Conformational stability and differential structural analysis of LcrV, PcrV, BipD, and SipD from type III secretion systems

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Abstract

Diverse Gram-negative bacteria use type III secretion systems (T3SS) to translocate effector proteins into the cytoplasm of eukaryotic cells. The type III secretion apparatus (T3SA) consists of a basal body spanning both bacterial membranes and an external needle. A sensor protein lies at the needle tip to detect environmental signals that trigger type III secretion. The *Shigella flexneri* T3SA needle tip protein, invasion plasmid antigen D (IpaD), possesses two independently folding domains in vitro. In this study, the solution behavior and thermal unfolding properties of IpaD's functional homologs SipD (*Salmonella* spp.), BipD (*Burkholderia pseudomallei*), LcrV (*Yersinia* spp.), and PcrV (*Pseudomonas aeruginosa*) were examined to identify common features within this protein family. CD and FTIR data indicate that all members within this group are α -helical with properties consistent with an intra-molecular coiled-coil. SipD showed the most complex unfolding profile consisting of two thermal transitions, suggesting the presence of two independently folding domains. No evidence of multiple folding domains was seen, however, for BipD, LcrV, or PcrV. Thermal studies, including DSC, revealed significant destabilization of LcrV, PcrV, and BipD after N-terminal deletions. This contrasted with SipD and IpaD, which behaved like two-domain proteins. The results suggest that needle tip proteins share significant core structural similarity and thermal stability that may be the basis for their common function. Moreover, IpaD and SipD possess properties that distinguish them from the other tip proteins.

Keywords: tip proteins; T3SS; biophysical analysis; IpaD; SipD; BipD; LcrV; PcrV

Gram-negative bacteria possess numerous complex mechanisms for transporting proteins into the extracellular milieu and into the cytoplasm of eukaryotic host cells to initiate bacterial infection (He et al. 2004; Yip and Strynadka 2006). Six major bacterial secretion pathways have been defined with the type III secretion system (T3SS) being a major contributor to mediating host–pathogen communication (Buttner and Bonas 2006). The T3SS is a common

theme among diverse Gram-negative bacterial pathogens including the animal pathogens *Shigella flexneri*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, and *Burkholderia pseudomallei*, and plant pathogens such as *Erwinia amylovora* and *Pseudomonas syringae* (Cossart and Sansonetti 2004; He et al. 2004; Buttner and Bonas 2006). Although these bacteria cause a wide spectrum of disease, each infection is established by the delivery of bacterial proteins through the type III secretion apparatus (T3SA) to the target eukaryotic cell membrane and cytoplasm to subvert normal cell mechanisms.

Sometimes described as a “molecular syringe and needle,” the T3SA is composed of 20–30 proteins that form a basal body spanning the inner and outer bacterial

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membranes and an external needle (He et al. 2004; Yip and Strynadka 2006). The external needle is a hollow tube ~50 nm long and 7 nm in diameter with an inner channel that is typically ~2.5 nm in diameter (Cordes et al. 2003). It is composed of >100 copies of a needle protein with 5.6 molecules per turn of the helix (Cordes et al. 2003). How proteins are prevented from free passage through the needle and how signals are sent from the needle tip to the base to trigger secretion are still unanswered questions.

Recently, a significant step toward answering these questions was made with the identification of a protein that resides at the T3SA needle tip (Mueller et al. 2005; Espina et al. 2006b). For *S. flexneri*, invasion plasmid antigen D (IpaD) was shown to stably reside at the tip of the TTSA needle, with this localization being required for T3SS secretion control and virulence-related functions (Espina et al. 2006b). Similarly, in *Y. enterocolitica*, LcrV localizes to the tip to form a distinct complex (Mueller et al. 2005). In addition to functioning as the needle tip proteins for their cognate T3SAs, IpaD and LcrV are required for translocator protein insertion into host cell membranes (Fields et al. 1999; Marenne et al. 2003; Picking et al. 2005).

Despite their functional homology as needle tip proteins, IpaD and LcrV share very little primary sequence similarity. There is significant sequence conservation, however, among members within the IpaD or LcrV needle tip protein subfamilies, respectively, especially at the C termini (see Fig. 1). The closest relatives of IpaD are the invasion proteins SipD and BipD of *S. typhimurium* and *B. pseudomallei*, respectively. Like *ipaD*-null mutants, *sipD*- and *bipD*-null mutants are noninvasive, with the *sipD* strain secreting massive amounts of T3SS effectors (Kaniga et al. 1995; Stevens et al. 2004). These three proteins share >25% sequence similarity, with >90% sequence similarity at their C termini (Kaniga et al. 1995). Similarly, *lcrV* and the *P. aeruginosa pcrV*-null mutants are defective for proper type III secretion and show 42% sequence identity, with most of this being within their C termini (Nanao et al. 2003). The crystal structures of LcrV (Derewenda et al. 2004) and, more recently, IpaD and BipD (Erskine et al. 2006; Johnson et al. 2007) have been solved and exhibit a dumbbell-like shape with two globular domains separated by a long coiled-coil. Previously, biophysical measurements have demonstrated that IpaD has two independently folding units with a thermally labile N-terminal and a more thermally stable C-terminal domain containing a coiled-coil (Espina et al. 2006a). Based on the structural and functional conservation between IpaD and LcrV and the high degree of sequence homology within the subfamilies, structural similarities may exist among all of the needle tip proteins that are required for their ability to localize to the needle tip.

In this study, the structure and conformational stability of recombinant SipD, BipD, LcrV, and PcrV are compared

to provide insight into the structural basis for their mechanistic similarities. Because IpaD was found to possess an independently folding N-terminal domain, N-terminal deletions were also introduced into these proteins so that the presence of a related domain could be assessed and compared to IpaD. All of the proteins were found to be α -helical with an intramolecular coiled-coil. Only SipD, however, appeared to possess an independently folding N-terminal domain that is analogous to that of IpaD. Based on these results, we propose that, although having divergent primary sequences, all T3SA tip proteins possess similar structural features that are required for their needle tip localization as well as distinct features necessary for their pathogen-specific activities.

