5.3 ± 1.3 nm and 5.0 ± 0.7 nm, respectively, consistent with IpaC monomers (Supplementary Table S1). IpaC in 2 M urea had a hydrodynamic radius of 12.7 ± 1.6 nm which is more than twice that found for IpaC in either of the detergents, suggesting that the protein is at least partially unfolded in the storage buffer conditions required to maintain IpaC solubility. Rapid dilution of IpaC in 2 M urea to a final urea concentration of 64 mM urea prior to DLS analysis resulted in a hydrodynamic radius of 6.3 ± 1.6 nm, suggesting that IpaC in urea may also be monomeric and is least partially refolded upon rapid urea dilution, though perhaps not fully. Urea dilutions greater than this were not practical due to limited IpaC solubility in urea.

Effects of purification conditions on IpaC secondary structure and stability

Far-UV CD analysis was used to determine the effects of OPOE and LDAO on IpaC secondary structure content and stability. The resulting scans exhibit strong minima near 208 nm and 222 nm and are nearly superimposable on one another (Fig. 3A). Secondary structure content analysis found that IpaC in both detergents contain primarily random coil secondary structure while supporting approximately 30% β -sheet, consistent with prior observation in the presence of urea (Table S1, and Ref^{30,45}). It is notable, however, that the urea clearly reduces the overall extent of observable secondary structure, reducing the CD signal intensity (Fig. 3A) and preventing reliable secondary structure prediction analysis in this study. Each condition of IpaC was then subjected to thermal unfolding using CD spectroscopy to determine thermal transition temperatures (T_m), defined as the inflection point of each transition (Fig. 3B). OPOE-IpaC exhibited a sharp T_m at $77 \pm 2^\circ\text{C}$ while urea-IpaC showed a much shallower transition at $48 \pm 1^\circ\text{C}$. LDAO-IpaC exhibited a gradual change in molar ellipticity as the temperature increased from 10°C to 90°C , but did not display a distinct transition or significant loss of CD signal, suggesting that it maintained much of its secondary structure content through the temperature range. After reaching solution temperatures of 90°C and completing the thermal unfolding analysis, the solutions

were returned to 10°C and far-UV CD scans repeated for the post-melt protein solutions (Fig. 3C). As expected, the post-melt urea-IpaC sample lacked evidence of remaining structure and the OPOE-IpaC displayed a marked decrease in CD signal, both consistent with the observed precipitate in the CD cuvette following thermal unfolding. LDAO-IpaC, however, did not precipitate and displayed almost no change in CD signal or secondary structure content following thermal unfolding analysis (Fig. 3C).

The CD results in Figure 3 show a remarkable thermal stabilization of IpaC in the presence of the detergent LDAO driving us to test the temporal stability of IpaC under identical conditions. Figure 4 contains a series of SDS-PAGE gels showing that the purified IpaC/IpgC complex and IpaC separated from IpgC using urea were no longer detectable in the supernatant of solutions stored at room temperature after only 2 days. IpaC isolated and stabilized using OPOE or LDAO remained soluble and stable for a minimum of 5 days in all tests. These results clearly show that LDAO not only provides thermal protection to IpaC's structure but additionally extends the temporal stability of IpaC in solution as well. We should note that visible precipitation of what appeared to be full length IpaC occurred over the same timeframe that the experiments were conducted (data not shown). Additionally, very little protein degradation was observed over the timeframe of the experiment, suggesting that the instability of the IpaC/IpgC complex and IpaC in urea is primarily the result of aggregation and precipitation and not short-term protein proteolysis/degradation.

