Studies of the conformational stability of invasion plasmid antigen B from *Shigella*

Shyamal P. Choudhari,¹ Ryan Kramer,² Michael L. Barta,³
 Jamie C. Greenwood II,¹ Brian V. Geisbrecht,³ Sangeeta B. Joshi,²
 William D. Picking,¹ C. Russell Middaugh,² and Wendy L. Picking^{1*}

¹Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, Oklahoma

²Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas

³School of Biological Sciences, University of Missouri-Kansas City, Kansas City, Missouri

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Abstract: *Shigella* spp. are the causative agent of shigellosis, the second leading cause of diarrhea in children of ages 2–5. Despite many years of research, a protective vaccine has been elusive. We recently demonstrated that invasion plasmid antigens B and D (IpaB and IpaD) provide protection against *S. flexneri* and *S. sonnei*. These proteins, however, have very different properties which must be recognized and then managed during vaccine formulation. Herein, we employ spectroscopy to assess the stability of IpaB as well as IpgC (invasion protein gene), IpaB's cognate chaperone, and the IpaB/IpgC complex. The resulting data are mathematically summarized into a visual map illustrating the stability of the proteins and their complex as a function of pH and temperature. The IpaB/IpgC complex exhibits thermal stability at higher pH values but, though initially stable, quickly unfolds with increasing temperature when maintained at lower pH. In contrast, IpaB is a much more complex protein exhibiting increased stability at higher pH, but shows initial instability at lower pH values with pH 5 showing a distinct transition. IpgC precipitates at and below pH 5 and is stable above pH 7. Most strikingly, it is clear that complex formation results in stabilization of the two components. This work serves as a basis for the further development of IpaB as a vaccine candidate as well as extends our understanding of the structural stability of the *Shigella* type III secretion system.

Additional Supporting Information may be found in the online version of this article.

Thermostability is a major aspect towards formulating a successful protein subunit vaccine. This article presents an assessment of the thermostability of IpaB, IpaB's cognate chaperone, IpgC and the protein-chaperone complex IpaB/IpgC from *S. flexneri*, using different spectroscopic techniques. To understand role of the cognate chaperone IpgC, the protective antigen IpaB and the chaperone were also studied individually. The results revealed greater thermostability of the proteins around neutral pH conditions. Furthermore the data can be used in designing screening studies for protein stabilizers.

Shyamal P. Choudhari and Ryan Kramer contributed equally to this work.

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*Correspondence to: Wendy L. Picking, Department of Microbiology and Molecular Genetics, Oklahoma State University, 307 Life Sciences East, Stillwater, OK 74078. E-mail: wendy.picking@okstate.edu

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Introduction

Shigella spp. are the causative agents of shigellosis, a severe form of bacillary dysentery and the leading cause of diarrheal disease in children between the ages 2 and 5.¹ The pathogenesis of *Shigella* is dependent upon the delivery of the effector proteins of the Type III secretion system (T3SS) to the host cytoplasm via the conduit of the Type III secretion apparatus (T3SA).² This nanomachine consists of a basal body spanning inner and outer bacterial membranes, an external needle and a tip complex. After needle polymerization, IpaD localizes to the needle tip³ and undergoes a conformational change to promote the mobilization of IpaB, the first translocator

protein to the tip.⁴ Upon host cell contact, IpaB undergoes a conformational change causing IpaC, the second translocator, to mobilize to the host cell membrane and form the complete translocon pore for the subsequent translocation of protein effectors and completion of the infection process.⁵ Because IpaD and IpaB are exposed prior to host contact, they represent potential candidates for a subunit vaccine to prevent shigellosis.

The current search for a protective vaccine has focused on formulations that only provide serotype specific protection such as live, attenuated strains or those containing LPS as the protective entity. We have demonstrated, however, that the highly conserved IpaD and IpaB are broadly protective, serotype-independent antigens against two of the major endemic *Shigella* serogroups, *S. flexneri* 2a and *S. sonnei*.⁶ IpaD is an easily purified, stable protein that we have previously studied by biophysical analysis.⁷ IpaB, however, is more complex. Purification of large quantities of IpaB in *E. coli* requires coexpression with its cognate chaperone, IpgC, forming a heterodimer.^{8,9} The IpaB/IpgC complex is then separated into an IpaB tetramer¹⁰ and an IpgC dimer with mild detergent.¹¹ A major challenge in formulating a subunit vaccine involving the hydrophilic IpaD and hydrophobic IpaB is the maintenance of these vastly different protective antigens in stable, biologically active states for extended times to permit their storage and subsequent delivery to diverse clinical settings worldwide. Thus, in this study, we have examined the biophysical properties of IpaB, IpgC, and the IpaB/IpgC complex for the purpose of providing preformulation characterization data for the development of a stable multivalent, broad-coverage vaccine against *Shigella* spp.

