Characterization of *Mycobacterium tuberculosis* EsxA Membrane Insertion

**ROLES OF N- AND C-TERMINAL FLEXIBLE ARMS AND CENTRAL HELIX-TURN-HELIX MOTIF**

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Received for publication, October 27, 2014, and in revised form, December 26, 2014. Published, JBC Papers in Press, February 2, 2015, DOI 10.1074/jbc.M114.622076

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**Background:** *Mycobacterium tuberculosis* EsxA exhibits a pH-dependent membrane-interacting activity.

**Results:** Both the N- and C-terminal flexible arms are required for membrane disruption, and the central helix-turn-helix motif of EsxA inserts into the membrane.

**Conclusion:** EsxA forms a membrane-spanning pore at low pH.

**Significance:** An understanding of EsxA membrane insertion facilitates development of novel therapeutics against tuberculosis.

EsxA (ESAT-6), an important virulence factor of *Mycobacterium tuberculosis*, plays an essential role in phagosome rupture and bacterial cytosolic translocation within host macrophages. Our previous study showed that EsxA exhibits a unique membrane-interacting activity that is not found in its ortholog from nonpathogenic *Mycobacterium smegmatis*. However, the molecular mechanism of EsxA membrane insertion remains unknown. In this study, we generated truncated EsxA proteins with deletions of the N- and/or C-terminal flexible arm. Using a fluorescence-based liposome leakage assay, we found that both the N- and C-terminal arms were required for membrane disruption. Moreover, we found that, upon acidification, EsxA converted into a more organized structure with increased α-helical content, which was evidenced by CD analysis and intrinsic tryptophan fluorescence. Finally, using an environmentally sensitive fluorescent dye, we obtained direct evidence that the central helix-turn-helix motif of EsxA inserted into the membranes and formed a membrane-spanning pore. A model of EsxA membrane insertion is proposed and discussed.

*Mycobacterium tuberculosis* infects one-third of the world’s population and kills over 1 million people each year (1). Although significant progress has been made over decades of research efforts, the cellular and molecular mechanisms of *M. tuberculosis* pathogenesis are still not fully understood. It is widely believed that after being internalized into host macrophages, *M. tuberculosis* establishes latent infection and remains inside the phagosomes through inhibiting phagosome maturation (2–4). Transition of latent infection to active tuberculosis requires *M. tuberculosis* to rupture the phagosomal membrane and translocate into the cytosol, where it replicates for cell-to-cell spreading (5–7). The ability of the pathogen to arrest phagosome maturation and to translocate from the phagosome to the cytosol has been attributed, at least in part, to a type VII secretion system named ESX-1 (5–9). Comparative genomic studies have revealed that the *esx-1* locus is present in the genome of *M. tuberculosis* but is absent in the genome of the attenuated vaccine strain *Mycobacterium bovis* bacillus Calmette-Guérin (10, 11). Deletion of *esx-1* from *M. tuberculosis* results in attenuation of virulence (12–14), whereas transfer of *esx-1* into bacillus Calmette-Guérin partially restores virulence (15, 16). The *esx-1* locus comprises 11 genes that encode the ESX-1 secretion system and two secreted proteins: ESAT-6 (6-kDa early secreted antigenic target; also called EsxA or Rv3875) (17, 18) and CFP-10 (10-kDa culture filtrate protein; also called EsxB or Rv3874) (19). EsxA and EsxB are secreted by the ESX-1 system in a codependent manner (20). Mutants of *M. tuberculosis* with either gene deletions or defects in secretion of EsxA and/or EsxB are not translocated into the cytosol and show reduced host cell lysis and cell-to-cell spreading (5–7, 12, 14, 21). Recently, it has been shown that apoptosis induction in host cells is clearly restricted to virulent *M. tuberculosis* strains and is associated with the capacity of *M. tuberculosis* to secrete EsxA (22). Biochemical analysis has shown that EsxA and EsxB form a 1:1 heterodimeric complex (23, 24), and EsxA exhibits membrane-lysing activity (12, 25). However, direct evidence that EsxA inserts into the membrane is lacking, and the molecular mechanism of EsxA membrane interaction has not been defined.

