Membrane-penetrating Domain of Streptolysin O Identified by Cysteine Scanning Mutagenesis*

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Michael Palmer‡§, Peter Saweljew‡, Ivan Vulicevic‡, Angela Valeva‡, Michael Kehoe‡, and Sucharit Bhakdi‡

From the ‡Institute of Medical Microbiology, University of Mainz, Augustusplatz D55101, Germany and §Department of Microbiology, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, United Kingdom

Streptolysin O (SLO), a polypeptide of 571 amino acids, belongs to a family of highly homologous toxins that bind to cell membranes containing cholesterol and then polymerize to form large transmembrane pores. A conserved region close to the C terminus contains the single cysteine residue of SLO and has been implicated in membrane binding, which has been the only clear assignment of function to a part of the sequence. We have used a cysteine-less active mutant of SLO to introduce single cysteine residues at 19 positions distributed throughout the sequence. The cysteines were derivatized with the polarity-sensitive fluorophore acrylodan, and the fluorescence emission of the label was examined at the different stages of SLO pore assembly. With several mutants, oligomerization on membranes was accompanied by emission blue-shifts, indicating movement of the label into a more hydrophobic environment. These effects were essentially confined to the range of amino acids 213–305. With oligomeric mutants L274C, S286C, and S305C, additional environmental alterations were induced when different non-denaturing detergents were used to dislodge the membrane lipids from the oligomers. The corresponding amino acid residues thus insert into the lipid bilayer during pore formation. Conversely, the spectra of oligomeric mutants A213C and T245C were not affected by detergents. Devoid of contact with the lipid bilayer, these amino acid residues probably participate in the interaction of SLO molecules within the oligomer.

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§To whom correspondence should be addressed. Tel.: 49 6131 17 3125; Fax: 49 6131 39 2359.

1 The abbreviations used are: SLO, streptolysin O; PBS, phosphate-buffered saline.
Binding and Oligomerization of Acrylodan-labeled SLO Mutants on Erythrocyte Ghosts—One hundred μl of the erythrocyte ghost suspension and 50 μg of the labeled mutant protein were mixed, brought to a volume of 0.3 ml with PBS, and incubated for 30 min at 37 °C. Membranes were pelleted by centrifugation (5 min at 10,000 × g), and resuspended with 1 ml of PBS. Wild-type SLO, which is inactivated by sulphydryl derivatization, was mixed with successively increasing amounts of mutant C530A to achieve incorporation into mixed oligomers.

Isolation of Oligomeric Labeled SLO Mutants—The membranes carrying labeled proteins were then solubilized by adding an equal volume of 10% (w/v) sodium deoxycholate, and samples were layered on top of linear sucrose density gradients contained in polyallomer tubes (10 to 50% (w/v), with 5 μl deoxycholate, 20 μl Tris/HC1, 1 mM EDTA, and 100 mM NaCl at pH 8.2). After centrifugation in a vertical rotor (Beckman VTI65–2, 50,000 rpm for 60 min in a Beckman Optima L60), five equal fractions were harvested from each gradient. Aliquots drawn from the fractions were diluted into 0.25% SDS, incubated at 37 °C for 15 min, and assayed for fluorescence to estimate the oligomer yield. The lower two gradient fractions were pooled and dialyzed against 5 mM deoxycholate, 20 μl Tris/HC1, 1 mM EDTA, and 100 mM NaCl, pH 8.2 (24 h at 4 °C).

Preparation of Membrane-bound Monomeric Labeled Toxin Mutants—Labeled protein (0.25 μg) was added to 20 μl of erythrocyte ghost suspension, brought to a volume of 1 ml with PBS, and incubated on ice for 5 min. Membranes were pelleted by centrifugation (5 min at 10,000 × g) and resuspended with 1 ml of PBS. The samples were kept at less than 4 °C throughout. To control for the functional activity of the protein, oligomer formation was then induced by adding a 10-fold excess of the unlabeled mutant C530A and by raising the temperature to 37 °C for 5 min.

Spectrofluorimetry—Emission spectra were recorded in a SPEX Fluoromax spectrofluorimeter (excitation wavelength, 365 nm; excitation and emission bandpasses, 2.1 nm; scanning interval, 1 nm). For each labeled mutant, samples of both monomeric protein in PBS and oligomeric protein on erythrocyte ghost membranes were examined. Where emission shifts were observed upon oligomerization, spectra were also recorded with membrane-bound monomers (in this case, the sample chamber was chilled to 3 °C) and, with isolated oligomers, solubilized with 2 μl deoxycholate both with and without the addition of the nonionic detergent ethylpolyoxyethylene glycol (1.25% by volume; Roth, Karlsruhe, Germany) or 10 μl octylthioglycoside (Sigma). Spectra were corrected for background fluorescence of the respective buffers and erythrocyte ghost suspensions.