Results

Generation of recombinant proteins

An earlier structural analysis of IpaD demonstrated that IpaD is a highly α -helical protein that contains two independently folding domains. The N-terminal domain (residues 1–120) is thermally labile, while the C-terminal domain (residues 121–332) is more stable and contains an intramolecular coiled-coil. Based on the functional similarities between IpaD and the putative T3SA tip proteins, a series of biophysical studies was initiated to compare the structures and conformational stabilities of SipD, BipD, LcrV, and PcrV. Shared structure–function relationships among these proteins might then be extrapolated to all T3SA tip proteins. To assess the impact of the N-terminal region on the four proteins, deletions were made to mimic the IpaD N-terminal deletion. Based on its crystal structure (Derewenda et al. 2004), an LcrV N-terminal deletion mutant was made by deleting the N-terminal domain prior to the intramolecular coiled-coil, giving rise to LcrV $^{\Delta 1-146}$. Although the crystal structure of PcrV has not been solved, *pcrV* can complement an *lcrV*-null mutant (Mueller et al. 2005), indicating that the core structure of the two proteins should be similar. Thus, the PcrV N-terminal deletion PcrV $^{\Delta 1-127}$ was based on an estimate of the location of the boundary between the putative N-terminal domain and coiled-coil region. In contrast, when this study was initiated, none of the crystal structures for the IpaD subfamily had been solved. Therefore, SipD $^{\Delta 1-121}$ and BipD $^{\Delta 1-123}$ were produced based on the previous IpaD $^{\Delta 1-120}$ results. Like the full-length proteins, the deletion mutants were highly soluble when made recombinantly in *Escherichia coli*.

Secondary structure analysis of SipD, BipD, LcrV, and PcrV

Far-UV CD was initially used to analyze the secondary structure and thermostability of SipD, BipD, LcrV, and

IpaD	-MNITTLTNSISTSSFPNNTNGSSTETVNSDIKTTTSSHPVSSLTMLNDTLHNIRTTNQALKKELSQKT
SipD	MLNIQNYASAPHPGIVAERPQTSPASEHVETAVVPSTTEHRTDIIISLSQAATKIHQAQQTQLQ---STPP
BipD	-MNMHVDMGRLTVRDWPALEALAKTMPADAGARAMTDDDLRAAGVDRRVPEQKLGAAIDEFASLRLPDR
	:* : : : : * : : : : :
IpaD	LTKTSLEEIALHSSQISMDVNKSAQLLDILSRNEYPINKDARELLHSAPKEAELDGD--QMISHRELWAK
SipD	ISEENNDETLARQQLTSSNLALAKSGVLSAEQENLRSAFSAPTSALFSASPMAPRTTISDAEIWDM
BipD	IDGRFVDG---RRANLTVFDDARVAVRGHARAQRNLLERLETELLGGTLDTAGDEGG---IQDPDILQG
	: : : : : : : : : * : * : :
IpaD	IANSSINDINEQYLKVYEHAVSSYTQMYQDFSAVLSSLAGWISPGGNDGNSVKLQVNSLKKALEELKEKY-
SipD	VSQNISAIGDSYLGVEYENVVAVYTDFFQAFSDILSKMGGWLLPG-KDGNVTVKLDVTSLNKDLNSLVKNYN
BipD	LVDVIG-QGKSDIDAYATIVEGLTKYFQSVADVMSKLDYISAK--DDKNMKIDGGKIKALIQQVIDHLP
	: : * . . . : * * * : * : : : : * : : * : : : :
IpaD	---KDPLYPANN---TVSQEQANKWLTGGLTGKVSQKNGGYVVSINMTPIDNMLKSLDNLGGNGE
SipD	QINSNTVLFPAQSGSGVKVATEAEARQWSELNLPNSCLKSYGSGYVVTVDLTPLQKMQVDIDGLGAPGK
BipD	-----TMQLPKG-----ADIAWRKELGDAVISISDSG---VVTINP---DKLIKMRDSLPPDG-
	: : : : : * * . . . * : : : : : * * *
IpaD	---VVLNNAKYQAWNAGFSAEDETMMNNLQTLVQKYSNANSIFDNLVKVLSSTISSCTDTKLFLHF 332
SipD	DSKLEMDNNAKYQAWQAGFKAEENMKTTLQTLTKYSNANSIYDNLVKVLSSTISSLETAKSFLQG 343
BipD	---TVWDTRYQAWNTAFSGQKDNQNDVQTLVEKYSHQNSNFNLVKVLSGAISTLDTAKSYLQI 310
	* : * : * : * : * : * : * : * : * : * : * : * : *
LcrV	-MIRAYEQNPQHFIEDLEKVRVEQLTGHGSSVLEELVQLVKDKNIDISIKYDPRKDEVFANRVITDDIE
PcrV	MEVRNLNAARELFLDELLAASAPASAE---QEELLALLRSEIRIVLAHAGQPLSEAQVLK-----
	: * : : * : * : * : * : * : * : * : * : * : *
LcrV	LLKKILAYFLPEDAILKGGHYDNLQNGIKRVKEFLES--SPNTQWELRAFMAVMHFSLTADRIDDILK
PcrV	---ALAWLLAANPSAPPGQ-----GLEVLREVLQARRQPGAQWDLREFLVSAFSLHG-RLDEEDVIG
	* : * : * : * : * : * : * : * : * : * : * : *
LcrV	VIVDSMNHGDARSKLREELAELETAEIKYSVIQAETINKHLSSTGTINIHDKSINLMDKNLYGYTD-EEI
PcrV	VYKDVLTQDQGRKALLDELKALTAELKVYSVQSINAALSAKQGIKIRIDAGGIDLVDPPTYGYAVGDPR
	* * : : . . * . * : * * * : * : * : * : * : * : * : *
LcrV	FKASAEYKILEKMPQTTIQVDGSEKKIVSIKDFLGSSENKRTGALGNLKNYSYNKDNELSHFATTCSDK
PcrV	WKDSPEYALLSNLDT---FSG---KLSIKDFLGSQPKQSGELKGLSDEYPFKDNPNVGNFATTVSDR
	: * * . * : * : : . . * : * : * : * : * : * : * : * : * : *
LcrV	SRPLNDLVSQKTTQLSDITSRFNSAIEALNRFIQKYDSVMQRLLDDTSGK 326
PcrV	SRPLNDKVNEKTTLLNDTSSRYNSAVEALNRFIQKYDSVLRDILSAI--- 294
	***** * : * * * * : * : * : * : * : * : * : * : * : *