Quantifying IpaC membrane interaction through liposome flotation

One of the key responsibilities of IpaC *in vivo* is interaction with the host cell and formation of the T3SS translocon pore in the host membrane.^{11,14} A liposome flotation assay was used to directly assess IpaC interaction with phospholipid bilayers of extruded asolectin liposomes under the tested IpaC stabilizing conditions. These conditions included IpaC as a part of the IpaC/IpgC complex and IpaC isolated and stabilized by urea, OPOE, or LDAO. Each form of IpaC was incubated with asolectin liposomes and subjected to ultracentrifugation through a discontinuous sucrose gradient to determine the extent of liposome interaction. In this assay, the dense proteins remain in the bottom fractions unless they interact with the more buoyant liposomes, causing them to migrate to the top lipid-containing fractions as we have shown previously for the *Shigella* protein IpaB.²¹ In the absence of liposomes, IpaC under all conditions remains in the bottom fraction of the gradient (Fig. 5). In contrast, ~70% of IpaC in urea and ~80% of IpaC in either OPOE or LDAO migrate to the top fractions following incubation with liposomes (Fig. 5B, red bars) demonstrating that IpaC under each of these conditions is able to efficiently interact with phospholipid membranes. The IpaC/IpgC hetero-complex, on the other hand, was unable to interact with lipid membranes and only a small fraction of IpaC which appears to have separated from IpgC is seen in the top fraction while the majority of the IpaC and all of the IpgC remains at the bottom of the gradient (Fig. 5A, blue bars). These findings not only show that IpaC in OPOE, LDAO, and refolded following denaturation with urea are capable of binding phospholipid bilayers, but also confirms that the chaperone IpgC prevents IpaC interaction with lipid membranes as we have previously shown for IpaB.²¹

Monitoring IpaC-mediated disruption of phospholipid bilayers

The precise role of IpaC in the formation of the translocon pore remains controversial, due in large part to challenges producing stable IpaC at levels and concentrations amenable for studies directly evaluating membrane incorporation. Accordingly, we have shown that detergent isolated and stabilized IpaC efficiently interacts with liposome membranes (Fig. 5) and additionally have tested its ability to disrupt phospholipid bilayers by monitoring the release of the fluorophore sulforhodamine B (SRB) from defined phospholipid vesicles. The high concentration of SRB in the liposomes results in fluorescence autoquenching that is relieved when IpaC disrupts the membranes and the dye is released. Liposome disruption assays have been central to several key IpaC studies investigating IpaC membrane disruption as a function of factors including membrane composition^{44,45} and pH⁴¹, providing an ideal mechanism to evaluate the influence of OPOE and LDAO on IpaC “membrane activity”. IpaC in both OPOE and LDAO quickly and efficiently disrupt asolectin liposome bilayers, releasing approximately 90% of the encapsulated SRB dye within 20 seconds of mixing (Fig. 6 and Table S2). In contrast, controls including PBS alone and PBS containing appropriate concentrations of detergent displayed little or no dye release even after 150 second exposures to liposomes. Not surprisingly, IpgC interaction with IpaC protected membrane integrity as the IpaC/IpgC complex failed to disrupt liposomes, consistent with our findings that the complex is unable to stably interact with asolectin liposome membranes (Fig. 5). IpaC stored in 2 M urea and rapidly diluted to 64 mM upon mixing with SRB filled liposomes displayed nearly identical disruption profiles and efficiencies as those for IpaC in OPOE or LDAO, suggesting that the refolded IpaC regains activity nearly equal to that of the detergent stabilized form upon dilution and interaction with lipid membranes. Together, these findings help us to better understand the ability of IpaC to disrupt phospholipid membranes and validate the use of the stabilizing detergents OPOE and LDAO with respect to maintaining IpaC membrane interaction and disruption characteristics.

DISCUSSION

Shigella is the causative agent of shigellosis, a severe form of bacillary dysentery responsible for an estimated 90 million annual cases and greater than 100,000 deaths each year.² Like many pathogenic Gram-negative bacteria, *Shigella* rely on a complex and dedicated secretion system (T3SS) to infect host cells,⁴⁶ evade host immune response,^{7,8} and spread throughout infected tissues.^{1,9,10} While the importance of the T3SS in *Shigella* virulence is becoming well-understood, improved imaging capabilities, the growing number of atomic-resolution structures, and advances in protein expression and purification techniques are just now beginning to uncover many of the mechanistic details surrounding the roles of individual proteins and apparatus components. This is especially true for the T3SA tip complex comprised of the invasion plasmid antigen (Ipa) proteins IpaD, IpaB, and IpaC. Groups including our own have uncovered key environmental conditions that stimulate the stepwise maturation of the *Shigella* T3SA tip complex, finding that a scepter-like pentamer of IpaD likely regulates protein secretion through the complex,⁴⁷ though the existence of a hetero-complex fulfilling the same role cannot be ruled out.^{14,48} From its position at the T3SA apex, IpaD acts as an environmental sensor for the bile salt deoxycholate, undergoing a conformational change that supports the secretion and stable