Results and Discussion

Circular dichroism

To better understand the conformational effects of chaperone binding to IpaB, the molar ellipticity and thermal unfolding profiles under varying pH conditions were examined for the IpaB/IpgC heterodimer, the IpaB tetramer and the IpgC dimer. The far-UV CD spectra (Supporting Information Fig. S1) of the IpaB/IpgC complex, IpaB and IpgC at 10°C are characteristic for the secondary structure of folded α -helical proteins. The differences in the absorbance at 222 nm are attributed to the unique α helical content of each protein. Compared to the IpaB/IpgC complex and IpgC, the molar ellipticity at 10°C of IpaB greatly varied with pH indicating that IpaB alone is more sensitive to pH alterations than the IpaB/IpgC complex. The thermal unfolding transition temperature (T_m) provides a quantitative measure of the loss of secondary structure and serves as a mark of stability at that pH (Table I). In summary,

sharp transitions at higher temperatures were seen for the IpaB/IpgC complex around neutral pH. For IpaB, the T_m values were seen between 56 and 58°C, independent of the pH condition examined. In acidic environments, however, protein instability is exhibited as a loss of secondary structure, which was also observed by dynamic light scattering where the hydrodynamic diameter for IpaB/IpgC showed a marked increase from ~21 nm at pH 7 and 8 to ~120 nm at pH 4. Similarly, the hydrodynamic diameter for IpaB increased from 45 nm at pH 6 to ~83 nm at pH 5. This instability is probably a result of structural perturbation and protein association in such environments. The thermal transition of IpgC was relatively sharp and consistent indicating its stability was not impacted between pH 6 and 8. As mentioned above, IpgC precipitates below pH 5. Taken together, the spectra and thermal unfolding results indicate that the IpaB/IpgC heterodimer is a more stable complex than either the IpaB or IpgC multimers. Yet, all three complexes exhibit decreased stability below pH 5.0.

Intrinsic fluorescence and static light scattering

Intrinsic fluorescence measurements were made to explore changes in the tertiary structure of the IpaB/IpgC complex as well as IpaB and IpgC alone (Supporting Information Fig. S2). At 10°C between pH 6 and 8, the emission maximum was seen near 338 nm for the IpaB/IpgC complex. Thus, the single tryptophan residue (Trp) of IpaB (IpgC does not contain Trp) appears to be extensively buried, which is consistent with its location in the partial crystal structure.¹² As the pH was lowered, the emission maxima shifted approximately 1–2 nm to a higher wavelength suggesting a subtle structural change in the complex. Since the peak positions are determined by a “mean spectral center of mass” method, the actual peak positions are approximately 10–12 nm lower than the actual peak position. This method produces values of peak position with a higher S/N ratio since it uses the area under the spectral curve rather than the noise-sensitive peak itself. As the temperature is raised at pH 6–8, a red shift is seen above 65°C and is accompanied by aggregation of the protein as indicated by the static light scattering data. Consistent with the CD data, fluorescence intensity data also indicated loss of structural stability at lower pH (Table I). This is accompanied by aggregation at pH 4 and 5, but only slight protein association is seen at pH 3 (Table I). In IpaB itself, the emission peak of the single Trp at 10°C is red shifted approximately 5 nm under all pH conditions examined suggesting that the Trp becomes exposed to solvent in the absence of its chaperone, IpgC. Furthermore, the effect of temperature on the fluorescence suggests small differences in stability of IpaB in its complex with IpgC,

Table I. Thermally induced unfolding^a of IpaB/IpgC, IpaB and IpgC at each pH by the three spectroscopic techniques

	CD	Peak position	SLS
IpaB/IpgC			
pH 3	46.9 ± 0.2	46.1 ± 0.1	49.6 ± 1.1
pH 4	64.2 ± 0.9/72.6 ± 0.8	58.6 ± 0.4	61.8 ± 0.4
pH 5	68.9 ± 0.7	65.4 ± 0.9	67.9 ± 0.1
pH 6	71.6 ± 0.7	68.7 ± 0.5	70.2 ± 0.2
pH 7	71.8 ± 0.6	68.8 ± 0.3	70.4 ± 0.3
pH 8	73.1 ± 0.4	67.7 ± 0.3	70.9 ± 0.1
IpaB			
pH 3	56.6 ± 0.2	56.7 ± 0.9	48.1 ± 0.1
pH 4	57.6 ± 0.1	61.3 ± 0.1	58.5 ± 1.5
pH 5	56.4 ± 1.6	61.4 ± 0.3	37.1 ± 1.1
pH 6	57.7 ± 0.2	62.1 ± 0.7	63.2 ± 0.4
pH 7	56.2 ± 0.1	62.3 ± 2.1	59.8 ± 0.5
pH 8	56.2 ± 0.2	63.2 ± 0.4	60.8 ± 1.8
IpgC ^b			
pH 6	41.2 ± 0.6	46.1 ± 0.7	47.4 ± 0.2
pH 7	47.6 ± 1.2	47.6 ± 1.4	49.0 ± 0.2
pH 8	45.5 ± 1.0	50.1 ± 0.6	51.4 ± 0.4

^a Midpoint of the transition (°C) determined by derivative analysis.