In our previous study, we characterized the pH-dependent membrane interactions and conformational changes of *M. tuberculosis* EsxA, and we showed that *M. tuberculosis* EsxA exhibits a unique membrane-interacting activity that is not found in its ortholog from nonpathogenic *Mycobacterium smegmatis* (26). To investigate the mechanism of EsxA membrane insertion further, in this study, we generated EsxA proteins with N- and/or C-terminal deletions and found that both the N- and C-terminal flexible arms were required for membrane disruption. Moreover, we used the environmentally sensitive fluorescent dye N,N-dimethyl-N-((iodoacetyl)-N-(7-ni-
trobenz-2-oxa-1,3-diazol)ethylenediamine (NBD)\(^2\) and the liposome-based NBD fluorescence assay to provide direct evidence that, upon acidification, the central helix-turn-helix motif of EsxA inserted into the liposomal membranes and formed a membrane-spanning structure.

**MATERIALS AND METHODS**

**Genes, Constructs, and Site-directed Mutagenesis**—The full-length esxA gene (WT, residues 1–95) immediately followed by a C-terminal His\(_{10}\) tag was cloned into pET22b at the NdeI/Xhol sites as described previously (26). An esxA gene fragment with C-terminal residues 86–95 deleted (ΔC) and also immediately followed by a His\(_{10}\) tag was obtained by PCR and cloned into pET22b at the NdeI/Xhol sites. esxA gene fragments with N-terminal residues 1–10 deleted (ΔN) or both N-terminal residues 1–10 and C-terminal residues 86–95 deleted (ΔN+ΔC) and immediately followed by a C-terminal His\(_{10}\) tag were obtained by PCR and cloned into the pGEX4T-1 vector at the BamHI/Xhol sites. All truncated constructs were confirmed by DNA sequencing.

To generate cysteine mutations for NBD labeling, pET22b-EsxA(WT) was used as the template. The selected residues of EsxA were mutated to cysteine by site-directed mutagenesis using a QuikChange kit (Agilent). All cysteine mutations were confirmed by DNA sequencing.

**Expression and Purification of the Recombinant Proteins of EsxA**—WT, ΔN, ΔC, and ΔN+ΔC—WT EsxA and ΔC were expressed and purified from bacterial inclusion bodies as described previously (26). Briefly, pET22b-EsxA(WT) and pET22b-EsxA(ΔC) were expressed in BL21(DE3) cells. The inclusion body was isolated and then solubilized in 8M urea. The proteins were refolded on a nickel column and eluted with an imidazole gradient. The eluted proteins were further clarified by size exclusion chromatography to ~90% purity. ΔN and ΔN+ΔC were expressed as GST fusion proteins following a similar protocol to that described previously for purification of GST-EsxB (26). Briefly, GST-ΔN and GST-ΔN+ΔC were expressed as soluble proteins in BL21(DE3) cells. The fusion proteins were purified on a glutathione-Sepharose 4B column. The purified GST fusion proteins were cleaved with thrombin, and His-tagged ΔN and ΔN+ΔC were purified on a nickel column, followed by size exclusion chromatography.

**Liposome Preparation**—Liposomes were prepared as described previously (26–30). Briefly, 1,2-dioleoyl-sn-glycero-3-phosphocholine (20 mg/ml in chloroform) was dried under nitrogen gas to form a lipid film, followed by vacuum overnight to remove residual solvent. For liposomes containing the dye/quencher pair 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)/p-xylene-bispyridinium bromide (DPX), the resulting dry lipid film was rehydrated by six freeze-thaw cycles in 50 mM ANTS, 50 mM DPX, and 5 mM HEPES (pH 7.3), followed by extrusion through a 200-nm Nucleopore filter in a mini-extruder (Avanti Polar Lipids). Subsequently, the liposome solution was applied to a Sephadex G-25 desalting column equilibrated with 10 mM HEPES (pH 7.4) and 150 mM NaCl for buffer exchange. The resulting liposome solution had ANTS/DPX inside the liposomes, whereas there was no fluorescent dye outside the liposomes. For liposomes used for NBD fluorescence, a regular buffer (20 mM Tris-HCl and 100 mM NaCl (pH 7.3)) was used to rehydrate the lipid film, and the liposomes were prepared with similar freeze-thaw cycles and extrusion but without desalting.