insertion of the translocator IpaB into the maturing tip complex.^{15,16,49,50} The *Shigella* T3SA now includes the translocator IpaB and is primed for interaction with host cell membranes. While the details of this interaction remain unclear, host cell contact appears to simultaneously induce full secretion of *Shigella* effectors and insert IpaC into the T3SA tip complex where it completes the translocon pore in the host membrane.^{1,9,10,17}

The translocators IpaB and IpaC are both hydrophobic membrane-associated proteins that are stabilized in the *Shigella* cytoplasm by association with the class II chaperone IpgC and while the IpaB/IpgC and IpaC/IpgC hetero-complexes can be stably expressed and purified from *E. coli*, obtaining and working with the isolated translocators has proven much more challenging. We recently investigated the role of mild detergents in the stabilization and activation of IpaB following isolation from the co-purified chaperone complex.²¹ Interestingly, we found that the nonionic detergent OPOE supported the formation of IpaB homo-tetramers which readily formed pores in phospholipid membranes while the zwitterionic detergent LDAO maintained IpaB as a monomer that was unable to disrupt membranes, supporting the model that IpaB forms a multimeric complex at the tip of the T3SA prior to membrane interaction and translocon formation. Here, we extended our previous work to include the *Shigella* translocator IpaC. While IpaC has been shown to incorporate into the mature T3SA¹⁷ and possess the ability to disrupt phospholipid membranes,^{41,42,44,45} previous work has relied on either collecting secreted IpaC from the *Shigella* culture supernatant or isolating recombinant IpaC from *E. coli* inclusion bodies under denaturing conditions. Here, we used the non-ionic detergent OPOE and the zwitterionic detergent LDAO at approximately four times their CMCs to isolate IpaC from the recombinant co-purified IpaC/IpgC complex. Both detergents worked equally well in removing IpgC, though the much lower CMC of LDAO allowed for a 10-fold lower detergent concentration compared to OPOE for isolation and stabilization of IpaC. Both detergents supported IpaC monomers with nearly identical secondary structure profiles.

Additionally, IpaC in both detergents as well as IpaC refolded following urea denaturation interacted with and disrupted liposome membranes though the IpaC/IpgC complex did neither, supporting the hypothesis that IpgC prevents premature membrane interaction of IpaC stores within the bacterial cytoplasm. Additionally, these results suggest that IpaC efficiently refolds following rapid urea dilution and that monomeric IpaC in either tested detergent condition is capable of both membrane interaction and disruption. This is in direct contrast to IpaB which is only capable of efficient membrane disruption in its oligomeric form.²¹ Further work is needed to evaluate the mechanism directly, but it seems increasingly likely that IpaC is capable of forming defined pores upon interaction with membranes or simply disrupting membrane via detergent like mechanism while IpaB requires a pre-formed complex that is supported by the scaffold of the T3SA tip itself. This would imply that secreted IpaC is capable of host membrane disruption independently of the T3SA while IpaB requires the T3SA to act as an organizational scaffold that promotes the formation of pore forming IpaB homo-tetramers at the apex of the maturing apparatus.