Figure 1. Protein alignments for the IpaD and LcrV subfamilies. IpaD, SipD, and BipD were aligned, as were LcrV and PcrV using ClustalW. The bars above amino acid sequences indicate the location of the putative intramolecular coiled-coil. At given positions, * shows amino acid sequence identity, : shows strong similarity, and • shows similarity.

PcrV as well as the N-terminal deletion mutants. The CD spectrum for each protein displays double minima at 208 nm and 222 nm, suggesting a significant amount of helical structure (Fig. 1). Their secondary structure contents were estimated from the far-UV CD spectra using the Dichroweb suite of algorithms (Lobley et al. 2002; Whitmore and Wallace 2004) with the program CDSSTR (Manavalan and Johnson Jr. 1987). As previously observed for IpaD (Espina et al. 2006a), these proteins appear to possess a high percentage of α -helical structure with some β -sheet and disordered structure being present (Table 1). Previous studies of PcrV estimated a lower percentage of helical structure (18%) compared to that obtained in the present work (58%) (Nanao et al. 2003). This may be at least partially due to the different experimental temperatures and methods used to estimate the secondary structure content. The N-terminal deletion

mutations of these proteins showed CD spectra similar to those of full-length proteins (Fig. 2), indicating fully folded proteins with no major changes in the global folding of the proteins as a result of the truncations. Like IpaD ^{Δ 1-120}, SipD ^{Δ 1-121} and PcrV ^{Δ 1-127} displayed a greater relative amount of helical structure than their full-length counterparts (Table 1), which may indicate that the N-terminal regions of these proteins possess less helical character (Espina et al. 2006a). In contrast, BipD ^{Δ 1-123} and LcrV ^{Δ 1-146} displayed a lower relative amount of α -helix with an increasing percentage of β -sheet and random structures.

The thermal stabilities of SipD, BipD, LcrV, and PcrV and the N-terminal deletion mutants were studied by monitoring the changes in the ellipticity at 222 nm as a function of temperature (Fig. 2). SipD displayed two distinct thermal transitions, one minor and one major, at ~57°C and 75°C, respectively, suggesting the presence of

Table 1. Secondary structure estimates for SipD, BipD, LcrV, and PcrV and their N-terminal mutants

Protein	α -Helix	β -Sheet	Turns	Random
SipD	55.0 ^a $\pm$ 1.4 ^b	19.0 $\pm$ 1.4	7.0 $\pm$ 1.4	18.0 $\pm$ 1.4
SipD Δ 1-121	61.5 $\pm$ 0.7	7.5 $\pm$ 0.7	10.0 $\pm$ 0.0	21.0 $\pm$ 0.0
BipD	76.3 $\pm$ 1.5	3.0 $\pm$ 0.0	7.0 $\pm$ 1.0	13.0 $\pm$ 0.1
BipD Δ 1-123	69.5 $\pm$ 0.7	5.0 $\pm$ 0.0	7.0 $\pm$ 0.0	17.5 $\pm$ 0.7
LcrV	68.3 $\pm$ 0.6	4.6 $\pm$ 0.5	8.3 $\pm$ 0.5	18.7 $\pm$ 0.5
LcrV Δ 1-146	54.0 $\pm$ 2.8	10.0 $\pm$ 1.4	13.5 $\pm$ 0.7	22.5 $\pm$ 0.7
PcrV	58.3 $\pm$ 0.5	9.3 $\pm$ 0.5	10.6 $\pm$ 0.5	22.0 $\pm$ 0.0
PcrV Δ 1-127	61.0 $\pm$ 0.0	9.0 $\pm$ 0.0	10.5 $\pm$ 0.7	20.5 $\pm$ 0.7

^aThe estimated percent content of each type of secondary structure is provided.

^bStandard error calculated from $n = 3$.

two independently folded domains (Fig. 2; Table 2). The SipD Δ 1-121 mutant only displayed the major transition, suggesting that the structural domain responsible for the low-temperature minor transition resides near the N terminus of SipD, which is similar to the transition pattern observed for IpaD (Espina et al. 2006a). In contrast, BipD, LcrV, and PcrV display a single transition, with BipD and LcrV being the most stable with transition midpoints around 80°C. In good agreement with previous results (Nanao et al. 2003), PcrV displayed a transition midpoint around 65°C. In general, N-terminal deletions introduced into BipD, LcrV, and PcrV resulted in a decrease in thermal stability (Fig. 2; Table 2).

