Streptolysin O: Inhibition of the Conformational Change during Membrane Binding of the Monomer Prevents Oligomerization and Pore Formation†

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ABSTRACT: Streptolysin O is a four-domain protein toxin that permeabilizes animal cell membranes. The toxin first binds as a monomer to membrane cholesterol and subsequently assembles into oligomeric transmembrane pores. Binding is mediated by a C-terminally located tryptophan-rich motif. In a previous study, conformational effects of membrane binding were characterized by introducing single mutant cysteine residues that were then thiol-specifically derivatized with the environmentally sensitive fluorophor acrylodan. Membrane binding of the labeled proteins was accompanied by spectral shifts of the probe fluorescence, suggesting that the toxin molecule had undergone a conformational change. Here we provide evidence that this change corresponds to an allosteric transition of the toxin monomer that is required for the subsequent oligomerization and pore formation. The conformational change is reversible with reversal of binding, and it is related to temperature in a fashion that closely parallels the temperature-dependency of oligomerization. Furthermore, we describe a point mutation (N402E) that, while compatible with membrane binding, abrogates the accompanying conformational change. At the same time, the N402E mutation also abolishes oligomerization. These findings corroborate the contention that the target membrane acts as an allosteric effector to activate the oligomerizing and pore-forming capability of streptolysin O.

Streptolysin O (SLO)[1] is secreted by *Streptococcus pyogenes* and belongs to the thiol-activated cytolysins of gram-positive bacteria, which among the bacterial pore-forming toxins are distinguished by their strict selectivity for membranes containing cholesterol. The requirement for the sterol comes to the fore already in the very first step of toxin action, i.e., in binding of monomeric SLO to the target membrane (*1*), which precedes formation of the exceptionally large oligomeric toxin pore (*2*).

The designation of the cytolysin family derives from the presence of a unique cysteine residue that, upon covalent modification, renders the molecules incapable of membrane binding and lysis. The cysteine is located within a C-terminally located conserved motif that is rich in tryptophans; these likewise participate in membrane binding as shown by mutagenesis experiments (*3*−*5*). The putative role in primary binding of this conserved motif fits well with the three-dimensional structure of the cytolysin monomer in solution, which can be inferred from that of the homologous toxin perfringolysin O (*6*) since both proteins share 65% of sequence identity (without any gaps or insertions; *7*) throughout the range covered by the perfringolysin O crystal structure. The cytolysin molecule exhibits an elongated shape comprised of four domains. The tryptophan-rich binding motif assumes an exposed position within the C-terminal domain 4 at the far end of the molecule (Figure 1).

Despite its sensitivity to covalent modification, the single cysteine residue of streptolysin O (C530; *8*) and pneumolysin (*9*) could be replaced with alanine without affecting function. This finding has provided molecules devoid of free sulfhydryl groups, hence permitting the introduction of mutant single cysteines, which in turn facilitate the site-directed, thiol-selective attachment of polarity-sensitive fluorescent probes. The latter approach has permitted identification of several amino acid residues that experience changes in environment concomitantly with membrane binding, among these amino acids A248 and A266 (*10*). These residues are located within domains 1 and 3, respectively, hence remote from the putative membrane-binding region (cf. Figure 1). This suggests that the effects of membrane binding to these labeled residues are due to local changes of conformation rather than to their immediate contact with the lipid bilayer. On the other hand, the amino acid residues in question are part of sequence elements which are involved in oligomer assembly (residue 248; *10, 11*) and concomitant membrane insertion (residue 266; *12, 13*). It has thus been proposed that the conformational changes upon monomer binding correspond to an allosteric transition which in turn triggers the subsequent event of oligomerization and pore formation. While this concept of allosteric activation by the target lipid bilayer is novel to the class of pore-forming proteins and possibly relevant to further members, the supporting experimental evidence has been incomplete. In the present study, the

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[1] Abbreviations: acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; BSA, bovine serum albumin; IAEDANS , *N*-(iodoacetaminoethyl)-1-naphthylamine-5-sulfonic acid; PBS, phosphate-buffered saline; SLO, streptolysin O.