Both OPOE and LDAO provided significant thermal and temporal stabilization of IpaC compared to urea purified conditions by preventing protein precipitation through what is likely protection of hydrophobic regions/domains. However, while IpaC exhibited similar

secondary structure, stoichiometry, hydrodynamic radii, temporal stability, and membrane interaction properties in both LDAO and OPOE, LDAO provided significantly improved thermal protection compared to OPOE. LDAO is a small (230 Da) zwitterionic surfactant possessing a polar head group with distinct positively and negatively charged regions while OPOE is a nonionic detergent with a heterogeneous size distribution ranging from about 162 to 450 Da. We previously speculated that the ionic character of LDAO supported IpaB monomers through direct interaction with charged sidechains in IpaB that are necessary for oligomer formation.²¹ The results presented here suggest that an alternate mechanism of oligomer (pore) formation would be required for IpaC as neither OPOE nor LDAO support the formation of higher order species in solution, though LDAO stabilization of IpaC surface charges in addition to protection of hydrophobic regions may explain the increased stability compared to the nonionic detergent OPOE. The observed stabilization of IpaC together with the maintained activity levels in LDAO opens many doors for follow-up mechanistic studies targeting membrane and protein interaction characteristics as well as potential avenues to support structural studies of IpaC both in solution and crystal form.

The demonstrated stability of IpaC in LDAO also provides a strong platform for *Shigella* vaccine formulation studies and animal trials taking advantage of the native immunogenicity of IpaC.^{24,27,51} As one of the conserved *Shigella* T3SA tip complex components, IpaC has been shown to provide a strong antibody response following an infection, making it an attractive cross-species *Shigella* vaccine candidate.⁵¹ The major challenge encountered in formulating an IpaC vaccine has been obtaining levels of soluble and stable protein necessary to perform the studies. The LDAO-based purification and stabilization of IpaC described here overcomes this challenge by increasing protein yield and stability while removing denaturants from the process. Furthermore, LDAO is an inexpensive, generally regarded as safe (GRAS) detergent that is effective at low levels and has been included in the successful formulation of *Shigella* vaccines containing a novel IpaD/IpaB fusion complex already.^{22,28} Determining the impact of a vaccine *a priori* is difficult, though the findings presented here together with previous vaccine studies involving *Shigella* T3SA tip proteins and the detergent LDAO suggest that a vaccine including IpaC-LDAO is not only feasible, but may provide a valuable addition to the currently available antigens used in anti-*Shigella* vaccine formulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FDA Federal Drug Administration

T3SS	type III secretion system
T3SA	type III secretion apparatus
LPS	lipopolysaccharide
DOC	deoxycholate
OPOE	<i>n</i> -octyl-oligo-oxyethylene
LDAO	<i>N,N</i> -dimethyldodecylamine <i>N</i> -oxide
GRAS	generally regarded as safe
IPTG	isopropyl β -D-1-thiogalactopyranoside
DSP	dithiobis(succinimidyl propionate)
DMF	dimethylformamide
DTT	dithiothreitol
CD	circular dichroism
SRB	sulforhodamine B
CMC	critical micelle concentration
SEC	size exclusion chromatography
DLS	dynamic light scattering

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Figure 1. Detergent isolation of IpaC from stable co-purified IpaC/IpgC complexes
SDS-PAGE gel showing the highly pure IpaC/IpgC complex resulting from co-purification with Ni^{2+} chelation chromatography targeting the N-terminal 6X histidine tag on IpaC followed by size exclusion chromatography of the complex. The purified IpaC/IpgC was re-bound to a Ni^{2+} column and IpgC removed by washing the column with binding buffer containing 6 M Urea, 0.1% LDAO or 1.0% OPOE to disrupt the IpaC/IpgC complex prior to elution of IpaC in buffer containing 400 mM imidazole and 2 M Urea, 0.05% LDAO or 0.5% OPOE to stabilize the isolated IpaC (left to right).

Figure 2. IpaC remains monomeric in LDAO and OPOE

A) Size exclusion chromatography analysis of IpaC in 2 M urea (dash), 0.5% OPOE (dot dash) and 0.05% LDAO (solid). Urea stabilized IpaC results in a main elution peak centered at 0.44 column volumes while IpaC in either 0.5% OPOE or 0.05% LDAO exhibit similar profiles that elute at approximately 0.49 column volumes. **B)** SDS-PAGE analysis of DSP crosslinked IpaC in 0.05% LDAO (left 3 lanes) and in 0.5% OPOE (right 3 lanes). The addition of DSP shows that IpaC is monomeric in both LDAO and OPOE as the protein remains centered at the 42 kDa denatured and reduced species in the first lane. The broadened band in the crosslinked IpaC conditions results from intramolecular crosslinking that is relieved upon the cleavage of the DSP internal disulfide with DTT (right lanes).