RESULTS

Construction and Localization of Cysteine Mutants—Based on the cysteine-less active SLO mutant C530A (12), the following 19 single cysteine replacement mutants were constructed: S101C, N110C, N155C, T188C, A213C, H237C, T245C, S259C, L274C, S286C, S305C, N310C, A334C, S358C, T376C, K403C, S423C, S457C, T495C.

Purification of the Mutant Proteins—The recombiant fusion proteins (consisting of maltose-binding protein fused N-terminally to the SLO moiety) were cleaved with trypsin, which releases two N-terminally truncated polypeptides of 490 and 489 amino acids, respectively. These molecules retain full hemolytic activity (18) and all sequence elements sharing homology—

Quality of Labeled Cysteine Mutants—Labeling with acrylodan was sulphydryl-specific, as was evident from the lack of reaction with the cysteine-less mutant C530A. Except wild-type toxin (which was inactivated), the labeled proteins retained unaltered specific hemolytic activities. With all mutants, oligomerization yields were above 50%.

Acrylodan Emission Shifting upon SLO Oligomer Formation—The fluorescence emission of thiol-derivatized acrylodan depends strongly on solvent polarity (19). Fig. 1A displays the emission spectra of the labeled mutant A213C both as monomer in solution and as membrane-associated oligomer. In the monomer, acrylodan emits maximally at 516 nm, indicating a hydrophilic environment consistent with a superficial location of the dye. Upon oligomerization on erythrocyte membranes, the emission is markedly blue-shifted (the maximum now observed was 489 nm), which means that the label has moved into a more apolar environment. By contrast, no significant spectral shift is seen with mutant T376C (Fig. 1B).

Fig. 2 displays the acrylodan emission maxima of all mutants as monomers in solution, and as membrane-associated oligomers, respectively. It is seen that, as monomers, most mutants afford a more or less hydrophilic environment to the probe. Upon membrane association and oligomerization, a small number of mutants display a markedly blue-shifted fluorescence emission, indicating that the probe has moved to a more hydrophobic environment. With the exception of mutant K403C, all of these effects cluster between amino acid residues 213 and 305.

The Changes in Environmental Polarity Are Linked to the Oligomerization Step of SLO Activation—To correlate the effects observed with the two basic steps of toxin action (i.e. monomer binding and oligomer formation), the respective mutants were also analyzed in a monomeric membrane-bound state. As dis-
played for mutant S286C (Fig. 3), monomer binding did not shift the acrylodan emission spectra; all of the characteristic shifts emerged, however, after oligomerization was induced by raising the temperature and the concentration of SLO.

Effect of Detergent Solubilization on Acrylodan Emission of Oligomers—It appears straightforward to interpret the apolar environment detected by some acrylodan-labeled mutants as the hydrophobic core of the lipid bilayer. Then, replacing the membrane lipids by detergents should impose another change of environment to the acrylodan molecules. Furthermore, this effect should vary with the particular detergent used.

All mutants that had yielded blue-shifts upon oligomerization on membranes were re-isolated by membrane solubilization with deoxycholate and density gradient centrifugation. The oligomers thus obtained were exposed to different non-denaturing detergents. Fig. 4 displays the acrylodan emission spectra of L274C in the presence of deoxycholate or ethylphenylpolyethylene glycol, respectively. The blue-shift arises concomitantly with oligomerization. The same was observed with all other blue-shifting labeled mutants (data not shown).

The original model assumes that the labeled amino acid residue had inserted into the membrane during oligomerization. The emission maxima of the detergent-solubilized oligomers are not significantly affected by detergent exchange. The acrylodan label is thus devoid of direct contact with detergent molecules, probably buried inside the oligomeric protein.

Fig. 5 summarizes the results obtained with the relevant mutants (as well as the wild-type toxin). Plotted are the emission maxima of detergent-solubilized oligomers obtained after detergent exchange. The detergent exchange is indicated by the labeled transition at the emission maxima. The same was observed with all other blue-shifting labeled mutants (data not shown).

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FIG. 2. Acrylodan emission maxima of labeled cysteine mutants in both free monomeric and membrane-associated oligomeric form. Positions of labeled amino acids that display blue-shifted emission spectra after oligomer formation on membranes are indicated. The most pronounced blue-shifts are observed with mutants L274C and S286C.

FIG. 3. Acrylodan emission spectra of labeled mutant S286C in free monomeric (fm), bound monomeric (bm) and membrane-associated oligomeric (ol) form. The blue-shift arises concomitantly with oligomerization. The same was observed with all other blue-shifting labeled mutants (data not shown).