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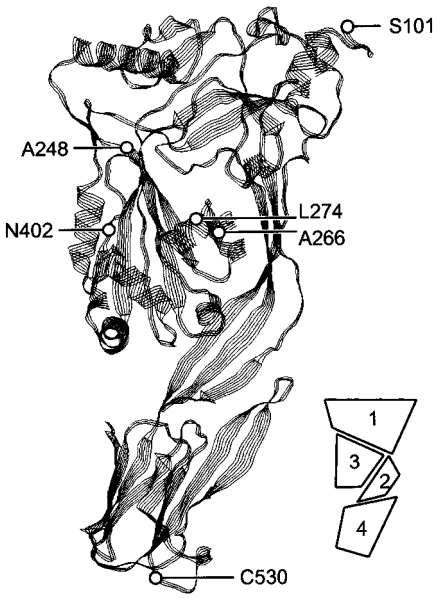

FIGURE 1: Structure of the streptolysin O monomer in solution (based on the crystal structure of the homologous molecule perfringolysin O; *6*). The molecule has an elongated shape comprised of four domains (see sketch). Indicated are the approximate locations of several point mutations employed in this study. Residue S101 of SLO corresponds to the far N-terminus of the crystallized perfringolysin O molecule. Residues A266, L274, and N402 are part of domain 3, whereas residue A248 is located near the "hinge" between domains 1 and 3. The tryptophan-rich motif within domain 4 (centered around the wild-type cysteine at position 530) is involved in primary binding to membrane cholesterol. This cysteine was first changed to alanine in order to enable introduction of unique cysteines to the other locations.

interaction of the streptolysin O monomer and its target membrane was characterized by additional spectroscopic experiments. The collective findings corroborate the hypothesis that the membrane behaves as an allosteric effector that renders the bound cytolysin molecule oligomerization-competent.

MATERIALS AND METHODS

Construction, Expression, Purification, and Fluorescent Labeling of Mutant Proteins. Mutagenesis was performed by polymerase chain reaction (*14*) and confirmed by DNA sequencing. The mutant proteins were expressed in fusion with the *Escherichia coli* maltose binding protein and purified by successive affinity and hydroxyapatite chromatography as described (*15*). Fluorescein-5-maleimide, *N*-(Iodoacetaminoethyl)-1-naphthylamine-5-sulfonic acid (IAEDANS) and 6-acryloyl-2-(dimethylamino)naphthalene (acrylodan) were obtained from Molecular Probes (Eugene, OR). For fluorescent labeling of protein thiol groups, the reagents were dissolved to 0.1 M in dimethyl sulfoxide and were then rapidly diluted to 0.25−0.5 mM with the mutant proteins dissolved in phosphate-buffered saline (PBS; pH 7.4). After incubation at 4 °C for 30−60 min, excess reagent was removed by hydroxyapatite chromatography (acrylodan) or gel filtration. Purity of the labeled proteins was verified by SDS−PAGE. The stoichiometry of labeling was determined spectrophotometrically and ranged from 0.7 to 0.9. Specificity of labeling for cysteines was confirmed by exposure to the reagents of the cysteine-less mutant C530A which was not appreciably labeled.

Hemolytic Titration. Serial 2-fold dilutions of the mutant proteins were prepared in a microtiter plate with PBS/0.1% BSA. Rabbit erythrocytes were admixed to 1.25% and plates incubated at 37 °C for 30 min before visual reading of the hemolytic titer.

Characterization of Oligomerization by Gel Permeation Chromatography. The IAEDANS-labeled mutant proteins (100 μg) were allowed to oligomerize for 15 min at 37 °C on liposomes consisting of egg yolk phosphatidylcholine with 40% cholesterol (2.5 mg total lipids) and prepared by membrane extrusion (*16*). The samples were solubilized in the cold by adding Triton X-100 to 0.5% and chromatographed over a Sephacryl S300-column (16 mm/60 cm) equilibrated with 20 mM Tris, 100 mM NaCl, 0.1% Triton $\times$100 (pH 8.3). Fractions of 2.8 mL were collected and analyzed for label fluorescence.

Preparation of Washed Sheep Erythrocyte Membranes. Five hundred microliters of pelleted sheep erythrocytes were washed 3 times in PBS and lysed osmotically in 5 mM Tris/HCl pH 7.5, and the membranes were pelleted by centrifugation (5 min, 10000*g). They were then repeatedly washed by centrifugation until the supernatant remained clear. The membranes were finally resuspended in 500 μL of PBS.

Assay of Binding of Fluorescently Labeled Streptolysin O mutants to Sheep Erythrocytes. To confirm binding of the nonlytic labeled mutants to membranes, the proteins were dissolved at 1 μg/mL in PBS/0.1% BSA, and sheep erythrocytes were added to 2.5%. The samples were incubated for 5 min on ice to allow for binding, and the cells were then pelleted by centrifugation. The fraction of unbound toxin in the supernatant was determined by label fluorescence (see below), whereby control samples to which no red cells had been added served as reference.