Figure 3. CD analysis of IpaC secondary structure content and thermal stability

A) IpaC was analyzed in the presence of 200 mM urea, 0.05% LDAO and 0.5% OPOE (black squares, blue circles and gray triangles respectively) showing that IpaC in both detergents exhibits strong CD profiles while urea results in significant unfolding of the protein. **B)** Thermal unfolding of IpaC prepared in 2 M urea, 0.05% LDAO and 0.5% OPOE (black squares, blue circles and gray triangles respectively) is shown based on monitoring the CD signal at 208 nm as the sample was heated from 10 $^{\circ}\text{C}$ to 90 $^{\circ}\text{C}$. **C)** Far-UV CD scans of IpaC in 200 mM urea, 0.05% LDAO and 0.5% OPOE (black squares, blue circles and gray triangles respectively) following the thermal melts displayed in panel B and return to 10 $^{\circ}\text{C}$, showing that IpaC in LDAO maintained nearly all native secondary structure content following heating to 90 $^{\circ}\text{C}$.

Figure 4. LDAO and OPOE enhance the temporal stability of recombinant IpaC

The co-purified IpaC/IpgC complex and isolated IpaC in 2 M urea, 0.05% LDAO, or 0.5% OPOE was maintained at room temperature and sampled for 6 days. The daily samples were analyzed by SDS-PAGE for both protein levels and degradation. The IpaC/IpgC complex and IpaC in 2 M urea are no longer observed in solution after 2 days (coincides with observed protein precipitation). Both OPOE and LDAO enhanced IpaC stability with IpaC in LDAO showing little change over the time course of the experiment.

Figure 5. Liposome flotation indicates that IpgC association prevents IpaC interaction with liposomes while IpaC isolation promotes liposome binding

Liposome flotation was carried out as described in the Methods section. Protein interacting with liposomes migrates to the top fraction while protein unable to bind liposomes remains in the bottom fraction of the sucrose gradient. The presence of protein in top, middle, and bottom fractions of the sucrose gradient was determined by SDS-PAGE and quantified by Oriole total protein staining followed by densitometry analysis. A) Representative gels used to determine the location of IpaC within the sucrose gradient. B) Quantitative densitometry results identifying the position of all tested IpaC conditions within the sucrose gradient. Red bars represent the percentage of the total protein in the top fraction of the sucrose gradient, black bars represent the percentage in the middle fraction, and blue is the relative amount of

protein that remained in the bottom fraction for each condition. The total amount of protein for each condition totals 100% and is plotted as the mean $\pm$ SD of 3 independent analyses. Co-purified IpaC/IpgC did not interact with liposomes and remained predominantly in the bottom gradient fraction while IpaC isolated from IpgC by urea, OPOE, or LDAO all efficiently bound liposomes and migrated to the top fraction of the sucrose gradient. In the absence of liposomes, all conditions resulted in the protein remaining in the bottom fraction.

Figure 6. Isolated recombinant IpaC promotes SRB release from liposomes

Disruption of liposomes and release of encapsulated SRB relieves autoquenching and results in an increase in rhodamine fluorescence emission. Representative time-dependent fluorescence curves following liposome exposure to tested conditions containing IpaC (filled shapes) and corresponding buffer/detergent only controls (hollow shapes). IpaC/IpgC complex in PBS, yellow diamond; IpaC in PBS with 2 M urea (final [urea] = 10 mM), black square; IpaC in PBS with 0.05% LDAO, blue circle; IpaC in PBS with 0.5% OPOE, gray triangle. The protein (or buffer control) was added seconds after fluorescence collection was initiated and Triton X-100 added after 180 seconds to fully disrupt remaining liposomes and provide a 100% lysis benchmark for quantitation of dye release.