^b IpgC precipitated during dialysis at and below pH 5. No data are available for those points.

although the temperatures at which transitions occur are overall similar (Table I). A dramatic difference in aggregation is seen at pH 5, however, with protein association initiating at a much lower temperature (i.e., 25°C at pH 5 vs. 60°C at other pH values). It is thus apparent that significant stabilization of IpaB occurs upon complex formation. IpgC lacks Trp and therefore the fluorescence observed presumably arises from the Tyr residues although the position of this peak (312 nm) is significantly higher than that normally observed for Tyr in proteins (302–305 nm). Despite the low sensitivity of Tyr to changes in its local environment, a structural change is still evident at 40°C at pH 6–8. This could correspond to the spectral changes seen near 40°C in the complex over the same range of pH. In the case of IpgC, marked aggregation is only seen at pH 6. Similar to CD studies, the fluorescence data suggest that the IpaB/IpgC complex is relatively more stable than the individual protein components.

Empirical phase diagrams

An empirical phase diagram (EPD) is a two-dimensional colored representation of the different physical states of a protein.¹³ In this method, experimental values from each technique employed are normalized from 0 to 1 for each pH/temperature condition. These values are used to construct a multi-dimensional vector at each point and a color assigned to each component using an RGB color scheme. A detailed discussion of the method is presented elsewhere.¹³ The physical origin of each phase is assigned by direct examination of the exper-

Figure 1. Empirical phase diagram. The EPDs generated for IpaB/IpgC complex (A), IpaB (B), and IpgC (C) using spectroscopic thermal stability data. The color of each phase is a synthesis of the individual colors assigned to each experimental component. The change of color is indicative of a change in the structure or aggregation state of the protein and the choice of colors is arbitrary.

imental data (see Supporting Information). The EPD of the IpaB/IpgC heterodimer [Fig. 1(A)] shows at least three apparent phases. Phase I corresponds to native protein-like state while Phase III represents the unfolded protein and insoluble aggregates. Between pH 6–8 an additional phase (Phase II) is observed from approximately 40–45°C and 70°C. This phase presumably results from the aggregation, indicated by the blue shift in the peak position data.

The EPD of the IpaB tetramer [Fig. 1(B)] shows at least six distinct apparent phases. Phase I corresponds to the most stable region. Phase V represents a transition region, whereas region VI indicates extensively unfolded protein. Phase IV is an aggregated phase as defined by the static light scattering data at pH 5. The decreased CD signal in the pre-transition region of the CD melt at pH 3 and 4 suggests that Phases II and III are non-natively folded protein.

At least five distinct apparent phases are observed in the EPD generated for the IpgC dimer as well [Fig. 1(C)]. Phase I corresponds to native protein and displays an apparent phase boundary at 40°C. Phase II corresponds to the broad transition region while Phase III, IV, and V correspond to more extensively structurally disrupted protein at pH 7 and 8.

Conclusion

In this study, we have performed a biophysical characterization of the T3SS first translocator, IpaB. Furthermore, we have compared IpaB characteristics with the IpaB/IpgC complex and isolated the IpgC protein as a method to better understand the effect of complex formation on the individual proteins. It is apparent that significant stabilization occurs when the complex is formed.

Since IpaB is currently thought to be one of two promising antigens for a broad-coverage vaccine, it may be necessary to further stabilize the protein for vaccine use by the addition of appropriate stabilizers. Understanding the effect of pH and temperature on the structural stability of IpaB could ultimately aid in the development of a successful vaccine against the diarrheal diseases caused by *Shigella* spp. Initially, the EPD provides structural transition conditions (i.e., pH and temperature) for assessing stabilizing excipients for use in formulation of a parenteral (IM or ID) vaccine. The current study was performed with IpaB in solution. The resultant formulations can directly be assessed for long-term stability at 4°C. Furthermore, should freezing be required, these methods will again be used to assess IpaB during freeze thaw cycles. It is, however, envisioned that a lyophilized or spray dried form may ultimately be required. The effect of freeze-drying cycles can be evaluated using these techniques to assess proper protein conformation and stability upon reconstitution. Thus, this study provides the first step in the preformulation of a stably formulated vaccine regardless of its physical state or administration route.

Materials and Methods

Preparation of recombinant proteins and biophysical methods

A more extensive methods section has been presented as a supplementary file. Cloning, expression

and purification schemes for IpgC and IpaB/IpgC have been described previously.^{6,11} Prior to spectroscopic measurements, each protein was dialyzed into 20 mM citrate phosphate buffer at 0.15 ionic strength at pH values ranging from 3 to 8. After dialysis protein was filtered using 0.22 μ m syringe filters to remove any aggregates. The protein concentration was determined by UV absorption at 280 nm and adjusted to 0.3 mg/mL for all measurements. Far-UV CD, intrinsic fluorescence and static light scattering data were collected and analyzed as previously described.⁷ The thermal stability data acquired using spectroscopy techniques was incorporated into EPDs using Matlab software package (The Mathworks, Natick, MA). Details of EPD construction have been discussed previously.¹³ Briefly, the data obtained from *n* techniques is represented as *n*-dimensional vectors and normalized to build a density matrix. The dataset is then converted into a three-dimensional vector, which is further converted into a colored map with each vector component assigned to an RGB (red, green or blue) color scheme.

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