Spectrofluorimetry. The composition of the samples is given in the Results section, whereby the buffers consisted of sodium chloride (150 mM) with 0.05 mg/mL bovine serum albumin buffered with 25 mM of either phosphate (pH 7.0 or 7.5) or Tris (pH 8 or above). Emission spectra were recorded in a SPEX Fluoromax spectrofluorimeter thermostated at the temperatures indicated in the Results section. Excitation was at 365 (*340, 488*) nm, and emission was recorded from 400 to 600 nm (400−600, 500−540 nm) for acrylodan (IAEDANS, fluorescein). Spectral band-passes were 8.5 or 4.2 (excitation) and 2.1 nm (emission). In the quenching experiments, 5-doxyl-stearic acid or 4-(*N*,*N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (Molecular Probes; dissolved to 5 mM in DMSO) were incrementally added to the samples; the label fluorescence was numerically corrected for dilution. Cuvettes with narrow lumen (2 mm in the emission axis) were used to minimize spectral distortions due to scattering of emitted light by the erythrocyte ghosts. Under these conditions, ghost spectra obtained with or without unlabeled SLO were virtually identical at both low and high temperature, indicating that light scattering by the membranes was only minimally affected by binding and oligomerization of the toxin. Accordingly, all spectra were subtracted with the appropriate buffers or membrane suspensions.

RESULTS

Amino Acid Residues 248 and 266 Do Not Contact the Lipid Bilayer in the Membrane-Bound State of the Monomer.

Table 1: Functional Properties of SLO Mutants Employed in This Study[a]

amino acid substitutions	activity without SH derivatization		activity with SH derivatization		ref
	B	O/H	B	O/H	
S101C	+	+	+	+	*12*
A248C	+	+	+	−	*10*
A266C	+	+	+	+	*10*
L274C	+	+	+	+	*12*
N402C	+	(+)	+	−	*33*, this study
N402E/S101C	+	−	+	−	this study
N402E/A248C	+	−	+	−	this study
N402E/A266C	+	−	+	−	this study

[a] All mutants were derived from the cysteine-less, hemolytically active mutant C530A (*8*), although this mutation is not referred to in the designations employed here. Thiol-specific (SH−) derivatization was performed with either acrylodan, IAEDANS, or fluorescein maleimide as indicated in the text. B: membrane binding; O/H: oligomerization and hemolysis. Brackets indicate reduced specific activity (with respect to wild-type SLO).

The present paper is concerned with the hypothesis of allosteric interaction between the streptolysin O monomer and its target membrane. This hypothesis arose from the observation of spectral shifts in the fluorescence emission of the polarity-sensitive dye acrylodan attached to several mutant cysteine residues that occur concomitantly with membrane binding (*10*; cf. Figure 6A and B).

The proposed interpretation of these spectral shifts depends on two conditions. The first prerequisite is that the membrane-associated labeled SLO under study does not form oligomers. The pore oligomers of functionally active SLO are stable in the presence of nondenaturing detergents, and they therefore can be detected by membrane solubilization and subsequent size fractionation of the labeled protein (*2, 17*). In this manner, it has previously been shown that the formation of detergent-resistant oligomers from membrane-bound monomers can be efficiently suppressed at the temperature conditions (4 °C) and toxin concentration also applied in this study (*10*; cf. Figure 5 for a similar experiment).

The second prerequisite states that, in the bound SLO monomer, the labeled residues in question must remain outside the lipid bilayer; otherwise, the emission shifts observed could not unambiguously be ascribed to alterations of protein conformation. We therefore employed the lipophilic molecule 5-doxyl-stearic acid to examine the spatial relationship between two of these amino acid residues (A248C and A266C) and the target membrane. 5-Doxyl-stearate inserts into lipid bilayers, where its nitroxide moiety quenches the fluorescence of neighboring fluorophores in a dose-dependent manner (*18*). This could be demonstrated with the acrylodan-labeled mutant L274C (*12*; Table 1) in its membrane-associated oligomeric form: with stepwise addition of quencher, the fluorescence of the label became progressively reduced in intensity (Figure 2B), thus confirming the previous finding that residue 274 inserts into the lipid bilayer during oligomerization (*12*). In contrast, when the labeled mutants A248C and A266C were bound to membranes as monomers, no fluorescence quenching occurred (Figure 2B). Very similar observations were made with 4-(*N*,*N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (not shown). The quenching nitroxide moiety of this molecule resides at the membrane

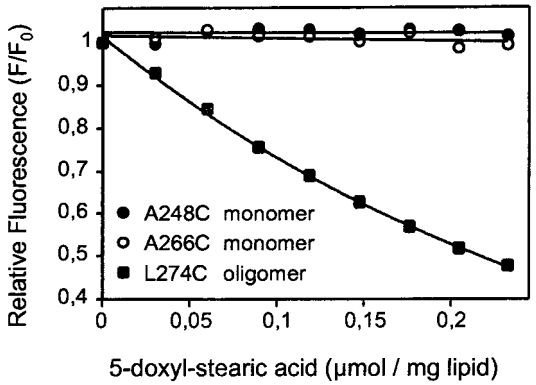

FIGURE 2: Fluorescence quenching of membrane-associated acrylodan-labeled cysteine mutants. The mutant proteins were bound to washed sheep erythrocyte membranes; the membranes were collected by centrifugation and resuspended. The samples were supplemented with 5-doxyl-stearic acid in a stepwise fashion, whereby the label fluorescence was assayed after each increment. Throughout the experiment, the samples were kept below 4 °C, with the sole exception that the initial incubation of L274C with the membranes was at 37 °C in order to induce oligomerization. With the latter mutant, the fluorescence intensity progressively decreased with increasing amounts of the quencher, indicating contact of the labeled residue with the quencher inside the lipid bilayer. No such decrease of fluorescence was apparent with the monomeric mutants A248C and A266C.