**Time-lapse Intensity Measurements of ANTS/DPX Dequenching**—ANTS fluorescence dequenching was measured in an ISS K2 multiphase frequency and modulation fluorometer with excitation at 380 nm and emission at 520 nm as described previously (26). Briefly, 100 μl of the liposomes containing ANTS/DPX after desalting was diluted into 1.3 ml of 50 mM sodium acetate and 150 mM NaCl (pH 5.0) with continuous stirring. After the baseline was stabilized, 100 μl of purified proteins (total of 100 μg) was injected into the cuvette, and the fluorescence signal was monitored in real time. Crossed polarizers on excitation and emission beams and a 395-nm-long path filter were used to reduce the background scatter.

**Circular Dichroism**—The purified EsxA proteins (WT, ΔN, ΔC, and ΔN+ΔC; 10 μM) were incubated in either a neutral pH buffer (20 mM Tris-HCl and 100 mM NaCl (pH 7.0)) or an acidic buffer (20 mM sodium acetate and 100 mM NaCl (pH 5.0)) for 30 min. The circular dichroism was measured in the ISS K2 fluorometer with excitation at 295 nm and emission at 310–450 nm. A 305-nm-long path filter was applied in the emission channel to reduce the background scatter. The emission spectra of the proteins were calibrated with the emission spectra of the same pH buffers without proteins using VINCi software.

**8-Anilino-1-naphthalenesulfonate (ANS) Fluorescence**—The purified EsxA proteins (WT, ΔN, ΔC, and ΔN+ΔC; 5 μM) were incubated with 100 μM ANS in either pH 7.0 or 5.0 buffer for 30 min in the dark. The ANS emission spectra of the samples were measured in the ISS K2 fluorometer with excitation at 380 nm and emission at 400–600 nm. A 395-nm-long path filter was placed in the emission path to reduce the background scatter. The emission spectra of the samples were calibrated with the same pH buffers without proteins using VINCi software.

2 The abbreviations used are: NBD, N,N-dimethyl-N-((iodoacetyl)-N-(7-nitrobenz-2-oxa-1,3-diazol)ethylenediamine; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, p-xylene-bispyridinium bromide; ANS, 8-anilino-1-naphthalenesulfonate.
Membrane Insertion of M. tuberculosis EsxA

FIGURE 1. Expression and purification of EsxA proteins with N- and/or C-terminal deletions. A, solution structure of the EsxA protein. The structural coordinates (Protein Data Bank ID 1WAB) of the EsxA/EsxB heterodimer were downloaded from the Protein Data Bank, and the structure of EsxA was displayed in Swiss-PdbViewer. B, diagrams of the full-length EsxA protein (WT) and the EsxA proteins with an N-terminal deletion (ΔN, residues 1–10 deleted), a C-terminal deletion (ΔC, residues 86–95 deleted), and an N- and C-terminal deletion (ΔN+C). C, SDS-PAGE of purified recombinant WT EsxA and truncated proteins. WT EsxA, ΔN, ΔC, and ΔN+C were expressed, purified to homogeneity, and examined by SDS-PAGE, followed by Coomassie Blue staining.

J-810 CD spectropolarimeter. The secondary structure content was analyzed by inputting the data into the K2D3 server.