FIG. 4. Acrylodan emission spectra of detergent-solubilized oligomeric labeled cysteine mutants. A, mutant L274C. Addition of the detergent ethylphenylpolyethylene glycol in excess to deoxycholeate-solubilized oligomers influences both the emission maximum and the fluorescence intensity. Detergents (having replaced the lipid bilayer) thus directly contribute to the environment of acrylodan, which indicates a membrane-embedded location of the labeled amino acid residue in the oligomer. B, mutant A213C. Neither fluorescence intensity nor emission maximum are affected by detergent exchange. The acrylodan label is thus devoid of direct contact with detergent molecules, probably buried inside the oligomeric protein.

FIG. 5. Summary of the results obtained with the relevant mutants (as well as the wild-type toxin). Plotted are the emission maxima of detergent-solubilized oligomers obtained after detergent exchange. The detergent exchange is indicated by the labeled transition at the emission maxima. The same was observed with all other blue-shifting labeled mutants (data not shown).
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which may face the cytoplasmic side of the target cell membrane. The extension of a probable additional membrane-associated domain around residue 403 remains to be elucidated, as does the spatial relationship of both membrane-contacting regions.

A detergent-exchange assay was used to discriminate contact of labeled amino acid residues with the lipid bilayer from their burial inside the oligomer. This assay yielded coincident variations with both intensity and spectral shifts of label fluorescence. However, although the fluorescence intensity of acrylodan regularly increases along with the spectral blue-shift caused by various organic solvents (19), no constant relationship of this kind was observed with the non-denaturing detergents used here. Presumably, this discrepancy is related to the anisotropic environment prevailing near the surface of detergent micelles as opposed to the homogeneous one afforded by organic solvents.

Residues 213 and 245 move to a proteinaceous hydrophobic environment during oligomerization. With the related pneumolysin, the epitopes of monoclonal antibodies interfering with the oligomerization of bound toxin have been mapped to this region (8). The most straightforward explanation would be that it participates in pore assembly and becomes occluded between adjacent subunits of the oligomer. Alternatively, it is possible that the environmental and functional effects observed are related to an internal conformational change of the monomer accompanying oligomerization. Additional experiments are under way to discriminate among these possibilities.

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FIG. 5. Effect of detergents on acrylodan fluorescence of oligomeric labeled cysteine mutants. To deoxycholate-solubilized oligomers, an excess of the detergent ethylphenylpolyethylene glycol was added. Shifts in emission maximum are given in nanometers (nm). As a relative measure of fluorescence intensity, the area ratio of the respective spectra (compare Fig. 4) is given ( ). With the two most N-terminally located mutants (A213C and T245C), either parameter remains largely unaffected by change of detergents. The other tested mutants (L274C, S286C, S305C, and K403C) and the labeled wild-type cysteine at position 530 display changes of both fluorescence intensity and emission maxima. These positions are thus in touch with detergents and probably were embedded in the lipid bilayer prior to detergent solubilization of membranes.

detein environment. By contrast, the residual mutants and the labeled wild-type SLO clearly display variation of both parameters depending on the detergent. We conclude that the respective amino acid residues attain a membrane-inserted localization concomitantly with SLO pore assembly. Entirely consistent results were obtained with a third detergent (octylthioglucoside; data not shown).

DISCUSSION

Oligomerizing, pore-forming toxin molecules like SLO must carry functional domains responsible for both membrane binding of monomers and intersubunit interaction within the oligomer. In addition, oligomers regularly display increased hydrophobicity (3, 20), which reflects their more intense membrane interaction and requires that, during oligomerization, parts of the polypeptide chain undergo conformational changes to expose additional hydrophobic surfaces. The functional domains involved in any of these steps should be subject to distinct environmental changes.

As a reporter signal of such changes, the polarity-sensitive fluorescence of IAEDANS (21) or acrylodan (14) attached to engineered single cysteine residues has been used. In the present study, a set of 19 cysteine replacement mutants distributed along the whole sequence at a distance of roughly 25 amino acids was constructed to screen for environmental effects of SLO pore assembly.

With the sole exception of mutant K403C, all of the environmental effects clustered between residues 213 and 305, a region that appears to be of crucial importance for SLO activity. Labeled residues 274, 286, and 305 become membrane-embedded concomitantly with toxin oligomerization. Within this part of the sequence, periods of roughly 3–4 amino acids display an alternating pattern of hydrophilicity and hydrophobicity (22). We thus propose that, within the SLO oligomer, the region comprising residues 274–305 forms an amphiphilic helix lining the aqueous lumen of the pore. The length of this stretch would suffice to traverse the lipid bilayer twice. Positioned in the middle is a cluster of four charged amino acids (K287–K291),

![Graph](https://example.com/graph.png)