surface (*19*). Nitroxide compounds should effect fluorescence quenching within a range of 1−1.2 nm (*20*). From the absence of quenching, we therefore conclude that residues 248 and 266 had neither penetrated nor contacted the lipid bilayer. In contrast, oligomerization of mutant A266C rendered the labeled residue accessible to the quenchers, albeit to a lesser extent than with mutant L274C (not shown). The collective findings substantiate the previous contention that domain 3 stays remote from the membrane in the bound monomeric state (*6, 12*) but partially inserts concomitantly with oligomerization (*12, 13*). They thereby support the view that the change in environmental polarity imposed to residues 248 and 266 by membrane binding is due to a conformational transition of the monomer.

Conformational Transition of the SLO Monomer upon Membrane Binding Is Temperature-Dependent and Reversible with Reversal of Binding. All spectroscopic experiments on the membrane-bound monomer reported so far had been facilitated by the fact that oligomerization (which proceeds rapidly at 37 °C) is suppressed at low temperature (*1*). The mechanism underlying this temperature-dependency has remained unclarified; however, the possibility now arose that the membrane-bound monomer might assume different conformations that in turn might differ in their capacity to oligomerize. To test this hypothesis, we employed the mutant A248C. The latter protein is rendered incapable of initiating oligomerization by derivatization with acrylodan (*10*), so that the monomer can be studied on membranes at both low and high temperatures. Figure 3A displays the acrylodan spectra of A248C in solution and on sheep erythrocyte ghosts at 4 and 36 °C. At either temperature, the emission maximum of the monomer in solution was around 504 nm, suggesting that its solution conformation is largely unaffected by temperature. Upon membrane binding, the emission spectrum was slightly blue-shifted at 4 °C but markedly shifted at 36 °C. Between these two extremes, the shift increased steadily with temperature, leveling off above 30 °C (Figure 3B). Thus,

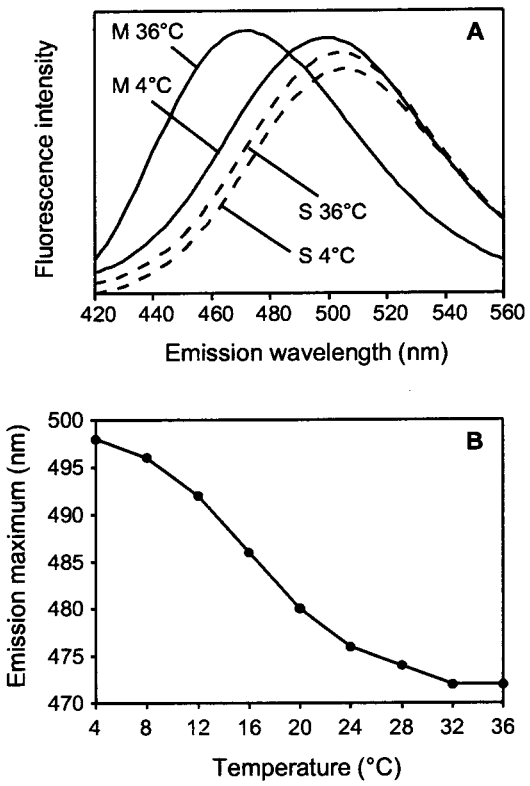

FIGURE 3: Effect of temperature on the conformation of monomeric mutant A248C. (A) Acrylodan-spectra of the labeled protein in solution (S) and on sheep erythrocyte membranes (M) at 4 and 36 °C. While in solution the spectrum was hardly affected by increasing the temperature, the emission blue-shift associated with the membrane-bound state was much more pronounced at 36 °C; the emission maxima were 472 nm at 36 °C and 494 nm at 4 °C. (B) Emission maxima of the membrane-bound monomer at varying temperatures.

it appears that the conformational transition invoked by membrane binding is curtailed in the cold and is only completed at physiological temperatures. The emission maximum returned to longer wavelengths when the samples were re-cooled (data not shown). These findings are in line with the assumption that the membrane-bound monomer partitions among (at least) two different conformational states and that the thermal modulation of this conformational equilibrium underlies the marked temperature-dependency of oligomerization.