RESULTS

Expression and Purification of the Truncated EsxA Proteins with Deletions of the N- and/or C-terminal Flexible Arm—In the reported solution structure of the EsxA/EsxB heterodimer, EsxA is featured as a major helix-loop-helix motif with two flexible arms at both the N and C termini (Fig. 1A) (23). To investigate the role of the N- and C-terminal arms in membrane disruption, we generated truncated EsxA proteins with deletions of the N- and/or C-terminal flexible arm: ΔN, ΔC, and ΔN+C (Fig. 1B). The deletions were generated based on the available structural information from both the solution structure and crystal structure of the EsxA/EsxB heterodimer (23, 31). ΔC was expressed from the pET22b vector in *Escherichia coli* and purified using a similar protocol to that described previously for purification of WT EsxA (26). Interestingly, however, ΔN and ΔN+C were not expressed from the pET22b vector even if the ATG start codon was present. Thus, we expressed both ΔN and ΔN+C as N-terminal GST fusion proteins. GST-ΔN and GST-ΔN+C were purified as described previously for purification of GST-EsxA (26), followed by thrombin cleavage and affinity chromatography on a nickel-Sepharose column. Finally, ΔN, ΔC, and ΔN+C were further clarified on a size exclusion column. The purified WT EsxA and truncated mutant proteins were examined by SDS-PAGE, and all of the purified proteins were shown to be pure and stable proteins (Fig. 1C).

Both the N- and C-terminal Flexible Arms Are Required for Membrane Disruption—To test the effects of N- or C-terminal deletions on EsxA membrane disruption, we applied the purified proteins to the ANTS/DPX fluorescence dequenching assay to measure liposome leakage. ANTS/DPX, the anion/cation fluorophore/quincher pair, is widely used for membrane leakage. ANTS fluorescence is quenched by DPX inside the liposomes, and it is dequenched upon release into the medium. We successfully used this assay previously to measure pH-dependent EsxA membrane disruption (26). Consistent with the previous results, at pH 7.0, WT EsxA and the truncated proteins were inactive in membrane disruption (data not shown). At pH 5.0, relative to WT EsxA, both ΔN and ΔC exhibited an ~40% reduction in membrane disruption, and ΔN+C exhibited a significant ~80% reduction (Fig. 2, A and B). These data strongly suggest that both the N- and C-terminal flexible arms are required for membrane disruption at low pH.

Deletion of Either the N- or C-terminal Flexible Arm Reduces Solvent-exposed Hydrophobicity at Low pH—The extrinsic fluorescence dye ANS is weakly fluorescent in an aqueous environment but becomes highly fluorescent when it binds to hydrophobic sites of proteins (32). In our previous report, we successfully used ANS to measure the solvent-exposed hydrophobicity of EsxA proteins upon acidification (26). Here, we used ANS to measure the solvent-exposed hydrophobicity of WT EsxA and its truncated mutants at either pH 7.0 or 5.0 (Fig. 3, A and B). Consistent with the previous results, at pH 5.0, WT EsxA demonstrated an increase in ANS fluorescence that was significantly higher than that at pH 7.0, suggesting that, upon acidification, WT EsxA undergoes a significant conformational change that results in increased solvent-exposed hydrophobicity. Upon acidification, the truncated proteins also showed an increase in ANS fluorescence to various degrees. Relative to WT EsxA, however, the ANS fluorescence intensity of ΔN, ΔC, and ΔN+C exhibited reductions of ~50, ~22, and ~75%, respectively. This result is consistent with the result obtained in the ANTS dequenching assay, suggesting that both the N- and C-terminal flexible arms are required for membrane disruption.

Upon Acidification, EsxA Converts into a Structure with Increased α-Helical Content—To rule out the possibility that deletions of the N and/or C terminus may cause protein unfolding, we analyzed the CD spectra of WT EsxA and the truncated proteins at both pH 7.0 and 5.0 (Fig. 4). In the reported solution structure and crystal structure of the EsxA/EsxB heterodimer, residues 11–43 and 49–85 of EsxA form two stable helices (23, 31), and a previous CD analysis showed that EsxA has a high helical content (24). Consistent with these studies, the CD spectrum of WT EsxA indicated a typical α-helical protein. As expected, the CD spectra of ΔN, ΔC, and ΔN+C also showed typical α-helical proteins at pH 7.0, suggesting that all of the truncated proteins are folded. Interestingly, WT EsxA, ΔN, and ΔN+C became even more helical at pH 5.0. Quantification of the secondary structure content showed that WT EsxA had an increase in α-helical content from 81 to 94% and that both ΔN

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and ΔN+C had an increase from 68 to 94% (Table 1). The data suggest that, upon acidification, EsxA converts into a more folded and organized molecule by adopting more α-helical structure.