Like IpaD, the deletion mutations all give rise to folded proteins possessing extensive secondary structure that could be attributed to a stable intramolecular coiled-coil (Fig. 1). Thus, the secondary structure of SipD, BipD, LcrV, and PcrV was analyzed by FTIR spectroscopy. The amide I' region of the full-length proteins at 25°C dissolved in D₂O was decomposed into its component peaks based on Fourier self-deconvolution and second derivative analysis (Fig. 3). The peak deconvolution for the four proteins showed three predominant bands around 1633, 1644, and 1653 cm⁻¹, with small variations depending on the protein under analysis (Table 3). This triplet of equally intense bands observed in the FTIR spectrum of the proteins has been taken as a unique spectral fingerprint of intramolecular coiled-coil proteins (Heimburg et al. 1996, 1999). The spectrum of BipD is slightly different from that of the other proteins since the high-frequency peak of the triplet possesses a higher intensity, a feature often seen with trimeric coiled-coils (Heimburg et al. 1996, 1999), while exhibiting a single thermal transition. In contrast, as was seen in the CD spectrum, SipD possesses two thermal transitions. LcrV and PcrV exhibit only one major transition (Fig. 3B,C). A progressive increase in breadth of the amide I' region along with the appearance of a sharp band at ~1616 cm⁻¹ and a weak signal at ~1680 cm⁻¹ were common features

for all proteins upon heating (Fig. 3B). These particular bands are characteristic of protein aggregation due to the formation of intermolecular β -sheets (Arrondo et al. 1994; Murayama and Tomida 2004). For BipD and SipD, the observed aggregation seems to be influenced by protein concentration, since it was not detected in more dilute solutions of the proteins during turbidity experiments (see below). Although FTIR experiments require samples with a much higher protein concentration than CD, an analysis of the thermal stability of the proteins based on the intensity of the 1616 cm⁻¹ band showed transition temperatures similar to those detected by CD spectroscopy (Fig. 3C).

Analysis of tertiary structure by fluorescence and second derivative UV absorption spectroscopies

Because SipD, BipD, LcrV, and PcrV possess four, four, one, and three tryptophan residues, respectively, intrinsic fluorescence spectroscopy was used to monitor changes in their tertiary structure. The four Trp residues of SipD and BipD are also present in the truncated forms of the proteins while LcrV Δ 1-146 does not contain a Trp residue and was therefore not further analyzed by this technique.

Figure 2. CD spectroscopy and thermal stability studies of SipD, BipD, LcrV, PcrV, and their N-terminal deletion mutants. (A) The far-UV CD spectra were recorded at 10°C. A scan rate of 20 nm/min with a resolution of 0.5 nm was used. All spectra presented are an average of three consecutive scans. (B) Ellipticity at 222 nm was monitored from 10°C to 90°C at 0.5°C intervals at a rate of 15°C/h. Protein concentrations were 0.1 mg/mL in 10 mM phosphate buffer (pH 7.4) for both CD studies.

Table 2. Thermally induced unfolding of Sip, BipD, LcrV, PcrV, and their N-terminal mutants evaluated by spectroscopic techniques

Protein	CD		Trp fluorescence		ANS fluorescence		UV Trp/Tyr peak position T_m 1	OD _{360 nm}	
	T_m 1 ^a	T_m 2	T_m 1	T_m 2	T_m 1	T_m 2		T_m 1	T_m 2
SipD	57.8 ^b ± 0.6 ^c	75.7 ± 0.8	57.6 ± 0.3	74.2 ± 0.3	49.3 ± 0.1	68.1 ± 0.0	72.8 ± 0.7	64.9 ± 1.6	74.3 ± 0.0
SipD ^{Δ1-121}		76.6 ± 0.9		73.0 ± 1.8		70.4 ± 0.3	71.9 ± 0.6		73.4 ± 0.6
BipD		83.7 ± 0.1		81.4 ± 0.2		76.7 ± 0.1	79.8 ± 0.2	ND ^d	ND
BipD ^{Δ1-123}		62.6 ± 0.3		78.5 ± 3.3		64.1 ± 0.1	72.6 ± 1.3	ND	ND
LcrV		81.9 ± 1.6		78.4 ± 0.3		74.3 ± 1.2	67.6 ± 0.6		69.0 ± 0.1
LcrV ^{Δ1-146}		50.0 ± 0.3	NT ^e			43.02 ± 0.2	61.4 ± 0.5		58.8 ± 0.2
PcrV		63.3 ± 0.1	ND			59.7 ± 0.1	61.4 ± 1.5		58.9 ± 0.0
PcrV ^{Δ1-127}		48.6 ± 0.1	ND			50.5 ± 0.4	54.0 ± 3.2		51.1 ± 0.1

^aTransition temperature in °C.^bMidpoint determined by derivative analysis.^cStandard deviation ($n = 4$).^dNot detected.^eNo Trp present in this protein.

Intrinsic Trp fluorescence is highly environmentally sensitive and is widely used to monitor changes in Trp microenvironments resulting from alterations in protein tertiary structure. Exposure to more polar environments generally results in Trp emission being shifted to a longer wavelength (red shift), while movement into more apolar regions produces a blue shift (Lakowicz 1983). The Trp emission spectra of the full-length proteins and three of the N-terminal deletion mutants manifest a maximum between 328 and 336 nm, indicating that the tryptophans are on average buried within the apolar core of the needle tip proteins. In contrast, the emission maximum for SipD^{Δ1-121} was red shifted ~5 nm with respect to the spectrum of the full-length protein (Fig. 4A), suggesting at least partial exposure of the Trp in this mutant. In response to increasing temperature, the Trp emission wavelength maximum for SipD (Fig. 4A) red-shifted significantly and displayed two thermal transitions centered at ~57°C and 74°C. These values agree well with those detected by CD analysis (Table 2). In contrast, SipD^{Δ1-121} only exhibited a single broad transition between 65°C and 82°C, which is again consistent with the CD thermal unfolding data (Fig. 4A; Table 2). The overall observed red shift is consistent with increased solvent exposure of the Trp residues resulting from temperature-induced unfolding (Fig. 4A). BipD^{Δ1-123} and PcrV^{Δ1-127} showed overall destabilization of the molecule with lower T_m values than the corresponding full-length proteins (Fig. 4A). The blue shift in the Trp emission maximum observed for LcrV and PcrV (Fig. 4A) at high temperatures is probably due to extensive aggregation of the proteins resulting from temperature-induced unfolding.