The concept of allostery would imply that the structural change induced by the ligand is reversed upon its dissociation from the protein. We therefore sought conditions that would permit observation of monomeric SLO following its dissociation from the target membrane. In the experiment depicted in Figure 4, sheep erythrocyte membranes were laden with acrylodan-labeled A248C at pH 7.0, pelleted, and then resuspended with buffers varying in pH. After incubating for 10 min at 37 °C to allow for equilibration, emission spectra were recorded and the membrane-associated toxin fractions determined. It is seen that, with increasing pH, the labeled protein is progressively desorbed from the membrane. Concomitantly, the emission maximum of the label was shifted back to longer wavelengths. This indicates reversal of the conformational transition associated with binding, which is in line with the allosteric character of the interaction between the monomeric SLO molecule and the target lipid bilayer.

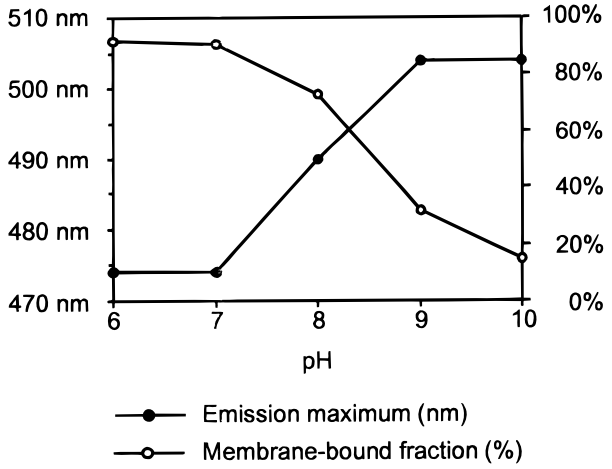

FIGURE 4: Dissociation of mutant A248C from sheep erythrocyte membranes is accompanied by reversal of the conformational change associated with membrane binding. The acrylodan-labeled protein was bound to the membranes at pH 7.0. The membranes were recovered by centrifugation and resupended at various pH values. Following incubation for 10 min, the fluorescence emission was examined and the bound fraction of the toxin was determined by centrifugation.

Conformational Transition of the Streptolysin O Monomer Associated with Membrane Binding Is Abolished by a Point Mutation (N402E) that also Inhibits Oligomerization. Our hypothesis of allosteric interaction between the SLO monomer and the target membrane would predict that if a mutation interfered with the conformational change of the toxin, it should also affect its capacity to oligomerize. In the following, evidence is presented that the predicted phenotype is displayed by the mutation N402E.

The functional importance of amino acid residue N402 was initially recognized by construction of a cysteine substitution mutant. When this mutation (N402C) was introduced into the cysteine-free background of the fully active mutant C530A (*8*), the ensuing protein exhibited a partial loss of pore-forming activity as revealed by hemolytic titration. Following modification of the mutant cysteine with fluorescein maleimide, hemolysis was entirely abrogated. Reduction of hemolytic activity to less than 0.1% was also observed upon introduction of glutamic acid instead of cysteine. To facilitate further functional characterization of the latter mutation (N402E), it was combined with the mutant cysteine residue S101C that can be labeled with a variety of fluorophores without affecting hemolysis (*12*). The fluorescein-derivatized combined mutant S101C/N402E readily bound to erythrocyte membranes (Figure 5A). The loss of hemolysis should therefore be related to the second step of toxin action, i.e., to oligomerization. The capacity of S101C/N402E to form oligomers was analyzed in the experiment shown in Figure 5B. The mutant protein, thiol-specifically labeled with *N*-(iodoacetaminoethyl)-1-naphthylamine-5-sulfonic acid (IAEDANS), was incubated with liposomes prepared from phosphatidylcholine and cholesterol. The sample was then solubilized with Triton X-100 and analyzed by gel filtration on Sephacryl S300. Formation of oligomers by the lytically active mutant S101C was evident from elution of the protein in the column exclusion volume. In contrast, the double mutant S101C/N402E was eluted in monomeric form. Thus, the N402E mutation indeed inhibits oligomerization.