**Intrinsic Tryptophan Emission Spectra of WT EsxA and the Truncated Proteins Are Blue-shifted at Low pH**—To further investigate the acidification-dependent conformational changes in WT EsxA, ΔN, ΔC, and ΔN+C, we measured the intrinsic Trp fluorescence of these proteins at either pH 7.0 or 5.0 (Fig. 5). The Trp emission spectrum is highly sensitive to solvent polarity. The emission of Trp may be blue-shifted (shorter wavelength) if the residue is buried within a native protein, and its emission may be red-shifted (longer wavelength) when the protein is unfolded (33). Consistent with our previous report (26), the Trp fluorescence spectrum of WT EsxA was blue-shifted upon acidification. Similar to WT EsxA, all of the truncated EsxA mutants exhibited a blue shift in their Trp fluorescence spectra. This result is in line with the CD data, and together, they strongly suggest that, upon acidification, EsxA undergoes a significant conformational change and converts into a more folded structure with increased α-helical content.

**Upon Acidification, Both Helices 1 and 2 Insert into Liposomal Membranes**—Although both the N- and C-terminal flexible arms are required for membrane disruption, it is not clear which part(s) of the molecule inserts into the membranes. To directly determine the membrane insertion domains(s) of EsxA, we performed the liposome-based NBD fluorescence assay. NBD is an environmentally sensitive dye that emits a strong fluorescence at 544 nm upon a shift from a polar to a nonpolar environment. Previously, we used NBD to monitor insertion of anthrax toxin into liposomal membranes (27, 28). In this study, we replaced 15 residues in EsxA with cysteine, one at a time, using site-directed mutagenesis. The cysteine mutations were randomly and widely distributed in the esxA gene. However, six EsxA cysteine mutants failed in expression, purification, or NBD labeling, presumably because these positions are critical to EsxA structure (Table 2). To test if the remaining nine NBD-labeled proteins were functional in membrane disruption, we applied them to the ANTS/DPX assay. The results showed that all of the NBD-labeled proteins disrupted liposomal membranes to a similar level as the non-NBD-labeled WT EsxA protein (data not shown). Thus, they were eligible for...
the NBD fluorescence assay. Of the nine NBD-labeled EsxA proteins, three (S35C, G45C, and A60C) showed rapid strong NBD emissions at low pH in the presence of liposomes but not in their absence (Fig. 6A). This clearly demonstrated that the NBD label at these positions inserted into the liposomal membranes and had direct contact with the lipid molecules. Ser-35, Ala-60, and Gly-45 are located in Helix 1, Helix 2, and the turn region between Helices 1 and 2, respectively (Fig. 7, A and B). Combined with the available structural data (see “Discussion” for more details), the NBD data confirm that both Helices 1 and 2 insert into the membrane and form a membrane-spanning pore (Fig. 7B). In this study, we have provided evidence that supports this model of EsxA membrane insertion.

Using nine functional and NBD-labeled EsxA cysteine mutants, we obtained essential evidence that supports the proposed model of EsxA membrane insertion. Ser-35 and Ala-60 are located in Helices 1 and 2, respectively (Fig. 7, A and B). When NBD was used to label S35C or A60C, it showed a rapid, strong, and liposome-dependent emission (Fig. 6), demonstrating that both Ser-35 and Ala-60 are lipid-facing residues. Interestingly, although Gln-34 and Thr-37 are adjacent to Ser-35, the NBD label in Q34C or T37C did not emit fluorescence (Fig. 6), suggesting that Gln-34 and Thr-37 are not lipid-facing residues. This result is in line with the fact that in the solution structure of EsxA, Gln-34 and Thr-37 are located on different helical faces from Ser-35 (Fig. 7B). Similarly, relative to Ala-60,
Val-54 is negative in NBD fluorescence and is located in a different face of Helix 2 (Fig. 7B). More interestingly, NBD emission in G45C was moderate, ~2–3-fold lower compared with S35C and A60C, suggesting that Gly-45 is partially embedded in the membrane. This result is consistent with the predicted position of Gly-45 after membrane insertion, which is at the edge of the membranes (Fig. 7B).

