Activation of *Serratia marcescens* Hemolysin through a Conformational Change

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Received 28 July 2003/Returned for modification 4 September 2003/Accepted 25 September 2003

For *Serratia marcescens*, secreted hemolysin/ cytotoxin is not only secreted but also activated by an outer membrane protein. Excluding posttranslational processing by mass spectrometry, the conformation of active and inactive ShlA derivatives strongly differed in electrophoretic mobilities, gel permeation chromatography, sensitivity to trypsin, circular dichroism, and intrinsic fluorescence. We concluded that ShlB interacts with ShlA during secretion and imposes a conformational change in ShlA to form the active hemolysin.

*Serratia marcescens* synthesizes a hemolysin (2, 3–9, 13, 18, 25, 28) encoded by the *shlA* gene. It also acts as a cytotoxin on epithelial cells and fibroblasts, where it causes ATP depletion and potassium efflux (15). The ShlA toxin is secreted across the outer membrane by the ShlB protein, encoded by the *shlB* gene (7, 19, 24, 27, 30), thereby distinguishing this type of secretion from all other known secretion systems (3, 4). Homologous hemolysins are formed by *Proteus mirabilis* (33), *Yersinia pestis* (23), *Haemophilus ducreyi* (22, 32), and *Edwardsiella tarda* (10, 17, 31). In addition, the FHA filamentous hemagglutinin of *Bordetella pertussis* (34) and the HMWP1 and HMWP2 adhesins of nontypeable *Haemophilus influenzae* (1, 11) require a B component for cell surface exposure. This kind of secretion has been designated the two-partner secretion pathway to distinguish it from other protein secretion mechanisms and to emphasize its particular properties (20). A conserved region in these proteins (3), which is contained twice in *S. marcescens* ShlA (68-ANPNL and 109-NPNGIS), is important for ShlA activity; replacement of N-69 and N-111 by isoleucine abolishes the hemolytic activity (29).

In addition to secretion of ShlA, ShlB converts ShlA into a hemolysin. In the absence of ShlB, nonhemolytic ShlA (termed ShlA*) remains in the periplasm and displays at most 0.2% of the ShlB-dependent and -secreted hemolytic activity of ShlA (27).

Activation of ShlA* in vitro can be uncoupled from secretion. Crude extracts of cells that synthesize only ShlB activate ShlA* contained in crude extracts of cells that synthesize only ShlA* (21). Highly purified ShlB does not activate highly purified ShlA* unless phosphatidylethanolamine is added (16). ShlA* can also be converted to a hemolysin by addition of an ShlB-secreted and -activated N-terminal fragment of ShlA consisting of 255 residues (ShlA255) (14, 21). ShlA255 itself is nonhemolytic because it lacks the remaining, C-terminal part of ShlA (1,576 residues), which is required for pore formation in erythrocyte membranes (26, 28). ShlA255 also restores the hemolytic activity of ShlAΔ3–97, in which residues 3 to 97 of ShlA are deleted (29).

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mentally more amenable ShlA242 fragment because of its better solubility and stability. Structural differences between active and inactive ShlA242 were examined using four constructs: (i) the secreted, active, N-terminal 242 amino acids plus six histidine residues at the C-terminal end (ShlA242-His6) and (ii) by mutated ShlB136ARSG-secreted but not activated ShlA242-His6, both isolated from the culture supernatant, (iii) ShlA242-His6 synthesized in cells lacking ShlB and therefore not secreted or activated (ShlA242*-His6), isolated from the periplasm, and (iv) ShlA242-His6 lacking a signal sequence, which therefore remained in the cytoplasm and formed inclusion bodies (C-ShlA242*-His6). All ShlA242-His6 derivatives contained an additional 33 amino acid residues between the His6 tag and ShlA242.