To further characterize changes in the tertiary structure of SipD, BipD, LcrV, and PcrV, the thermal unfolding of the proteins was evaluated in the presence of 1-amino-8-naphthalenesulfonate (ANS), an extrinsic probe that often

binds to apolar regions of proteins. The fluorescence of ANS is highly quenched in aqueous solution but can increase dramatically upon binding to protein apolar regions (Rosen and Weber 1969). SipD showed two well-defined transitions at ~49°C and 68°C which were attributable to exposure of apolar regions caused by the conformational alterations within the two individual domains (Fig. 4B; Table 2). SipD^{Δ1-121} showed a single transition near 70°C (Fig. 4B; Table 2), confirming the CD and intrinsic fluorescence observations (Fig. 4A). When the unfolding of full-length and N-terminal mutants of BipD, PcrV, and LcrV were evaluated by ANS fluorescence, the truncated proteins showed lower thermostability as evaluated by their T_m values (Fig. 4B; Table 2), which again indicates a destabilizing effect on the proteins after truncation.

SipD, BipD, LcrV, and PcrV also possess substantial numbers of the aromatic amino acids tyrosine and phenylalanine. Changes in the microenvironments of these residues also induce changes in their spectral characteristics, thus providing a means for monitoring tertiary structure stability that is not completely dependent on the presence of scarce Trp residues. The second derivative spectra of the proteins showed five to six negative peaks with the following general assignments: Phe (~253 nm and 259 nm), Tyr (at 274 nm and 279 nm), an overlapping Tyr/Trp signal (at 282 nm), and Trp (at 291 nm) (spectra not illustrated). In general, exposure of aromatic amino acid side chains to a more polar environment causes a blue shift in the absorbance minimum, whereas shifts to longer wavelengths suggest that the residues are present in a more buried, less polar environment (Mach and Middaugh 1994). The Tyr/Trp signal was found to be the most well-behaved and superior in signal-to-noise ratio to the other peaks in the analysis of the protein's stability (Fig. 5). The Tyr/Trp peak positions of SipD and

SipD Δ^{1-121} show a quasilinear temperature-dependent decrease in wavelength before the thermal transition. Broad transitions around 71°C and 74°C were observed for SipD and SipD Δ^{1-121} , respectively, with an overall blue shift (Fig. 5A; Table 2). Surprisingly, this is the only technique of those used that does not clearly demonstrate a double transition for full-length SipD, suggesting that the N-terminal portion does not strongly impact the thermal response of this particular second derivative minimum. The Tyr/Trp peak positions of BipD and BipD Δ^{1-123} remain relatively constant until 65°–75°C, respectively, suggesting that the tertiary structure of the protein undergoes negligible changes up to this temperature (Fig. 5A). Above these temperatures, the Tyr/Trp peak positions shift to lower wavelengths, suggesting that the aromatic residues are becoming more exposed to the solvent, in good agreement with the intrinsic fluorescence observations (Fig. 4A). The noise in the signals observed in LcrV Δ^{1-146} and PcrV Δ^{1-127} at temperatures above the transition is almost certainly a consequence of the extensive aggregation detected for these proteins (Fig. 5B). In general, the transition temperatures agreed well with those seen for the transitions detected by CD spectroscopy (Table 2).

SipD, BipD, LcrV, PcrV, and their N-terminal mutants' associative behavior was evaluated by monitoring the turbidity (OD) at 360 nm as the temperature was increased (Fig. 5B). In general, all of the proteins showed an increase in turbidity starting at ~50°–65°C depending on the protein, with well-defined transitions (Fig. 5B; Table 2). The drop in turbidity after the transition is due to settling of the precipitated aggregates within the cuvette. It is noteworthy that no aggregation was detected for SipD, BipD, and BipD Δ^{1-123} , which is similar to previous findings for IpaD and IpaD Δ^{1-120} (Espina et al. 2006a).

Analysis of thermal unfolding by differential scanning calorimetry

Differential scanning calorimetry (DSC) is another useful method for analyzing the thermal unfolding of proteins with the potential to provide a direct energetic description of distinct protein unfolding events. The SipD thermogram was quite complex showing high-temperature (~85°–90°C) transitions not detected by most spectroscopic techniques (Fig. 6). Although the protein displayed five distinct transitions, the T_m for the first and third transitions agreed well with those values for the double transition detected by CD and fluorescence experiments (Table 2; Fig. 5). SipD Δ^{1-121} displayed only the major and high-temperature transitions (Fig. 6). The rest of the proteins under study showed one endothermic transition (Fig. 6). Except for the BipD thermogram, the best fit using a non-two-state model was composed of two peaks for each protein (Table 4). N-terminal truncation of BipD, LcrV, and PcrV caused destabilization of the

Figure 3. (Legend on next page)

Table 3. Peak position and percentage of area corresponding to the curve fitting Gaussian components of the amide I' band of BipD, SipD, LcrV, and PcrV at pH 7.4 and 25°C

BipD		SipD		LcrV		PcrV	
Peak position (cm ⁻¹)	Peak area (%)	Peak position (cm ⁻¹)	Peak area (%)	Peak position (cm ⁻¹)	Peak area (%)	Peak position (cm ⁻¹)	Peak area (%)
1683.4	0.8	1690.9	0.2	1690.2	0.5	ND ^a	ND
1674.5	7.3	1675.0	9.3	1678.8	4.6	1677.6	3.7
1663.3	12.7	1662.4	13.5	1667.1	12.1	1665.6	12.9
1653.0	28.3	1652.0	21.0	1654.7	24.2	1654.1	24.2
1643.6	22.4	1642.9	23.0	1644.3	26.8	1643.8	27.6
1634.1	18.0	1633.2	17.4	1633.4	20.4	1633.3	19.5
1624.3	8.7	1624.3	10.1	1623.3	8.1	1623.9	7.5
1612.6	1.7	1614.0	4.0	1610.9	3.3	1613.1	3.8
1606.9	0.1	1605.0	1.5	ND	ND	1604.4	0.7

^a Not detected.

proteins with a 10°–20°C drop in the T_m values compared to those found for the respective full-length protein thermograms (Fig. 6; Table 4). Thus, DSC confirms the high thermal stability of these proteins, which is probably at least partially a result of the intramolecular coiled-coil. It also illustrates the presence of two independently folding domains that appears to be unique to IpaD and SipD. Lack of complete reversibility prevented calculation of the enthalpies of unfolding. Hints of a second transition are for the first time present using this technique for the other proteins.