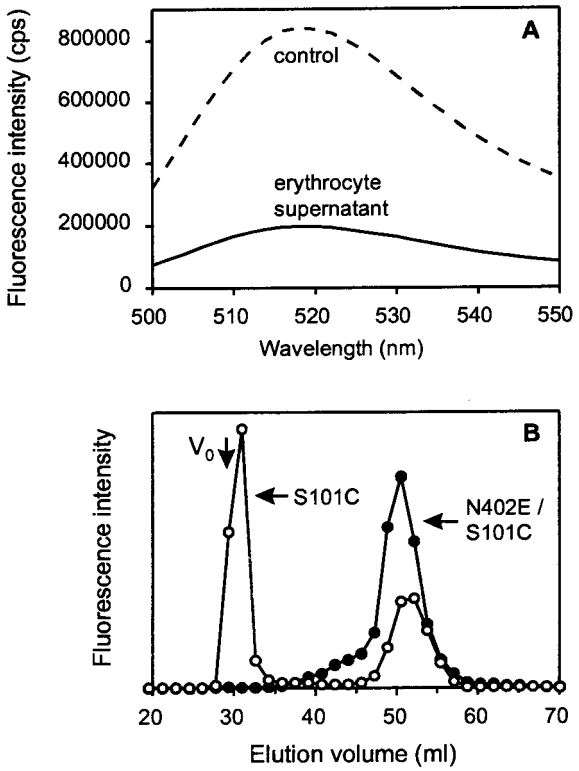

FIGURE 5: Nonlytic N402E substitution mutants bind to cell membranes but do not oligomerize. (A) The double mutant N402E/S101C, thiol-specifically derivatized with fluorescein maleimide, was incubated at 5 μg/mL with 2.5% (v/v) sheep erythrocytes. After 5 min, the cells were removed by centrifugation and the supernatant fluorimetrically assayed for unbound protein. Control: labeled toxin that had not been incubated with erythrocytes. (B) N402E/S101C, thiol-specifically derivatized with IAEDANS, was added to liposomes containing cholesterol and incubated at 37 °C. The sample was solubilized with Triton X-100 and chromatographed on Sephacryl S300, and the column fractions were analyzed for AEDANS fluorescence. The mutant protein peak was virtually indistinguishable from a monomer control sample not incubated with membranes (not shown). In contrast, the lytically active protein S101C-AEDANS, following incubation with liposomes and solubilization, was largely recovered in the exclusion volume (V_0), indicating oligomerization.

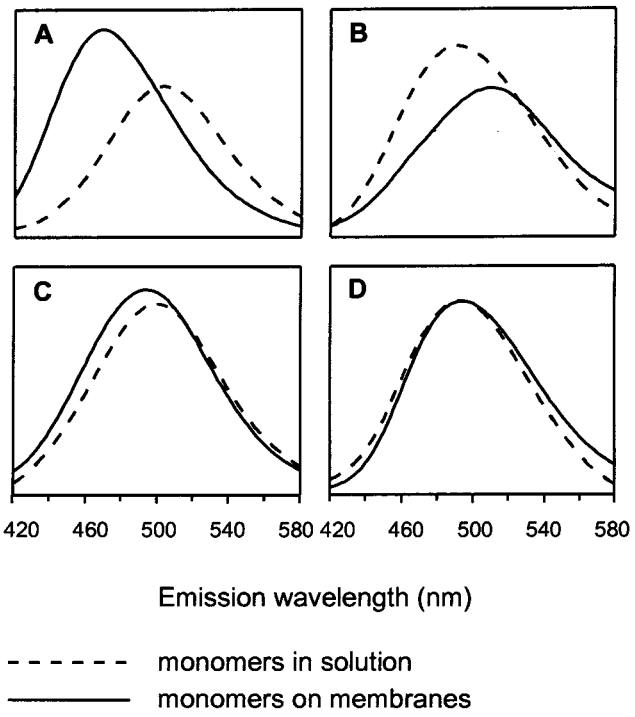

FIGURE 6: Acrylodan emission spectra of monomeric mutants (A) A248C, (B) A266C, (C) N402E/A248C, (D) N402E/A266C, in solution or bound to sheep erythrocyte membranes. Mutant A266C (B) was examined at 4 °C in order to suppress oligomerization; all other spectra were acquired at 25 °C. The two single mutants A248C (A) and A266C (B) exhibited pronounced emission shifts upon membrane binding, indicative of concomitant conformational changes (*10*). In contrast, only very slight spectral effects of binding were detected with the two combined mutants (C and D).

To examine its effect upon the conformation of the membrane-bound monomer, we transferred the N402E substitution into the mutant proteins A248C and A266C. As expected, the respective double mutants readily bound to erythrocytes but were again nonhemolytic. In the case of the double mutant A266C/N402E, this phenotype could again be assigned solely to the N402E substitution, since the corresponding single mutant A266C retains full hemolytic capability (*10*). The double mutants were thiol-specifically derivatized with acrylodan and then bound to sheep erythrocyte ghosts. The respective acrylodan emission spectra in solution and on membranes are given in Figure 6; for comparison, the spectra of the corresponding single mutants A248C (A) and A266C (B) are also shown. With the latter, the spectral shifts induced by binding to the membrane indicate changes in environmental polarity which reflect the concomitant conformational transition. That the shifts occurred in opposite directions indicated that the labeled residue had moved to a less polar environment with A248C but to a more polar one in the case of A266C. In contrast, the acrylodan spectra of both A248C/N402E (C) and A266C/N402E (D) were only minimally influenced by interaction of the proteins with the membrane. Thus, the local change of conformation, which is normally imposed upon amino acid residues 248 and 266 by membrane binding, is essentially abolished by the N402E mutation. In conclusion, inhibition of the conformational transition coincided with the loss of oligomerizing capacity.