It is worth mentioning that Ser-35 is located in the contact interface between EsxA and EsxB in the heterodimer structure (23, 35). Thus, these data provide indirect evidence supporting the notion that, upon acidification, EsxB is dissociated from EsxA, allowing EsxA to insert into the membranes (25). However, current data from the literature are conflicting as to whether or not the EsxA/Esb heterodimer dissociates at acidic pH. Using the native proteins extracted from the culture filtrate of \( M. \)\( \text{tuberculosis} \), de Jonge et al. (25) showed that EsxA and EsxB dissociated at low pH. In our previous study, however, the recombinant heterodimer was inactive in membrane disruption and showed no aggregation at acidic pH, suggesting that EsxA and EsxB did not dissociate at low pH (26). Consistent with our results, the CD study by Lightbody et al. (35) showed that the complex formed by EsxA and EsxB (proteins purified from \( E. \)\( \text{coli} \)) was too stable to dissociate at low pH. One possible explanation for this discrepancy could be that the mycobacterium-produced proteins possess unique properties that are not present in the \( E. \)\( \text{coli} \)-produced proteins, allowing EsxA and EsxB to dissociate at low pH. These properties might include post-translational modifications, such as N-acetylation of Thr-2 in EsxA. It has been shown that acetylated and non-acetylated EsxA differentially bind to EsxB (36). Moreover, a recent study showed that homeostasis of N-acetylation of EsxA correlates with virulence of \( M. \)\( \text{marinum} \) (37). Although N-acetylation of Thr-2 in EsxA might play a role in dissociation of EsxA and EsxB at low pH, other post-translational modifications and other bacterial and/or cellular factors may be involved in this process.

A striking feature of EsxA structure is the flexible N- and C-terminal arms, in which a number of hydrophobic and aromatic residues play no structural role (23). The data showing

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**FIGURE 5.** Trp fluorescence spectra of WT EsxA and the truncated proteins show a blue shift at pH 5.0. The purified EsxA proteins (WT (A), \( \Delta \)N (B), \( \Delta \)C (C), and \( \Delta \)N+C (D); 10 \( \mu \)M) were incubated in either pH 7.0 or 5.0 buffer for 30 min. The samples were excited at 295 nm, and the emission spectra of Trp fluorescence were recorded at 310–450 nm. The emission spectra of the proteins were calibrated with the emission spectra of the same buffers without proteins. Representative data from at least three independent experiments are shown.

**TABLE 2**

| Mutant | Purification | NBD label | Pore-forming activitya | Membrane insertionb |
|--------|--------------|-----------|------------------------|---------------------|
| Q4C    | +            | +         | +                      | –                   |
| A9C    | +            | +         | +                      | –                   |
| G10C   | +            | +         | +                      | –                   |
| A14C   | +            | –         | –                      | –                   |
| N21C   | –            | –         | –                      | –                   |
| V22C   | +            | +         | +                      | –                   |
| Q34C   | +            | +         | +                      | –                   |
| S53C   | +            | +         | +                      | +                   |
| T37C   | +            | +         | +                      | –                   |
| G45C   | +            | +         | +                      | +                   |
| Q55C   | +            | +         | +                      | +                   |
| W58C   | –            | –         | –                      | –                   |
| A65C   | +            | +         | +                      | –                   |
| A79C   | +            | +         | +                      | –                   |
| G88C   | +            | +         | +                      | –                   |

a Pore-forming activity was measured by the ANTS fluorescence dequenching assay as described under “Materials and Methods” (data not shown).

b Membrane insertion was measured by NBD fluorescence as described under “Materials and Methods,” and the results are shown in Fig. 6.
that ΔN, ΔC, and ΔN+C were defective in membrane disruption (Fig. 2) demonstrated that both the N- and C-terminal flexible arms were required for EsxA to disrupt the membranes. Because the NBD label in Q4C, G10C, or G88C did not emit liposome-dependent fluorescence (Fig. 6), the N- and C-terminal arms are not likely to insert into the membranes. However, we do not exclude the possibility that parts of the N- or C-terminal arm have transient dynamic contacts with the lipid membrane to anchor the molecule on the membranes and/or support insertion of Helices 1 and 2 into the membranes. Current data from our laboratory and others suggest that EsxA forms a pore on the membranes (26, 38). Because the EsxA monomer has a limited molecular size, one can image that EsxA must assemble into an oligomeric complex to form a membrane-spanning pore on the membranes. In this scenario, both the N- and C-terminal arms might play an essential role in intermolecular interactions within the oligomeric pore complex.