Discussion

In this study, we have extensively characterized the conformational stability of SipD, BipD, LcrV, and PcrV, all of which have either been found or are proposed to be located at the tip of the T3SA needles of *S. typhimurium*, *B. pseudomallei*, *Y. enterocolitica*, and *P. aeruginosa*, respectively. Their structural properties were then compared to the recently well-characterized properties of IpaD from *S. flexneri*. These needle tip proteins appear to fall into two main subfamilies: the IpaD subfamily consists of IpaD, SipD, BipD, and possibly other proteins that belong to related T3SSs that are largely involved in cellular invasion; and the LcrV subfamily, which consists of LcrV, PcrV, and other proteins, many of which are involved with modifying eukaryotic cell behavior for

other purposes (e.g., prevention of phagocytosis and cell death).

Based on the initial biophysical characterization of IpaD, it was anticipated that this subfamily of needle tip proteins would be highly α -helical and possess two independently folding domains, with the N-terminal third of the protein forming a thermally labile domain and the C-terminal two-thirds being more heat stable and containing a large intramolecular coiled-coil. SipD followed this pattern, but surprisingly BipD did not. BipD shares significant sequence identity with IpaD and SipD, especially within the C-terminal half of the protein, but it exhibits only one thermal unfolding transition that is even more stable than the higher of the two transitions seen for IpaD and SipD. FTIR analysis suggests that BipD possesses an intramolecular coiled-coil, but it may be somewhat different from that of IpaD and SipD because it exhibits spectral characteristics between that of a double- and triple-stranded coiled-coil (Heimburg et al. 1996, 1999). It is possible that the N-terminal region of BipD interacts more extensively with the intramolecular coiled-coil than do the N-terminal regions of IpaD or SipD. As a result, not only does the FTIR spectrum of BipD resemble a triple coiled-coil, but interaction of the N-terminal region with the coiled-coil may prevent the formation of a fully independently folding domain as seen with IpaD and SipD. This idea is corroborated by an overall thermal destabilization of BipD when the N-terminal region is removed. Indeed, during the preparation of this manuscript, the crystal structures of IpaD and BipD were solved (Erskine et al. 2006; Johnson et al. 2007). Like LcrV, IpaD and BipD have a dumbbell-like shape with globular domains flanking an intramolecular coiled-coil, which forms the handle. The N-terminal domain of IpaD forms a helix–turn–helix that is almost identical in structure to MxiH, its cognate T3SA needle protein, followed by a short α -helix that attaches the domain to

Figure 3. FTIR study of SipD, BipD, LcrV, and PcrV in D₂O. (A) Peak fitting of the amide I' of the full-length proteins (~15–18 mg/mL, pD 7.0) in solution measured by ATR-FTIR. A second derivative trace and Fourier self-deconvolution analysis of the amide I' were used for initial positioning of the individual Gaussian components. (B) Fourier self-deconvolution of the amide I' region of the spectra of each protein as a function of temperature (bandwidth = 17 cm⁻¹; band-narrowing factor of 2.0). (C) Thermal unfolding of the proteins was evaluated by the intensity of the peak at 1616 cm⁻¹ (indicating intermolecular β -sheet) as a function of temperature. All spectra presented are an average of 256 scans.

Figure 4. Analysis of the tertiary structure stability of SipD, BipD, LcrV, and PcrV and its N-terminal mutants using intrinsic fluorescence and ANS spectroscopy. (A) Effect of temperature on the Trp fluorescence emission wavelength maxima. Spectra were collected from 10°C to 90°C at 2.5°C intervals with a 3-min equilibration time at each temperature. Samples were excited at 280 nm, and the wavelength maximum at each temperature was calculated by derivative analysis. (B) Effect of temperature on ANS binding. A solution containing SipD, BipD, LcrV, PcrV, or an N-terminal deletion mutant in the presence of 40 μ M 8-anilino-1-naphthalene sulfonate (ANS) was excited at 375 nm, and the fluorescence intensity at 485 nm was monitored as a function of temperature. The protein concentration was 0.1 mg/mL for both experiments.

the coiled-coil. Based on the biophysical characterization, it appears that this domain is able to unfold and fold independently of the remainder of the protein. This property may be important for the virulence of *Shigella*. In contrast, the N-terminal domain of BipD is longer, with more of a loop rather than a turn. This loop appears to form a stronger interaction with the coiled-coil, which accounts for the apparent triple-stranded coiled-coil of BipD. Thus, although BipD does not possess the two independently folding domains structure of IpaD and SipD, it may have an N-terminal domain whose unique structural characteristics point to a unique biological function for this region of the protein.

The LcrV subfamily of needle tip proteins differs significantly from the members of the IpaD subfamily based on sequence, but the members share significant sequence similarity among themselves, especially in their C-terminal halves. In considering the published crystal structure of LcrV (Derewenda et al. 2004) in the context

of the biophysical characteristics of the two subfamilies, it appears likely that the N-terminal domain of the LcrV subfamily does not exist as a well-defined, independently folding domain, making the native organization of the proteins of the two subfamilies different from one another. This may directly reflect the divergence in function between the two subfamilies. Furthermore, the lack of independent folding domains would also readily explain why deletion of the LcrV N-terminal domain and the putative PcrV N-terminal domain results in overall protein destabilization. Nevertheless, even these proteins continue to form highly folded, stable structures when the N-terminal deletion is introduced. This is probably because of the stable nature of the intramolecular coiled-coil that all of these tip proteins seem to possess.