DISCUSSION

The formation of transmembrane pores is widespread as a mode of action among cytotoxic proteins of both bacterial and eukaryotic origin. In particular, it is one of the key principles operative in the struggle between microbes and the mammalian immune system, and the first prototypes of pore-forming proteins to be discovered were the complement C5b-9 complex and staphylococcal α-toxin (*21*). Both C9 and α-toxin exist as monomers in solution until they bind to their respective target membranes. They then rapidly assemble into ring-shaped oligomers surrounding central aqueous pores. This usual sequence of events is disturbed with mutants of staphylococcal α-toxin (*22*, *23*) and likewise of complement factor C9 (*24*) that spontaneously form oligomers in solution, thereby losing their capacity to attack membranes. The deficient phenotype of these mutants may be regarded as the disinhibition of slow, tick-over oligomerization that is observed with highly concentrated solutions of various wild-type proteins (*25*−*27*). It therefore appears that oligomerization is an exergonic process, a conclusion that is supported by the fact that the oligomers of many pore-

formers are sufficiently stable to resist dissociation by SDS at room temperature. These considerations highlight a demand that probably must be met by most pore-forming proteins: oligomerization must be inhibited in solution and only be permitted to proceed upon binding to the target lipid bilayer.

Functional activation of a protein by ligand binding usually occurs by an allosteric conformational transition. Application of this concept to a pore-forming protein would assign the role of the allosteric effector to the target membrane. The arguments in support of an allosteric interaction between the SLO monomer and its target membrane may be summarized as follows:

(1) The interaction between the "thiol-activated, cholesterol-binding cytolysins" (*1*) and membrane cholesterol exhibits a high degree of mutual stereospecificity, since it is strongly affected by circumscribed modifications of either the sterol (e.g., by derivatization or isomerization of the 3-β-hydroxy group; *28*) or of the cytolysin. The latter is prevented from membrane binding upon derivatization of the single cysteine residue of the wild-type molecule (*29*), which is part of a conserved motif located near the C-terminus. Binding affinity is also reduced upon substitution of several neighboring tryptophans (*5*).

(2) The amino acids 248 and 266, which in the monomer reside at a distance of approximately 6−7 nm from the tryptophan-rich motif (*6*), are subject to a change in environmental polarity during membrane binding (*10*). It was demonstrated here that this is not due to immediate contact of these residues with the target lipid bilayer. Moreover, the environmental change experienced by these residues can be dissociated from membrane binding by a point mutation (N402E). These data are consistent with a conformational change which arises in the primary binding site and is transmitted along the molecule to the distantly located residues 248 and 266.

(3) The conformational transition of the monomer evoked by membrane binding is functionally related to oligomer and pore formation. This was demonstrated here by the finding that replacement of residue N402 curtails or abrogates the change of conformation and thereby inhibits oligomerization.

(4) Membrane binding of thiol-activated toxins is reversible as long as these remain in the monomeric form (*1*, *17*). Here we confirmed that dissociation from the membrane is also accompanied by reversal of the conformational transition.

(5) The conformational transition of the SLO monomer is inhibited in the cold. At the same time, oligomerization is also impeded at low temperature (*1*, *17*). In this context, it is noteworthy that, with perfringolysin O, a proteolytically nicked variant has been described that is fully hemolytic at 37 °C but ceases to oligomerize at 20 °C, a temperature at which the uncleaved protein is still functional (*30*). These findings suggest that the temperature-sensitivity of pore formation is due to the cytolysin molecules themselves, rather than to the physical properties of the target lipid bilayer.

The above synopsis shows that there is now a considerable amount of data in support of our hypothesis. Nevertheless, several issues remain that cannot be definitively settled at present:

(1) What is the molecular basis of the pH-dependence of monomer binding? Among the charged amino acid residues, histidine is closest in pK_a to the critical pH value of 8 above which reversal of monomer binding is observed. Indeed, with the related toxin pneumolysin, membrane binding has been abolished by selective chemical modification of histidines with diethylpyrocarbonate. Replacement by arginine of the unique highly conserved histidine residue (which corresponds to residue H469 of SLO and belongs to domain 4) abrogated hemolysis (*31*). Although membrane binding of this nonlytic mutant was not separately determined, the available data are compatible with a role of the conserved histidine in binding.