One interesting finding in this study is that unlike many other pore-forming proteins, which usually become unfolded (at least in part) upon acidification, EsxA became more folded with increased α-helical content. This finding was evidenced by CD analysis (Fig. 3 and Table 1) and Trp fluorescence (Fig. 4). Upon acidification, the α-helical content of EsxA increased from 81% (pH 7.0) to 94% (pH 5.0). The increase in α-helical content was dependent on the presence of the C-terminal arm because ΔC did not show any increase in α-helical content. By calculation, a 13% increase in α-helical content represents 12–13 amino acids adopting α-helical structure. Coincidently, a recent study suggested that EsxA and other WXG100 proteins have a C-terminal conserved residue pattern (residues 83–95 in EsxA, MASTEGNVTGMFA) (31). The underlined residues are spaced about three residues apart and correspond to a turn of the α-helix, placing them all on the same face of the helix. Our data are consistent with this prediction and suggest that although these residues are shown as a disordered arm in the solution structure of EsxA at pH 7.0, they may adopt α-helical structure upon acidification.

It is also worth mentioning that compared with WT EsxA, both ΔN and ΔN+C showed a decrease in α-helical content from 81 to 68% at pH 7.0 (Table 1). This may be explained by fact that the N-terminal deletion caused partial unfolding of the adjacent helical structure. This may also explain why ΔN and ΔN+C were not expressed in the pET22b vector. Thus, the N-terminal flexible arm appears to have effects on the stability of the EsxA molecule. However, at pH 5.0, the α-helical content

FIGURE 6. The NBD label at Ser-35, Gly-45, and Ala-60 of EsxA proteins exhibits a rapid, strong, and membrane-dependent fluorescence emission. The EsxA proteins were labeled with NBD at the indicated positions and incubated with or without liposomes (Lip) in pH 7.0 buffer for 30 min. Subsequently, the pH was rapidly lowered by adding 0.1 volume of 1 M sodium acetate (pH 4). The solution was continuously stirred in the cuvette. NBD fluorescence emission was recorded as a function of time (excitation at 488 nm and emission at 544 nm). The representative NBD fluorescence curves of S35C, G45C, and A60C are shown in A. The NBD emission at 20 s post-acidification was calculated from three independent measurements and then normalized to that of G45C (set as 1). The relative NBD emission at the indicated positions is shown in B.
of both $\Delta N$ and $\Delta N+C$ increased up to $\sim$94%, suggesting that the majority of the molecules adopted $\alpha$-helical structure.

In summary, this study provides, for the first time, direct evidence that, at low pH, EsxA converts into a membrane protein, with the central helix-turn-helix motif inserting into the membrane and forming a membrane-spanning pore. We have also shown that both the N- and C-terminal flexible arms are required for EsxA membrane disruption. Upon acidification, EsxA converts into a more organized helical structure, with the C-terminal flexible arm adopting $\alpha$-helical structure. This study has provided a foundation for in-depth characterizations of the EsxA pore on membranes in the future. An understanding of the mechanism of EsxA pore formation and the structure of the EsxA pore will facilitate development of novel therapeutics against tuberculosis. Moreover, EsxA is the prototype of the WXG protein family, which includes hundreds of bacterial proteins without known structure and function (31). The knowledge obtained from EsxA can be applied to the study of other WXG proteins.

Acknowledgment—We thank Dr. Barbara Lyon (Department of Chemistry and Biochemistry, New Mexico State University) for providing assistance with using the Jasco J-810 CD spectropolarimeter.

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