It has been previously noted that the tip proteins can exist in various multimerization states. LcrV has been described to be a dimer and multimers thereof (Lawton et al. 2002). Similarly, the N-terminal truncation of IpaD that provided its original crystal was present as a dimer (Johnson et al. 2007). In this study, gel filtration chromatography showed that IpaD, SipD, and BipD elute as

Figure 5. UV second derivative from Tyr/Trp peak position of SipD, BipD, LcrV, PcrV, and N-terminal mutants and turbidity as a function of temperature. (A) The wavelength minima of the Tyr/Trp peaks were monitored as a function of temperature. Spectra were collected from 10°C to 90°C at 2.5°C intervals with a 3-min equilibration time before collection of each spectrum. (B) The turbidity was studied by monitoring the OD at 360 nm as a function of temperature. The protein concentration was 0.1 mg/mL, and a 20 mM phosphate buffer (pH 7.4) was used.

Figure 6. Differential scanning calorimetry thermograms of full-length and N-terminal deletion mutants. The thermal transitions are reported in Table 3 and were obtained by fitting the data using a non-two-state model (dashed line). A protein concentration of 2 mg/mL was used.

monomers, PcrV was primarily monomeric, and LcrV appeared to be present as both a monomer and dimer. In contrast, all of the N-terminal deletion mutants appear to behave as oligomers. Although oligomerization has been reported to occasionally provide increased thermal stability to a protein, this property was not apparent for the tip protein family upon deletion with the N-terminal domain since the second melting transition is lower than that of the full-length protein. Owing to the presence of multiple oligomerization states for LcrV, the impact of this property on the biophysical characterization of this protein cannot be directly assessed. The multimeric state of these proteins and deletion mutants is likely due to interactions involving the coiled-coil (Lawton et al. 2002; Johnson et al. 2007), a property required for proper oligomerization

at the tip of the needle (Deane et al. 2006). Once the N-terminal domain has been removed, the coiled-coil is exposed, promoting spontaneous oligomerization.

The presence of coiled-coils and/or helices that fold back upon themselves appears to be a recurring theme for many T3SS secreted proteins. All of the needle monomer structures solved to date have a helix–turn–helix structure that centers around a PXXP turn motif that is shared by almost all known needle protein monomers (Deane et al. 2006; Zhang et al. 2006). At the end of the needle resides a tip protein that possesses a prominent intramolecular coiled-coil that is not only required for the structure of the tip protein but for the interaction of the tip protein with the needle itself (Deane et al. 2006; Espina et al. 2006b). Within the IpaD tip protein subfamily, it appears that the N-terminal domain mimics the needle protein to chaperone the coiled-coil (Johnson et al. 2007). After exit from the needle, the domain is repositioned to allow the coiled-coil to dock onto the needle. In *Shigella*, the next secreted protein to localize at the needle tip is IpaB (Olive et al., in press), one of the two translocator proteins, which is also predicted to possess a coiled-coil motif (Johnson et al. 2007). Similarly, the other *Shigella* translocator protein, IpaC, is predicted to contain a coiled-coil that may be involved in protein–protein interactions (Pallen et al. 1997). As a rule, disruption of these helical structures results in a loss of T3SS function (Kuelto et al. 2003b; Picking et al. 2005; Espina et al. 2006b). Regions outside the helical core of these proteins appear to impart specific pathogen functions (Kuelto et al. 2003b; Picking et al. 2005; Espina et al. 2006b; Johnson et al. 2007).

This study provides the first detailed biophysical characterization of a family of T3SA needle-associated proteins and builds on the recent crystal structures of three of the tip proteins. It is now clear that there are certain structural features that seem to generally apply to this group of proteins. The secondary structure analyses of these five T3SA needle tip proteins suggest that all of the T3SA needle tip proteins are highly α -helical and contain an intramolecular coiled-coil located within the

Table 4. Thermal unfolding of SipD, BipD, LcrV, PcrV, and $\Delta 1$ –120 N-terminal mutants by DSC

	T_m 1 ^a	T_m 2	T_m 3	T_m 4	T_m 5
SipD	56.74 $\pm$ 0.23 ^b	67.98 $\pm$ 0.29	73.62 $\pm$ 0.07	83.09 $\pm$ 0.18	88.97 $\pm$ 0.15
SipD $\Delta 1$ –121	64.64 $\pm$ 0.40	70.39 $\pm$ 0.17	88.04 $\pm$ 0.44		
BipD	—	80.28 $\pm$ 0.00			
BipD $\Delta 1$ –123	59.86 $\pm$ 0.01	68.84 $\pm$ 0.06			
LcrV	67.28 $\pm$ 0.49	76.16 $\pm$ 0.02			
LcrV $\Delta 1$ –146	48.56 $\pm$ 0.29	56.78 $\pm$ 0.15			
PcrV	57.05 $\pm$ 0.40	60.52 $\pm$ 0.13			
PcrV $\Delta 1$ –127	42.96 $\pm$ 0.40	49.55 $\pm$ 0.01			

^aMidpoints, in $^{\circ}\text{C}$, were determined by fitting the thermograms to a non-two-state unfolding model.

^bStandard deviation ($n = 3$).

C-terminal two-thirds of the protein that dictates the nature of the tip protein interaction with the needle tip. The independently folded N-terminal domain of IpaD and SipD is not clearly identifiable within all of tip proteins within the resolution of the approaches used here. The presence or absence of an independently folding N-terminal domain may be associated with biological functions, in addition to needle tip localization, that are unique to the individual proteins. The lack of multiple transitions, however, does not exclude the presence of distinct domains having similar thermal stabilities. Since it appears that IpaD and the other needle tip proteins are a structural component of the T3SA needle, it is noteworthy that a major feature of flagellin is the existence of a coiled-coil structure that contributes to flagellar filament polymerization. This may reflect a similarity in the mechanism by which the helix–turn–helix region of the needle proteins mediates needle filament assembly (Deane et al. 2006). In this respect, the needle tip proteins may represent a short extension of the T3SA needle that is capable of sensing the environment to control the subsequent secretion of T3SS secretion substrates (Olive et al., in press). While speculative, this model for assembly of the external portion of the T3SA provides a basis upon which future investigation can be built.