(2) What is the extent of the translational or rotational movements involved in the conformational change of the SLO monomer? This question cannot be assessed by the spectrofluorometric method applied here, since it solely pinpoints environmental changes to single amino acids which are equally well compatible with limited or more thorough changes to the structure of the monomer. Nevertheless, the essential feature of a conformational change that qualifies it as an allosteric transition is that it provides communication among two distantly located and functionally distinct sites of the protein molecule. This criterion is clearly fulfilled by the SLO monomer.

(3) Does monomeric cholesterol suffice to bring about the conformational transition of SLO or does the sterol have to be part of an aggregate or a mixed lipid bilayer? Early studies have shown that dispersed cholesterol transforms SLO into rings and arcs (*32*) now known to represent the oligomeric state of the toxin (*2*). This transformation most probably proceeds via the same conformational transition that also ocurs on membranes, since thiol-specifically modified derivatives of mutant N402C fail to form arcs on cholesterol crystals (*33*). These findings suggest that the sterol alone is sufficient to induce the conformational change of the monomer. However, due to the very low aqueous solubility of cholesterol or other sterols that fulfill the steric requirements for interaction with SLO (*28*), it is difficult to characterize stoichiometric complexes of sterol and toxin and it cannot be excluded that an aggregated state of the sterol is needed to induce the conformational transition.

(4) What is the cause of the slow "tick-over" oligomerization that occurs with concentrated solutions of monomeric SLO (*26*)? Even in solution, the thermodynamic equilibrium should sustain a minor fraction of molecules in the oligomerization-competent conformation. Moreover, the "incompetent" conformation of the SLO monomer might still be endowed with a residual propensity to oligomerize. With the methods presently available, it is not possible to assess the relative contributions of these two conceivable effects.

It should be mentioned again that several of the experiments presented here were conducted with a conformationally mobile but hemolytically inactive labeled mutant (A248C). This may be regarded as a mixed blessing: on one hand, this protein allows one to study the monomer on the membrane at elevated temperature and in the absence of oligomer formation; on the other hand, it may be argued that the conformational change undergone by the A248C monomer may be stalled, since it is not sufficient to render it oligomerization-competent. However, although this possibility has to be admitted, the abortive conformational transition should share with the entire one essential characteristics such as reversibility and temperature-dependency.

Another possible objection to the proposed allosteric model merits consideration: the observed conformational changes to the SLO monomer might not immediately be imposed by the target membrane but rather be due to a subsequent mutual interaction of the membrane-bound toxin molecules. This would imply that the bound monomers would initially assemble into a clandestine, loosely bonded oligomeric form which would succumb to solubilization with Triton or deoxycholate and hence escape experimental detection. The immature oligomer would then turn into its mature, detergent-resistant form concomitantly with membrane insertion. In fact, a similar mechanism applies to several pore-forming proteins, e.g., staphylococcal α-toxin and aerolysin. With these toxins, oligomerization precedes pore formation and at low temperature they accumulate on membranes as immature "pre-pore" oligomers (*34*, *35*). However, with SLO, this pre-pore mechanism is at odds with the observed kinetic characteristics of oligomerization (*17*). Moreover, with perfringolysin O, the oligomerization on membranes has been studied by a fluorescence energy transfer method that should have detected both mature and immature oligomers (*36*). Importantly, with varying temperature, a very close correlation was maintained between oligomerization and pore formation, indicating that under no condition there was any detectable accumulation of oligomeric pre-pores. This indicates that, prior to their incorporation into the detergent-stable pore oligomer, the membrane-bound toxin molecules remain in the monomeric state, a conclusion that is in line with the easy reversal of their membrane binding. The latter feature applies to both conformationally mobile mutant proteins (A248C; cf. Figure 4) and immobile ones (the N402E combined mutants; data not shown). This suggests that the latter also do not form detergent-sensitive oligomers under the present experimental conditions. However, in the morphological study cited above, a functionally equivalent derivative of mutant N402C was found to pack into linear arrays on the surface of cholesterol crystals (*33*), which raises the possibility that the N402 mutant proteins might possess a residual oligomerizing capacity that materializes under appropriate circumstances.

In our view, the functional role of the proposed allosteric control of SLO by its target membrane is to avoid premature oligomerization of the monomer in solution or on inappropriate surfaces. Although less readily amenable to observation than SLO in their membrane-associated, monomeric states, many other pore formers might interact with their target membranes in a similar manner. *Vibrio cholerae* cytolysin, for example, requires membranes containing both cholesterol and sphingolipids for efficient oligomer formation (*37*). In contrast, oligomerization of staphylococcal α-toxin has been induced with deoxycholate (*38*) and with micellar (*39*) or liposomal (*40*) phosphatidylcholine, and insertion into lipid monolayers (which most probably corresponds to oligomerization) has been observed with a wide variety of pure lipid species (*41*). It thus appears that not all pore-forming toxins interact with their target lipid bilayers according to the "lock and key" paradigm that belongs to the characteristics of allosteric regulation.

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