ShlA242-His6 was precipitated by (NH₄)₂SO₄ from the culture supernatant and purified on a Ni²⁺-nitrilotriacetic acid (NTA) affinity column. Periplasmic ShlA242*-His6 was isolated from the (NH₄)₂SO₄ precipitate of the periplasmic extract and purified by affinity column chromatography as described above. Cytoplasmic C-ShlA242*-His6 was isolated from the French press lysate of cells in the presence of 6 M urea and then purified by affinity column chromatography. The
N-terminal sequence of all of the isolated derivatives started with AEIVAANGA (determined by Edman degradation), which is identical to the determined ShlA sequence (24). In addition, inactive ShlA242-His₆, which is secreted but not activated by mutant ShlB136 (contains an insertion of the tetrapeptide ARSG between residues 136 and 137), was studied. Only ShlA242-His₆ complemented ShlAΔ3–97 to an active hemolysin (82% lysed erythrocytes, compared to 0% for ShlAΔ3–97 alone). This result shows that the His tag and the additional amino acids in the linker region did not affect the complementing activity of ShlA242.

The electrophoretic mobility of active ShlA242-His₆ in a native polyacrylamide gel, without addition of sodium dodecyl sulfate or mercaptoethanol and without heating, was much higher than that of the three inactive ShlA242-His₆ derivatives isolated from the periplasm and cytoplasm and of an ShlA242-His₆ derivative that was secreted but not activated by the mutant ShlB136 protein (35) (Fig. 1). Upon incubation with trypsin, ShlA242-His₆ was truncated to approximately 90% of the nondegraded size, in contrast to the inactive ShlA242-His₆ derivatives, which were completely degraded (Fig. 2). The differences between secreted and nonsecreted ShlA242-His₆ indicated changes in the primary or secondary structures of cytoplasmic ShlA242-His₆ during export across the cytoplasmic membrane, within the periplasm, during secretion across the outer membrane, or in the culture medium.

The molecular masses of the ShlA242-His₆ derivatives were estimated by gel permeation chromatography in the presence of 6 M urea to avoid aggregation and were found to be 41 kDa for active ShlA242-His₆ and 64, 66, and 60 kDa for ShlA242*-His₆, C-ShlA242-His₆, and secreted but inactive ShlA242-His₆, respectively. None of these values reflect the calculated molecular mass of 29 kDa. They suggest different shapes of the noncomplementing ShlA242 derivatives compared to complementing ShlA242 as a result of distinct conformations. The same chromatography with the buffer containing dithiothreitol did not much alter the chromatographic properties, excluding various disulfide bridges as causes of the distinct elution positions. Active ShlA242-His₆ and inactive ShlA242*-His₆ eluted at positions equivalent to proteins of 38 and 63 kDa, respectively (compared to 41 and 66 kDa under nonreducing conditions). The aberrant chromatographic behavior could also be the result of a covalent modification during the activation. Therefore, the molecular weights of the four ShlA242-His₆ derivatives were determined by quadrupole electrospray mass spectrometry (12, 14). Each of the derivatives had a molecular weight of the calculated size (calculated value for ShlA242-His₆, 29,069; determined value for active ShlA242-His₆, 29,069, and for the inactive derivatives, 29,067, 29,067, and 29,066). This result excludes a covalent modification larger than the conversion of a carboxyl group to an amide. It is unlikely that such a small alteration would affect the properties of the ShlA242-His₆ derivatives as profoundly as has been observed.

The altered electrophoretic mobility, the deviation of the size obtained by gel permeation chromatography from the calculated size, the exclusion of a covalent modification, and the trypsin sensitivity suggested that the inactive ShlA242*-His₆ derivatives assume a conformation different from that of active ShlA242-His₆. To evaluate conformational differences directly, the circular dichroism (CD) was determined. Profound differences between active ShlA242-His₆ and the inactive ShlA242*-His₆ derivatives assume a conformation different from that of active ShlA242-His₆. To evaluate conformational differences directly, the circular dichroism (CD) was determined. Profound differences between active ShlA242-His₆ and the inactive ShlA242*-His₆ derivatives and smaller differences among the inactive ShlA242-His₆ derivatives were found (Fig. 3). The spectra were compared with spectra of standard peptides to estimate the proportion of secondary-structure elements. Only the curves of ShlA242-His₆ and ShlA242*-His₆ were close enough to the calculated curves to allow a comparison. The
We thank R. Süssmuth, Department of Organic Chemistry, University of Tübingen, for the N-terminal protein sequencing and mass spectrometry; A. Kapurniotu, Department of Biochemistry, University of Tübingen, for help with CD spectroscopy; and Karen A. Brune for critical reading of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (BR 3110-2-1) and the Fonds der Chemischen Industrie.

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