Materials and Methods

Materials

The pET15b plasmid containing *pcrV*, pET/V, was a gift from I. Attree (CEA-Grenoble, Grenoble, FR) (Nanao et al. 2003). The pET9b plasmid containing *lcrV* was donated by M. Nilles (University of North Dakota, Grand Forks, ND). Chromosomal DNA from *B. pseudomallei* (strain K96243) was a gift from D. DeShazer (United States Army Medical Research Institute of Infectious Disease, Fort Detrick, Frederick, MD). pET15b and competent *E. coli* were from Novagen. Oligonucleotides were obtained from IDT.

Preparation of affinity-purified recombinant proteins

The *sipD* gene was subcloned from pwpsf4 (Picking et al. 2005) into pET15b by previously described methods (Harrington et al. 2003). The *bipD* gene was copied from *B. pseudomallei* chromosomal DNA by PCR using a 5′-primer containing GAGAGA, an NdeI restriction site, and the first 14 bases of the gene; and a 3′-primer containing GAGAGA, a BamHI site, and the last 14 bases of the gene. The resulting PCR fragment was digested with NdeI/BamHI and ligated into pET15b, and the ligated plasmid was transformed into *E. coli* NovaBlue. The N-terminal deletion mutants were made by copying the truncated gene from the plasmid containing the full-length gene using a T7 terminator primer as the 3′-primer and the following as the 5′-primers: *sipD*^{Δ1–121}, GAGAGACATATGGCGCAGCC GAGAAC; *bipD*^{Δ1–123}, GAGAGACATATGATCCAGCCGGACC CGA; *lcrV*^{Δ1–146}, GAGAGACATATGCATGGTGATGCCCGTAG; *pcrV*^{Δ1–127}, GAGAGACATATGAAGCGCAAGGCGCTGC. The

resulting PCR products were digested with NdeI and BamHI or XhoI for *sipD* and ligated into pET15b, and the ligation products were transformed into *E. coli* NovaBlue.

The expression plasmids were transformed into *E. coli* Tuner (DE3) with the recombinant proteins purified as previously described using nickel chelation chromatography (Marquart et al. 1995), dialyzed against 10 mM Na₂HPO₄ (pH 7.0), 150 mM NaCl (PBS), and stored at –70°C. Protein concentrations were determined by measuring the absorbance at 280 nm using extinction coefficients based on the amino acid composition of each protein (Mach et al. 1992).

Far-UV circular dichroism spectroscopy

Far-UV CD spectra were collected in a Jasco J720 spectropolarimeter (Jasco, Inc.) as previously described (Ausar et al. 2005; Espina et al. 2006a) using a protein concentration of 0.1 mg/mL. Spectra were collected at 10°C in 0.1-cm path-length cuvettes using a resolution of 0.5 nm, 2-sec response time, and a scanning speed of 20 nm/min. Thermal unfolding was followed by monitoring the ellipticity at 222 nm from 10°C to 90°C. Data analysis was performed as previously described (Espina et al. 2006a).

FTIR spectroscopy

The secondary structure of the full-length proteins in solution was also assessed using Fourier transform infrared (FTIR) spectroscopy with an ABB Bomen FTIR MB series spectrometer (ABB Bomen). Samples of 1 mL of protein in D₂O (15–18 mg/mL) were placed onto an attenuated total reflectance (ATR) crystal equipped with an A.R.K. temperature controller (Spectra-Tech). Spectra were collected under dry air purge using a resolution of 4 cm^{–1} and the co-addition of 256 interferograms. Data analysis was performed as described previously (Espina et al. 2006a).

UV absorbance spectroscopy

High-resolution absorbance spectra were collected as described (Kuelto et al. 2003b; Ausar et al. 2005; Espina et al. 2006a) using a protein concentration of 0.1 mg/mL in a Hewlett-Packard 8453 UV-Visible spectrophotometer (Agilent). Spectra were analyzed between 200 nm and 400 nm with an experimental resolution of 1 nm over a temperature of 10°–90°C at 2.5°C intervals. A 3-min equilibration time was used before collection of each spectrum. The optical density at 360 nm was simultaneously recorded as the measure of protein association/aggregation.

Intrinsic and extrinsic fluorescence spectroscopy

Tryptophan fluorescence and externally bound 8-anilino-1-naphthalene sulfonate (ANS) were monitored as previously described (Kuelto et al. 2003a; Ausar et al. 2005; Espina et al. 2006a) using a PTI QuantaMaster spectrophotometer (Photon Technology International) equipped with a Peltier controlled cuvette holder. The protein concentration used was 0.1 mg/mL. A 1-cm path-length cuvette with Teflon cap was used in all experiments. Spectra were collected from 10°C to 90°C at 2.5°C intervals with a 3-min equilibration time at each temperature. The excitation wavelengths for Trp and ANS were set at 280 and 385 nm, respectively.

Differential scanning calorimetry (DSC)

Calorimetric experiments were performed as previously described (Espina et al. 2006a) using a protein concentration of 2 mg/mL with a MicroCal VP-DSC high-throughput capillary differential scanning calorimeter (Northampton). DSC thermograms were obtained from 10°C to 115°C at a scan rate of 1°C/min with a protein concentration of 2 mg/mL in PBS. Baseline correction was performed by subtracting a buffer thermogram obtained under identical conditions. The data were analyzed using MicroCal Origin 7.0 (Origin-Lab Corporation) assuming a non-two-state unfolding model (Sanchez-Ruiz 1992).

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