Streptolysin O: A Proposed Model of Allosteric Interaction between a Pore-Forming Protein and Its Target Lipid Bilayer†

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ABSTRACT: Streptolysin O, a polypeptide of 571 amino acids, belongs to the family of thiol-activated toxins that permeabilize animal cell membranes. The protein binds as a monomer to membrane cholesterol. Binding involves a conserved region close to the C-terminus and triggers subsequent polymerization into large arc- and ring-shaped structures surrounding pores of up to 30 nm. Besides the C-terminus, a distantly located region spanning residues 213−305 is involved in oligomerization and in membrane insertion. Here, we searched for conformational effects of monomer binding to the latter functionally important region. To this end, single cysteine substitution mutants were produced and derivatized with the polarity-sensitive fluorophore acrylodan. Fluorimetric measurements revealed that binding of the monomer to membranes is accompanied by distinct environmental changes at amino acid residues 218, 248, 266, and 277. Conspicuously, the environment of residues 218 and 266 became more hydrophilic, suggesting movement of these residues out of hydrophobic protein pockets. Upon oligomerization, further alterations in all side-chain environments were observed. The membrane-bound monomer thus differs in conformation from both the monomer in solution and the subunit of the oligomer. The putative binding site of the molecule is linked to remote domains involved in oligomerization and membrane insertion in an apparently allosteric fashion. It is proposed that allostery is responsible for restricting oligomerization to the membrane-bound state of the toxin.

Streptolysin O (SLO) is a member of the homologous group of thiol-activated toxins (*1*) that are produced by a wide variety of Gram-positive bacteria. The toxin molecules bind as monomers to cholesterol in the target lipid bilayer (*2*). Upon binding, they oligomerize into arc- and ring-shaped structures comprising about 50−80 subunits (*3*, *4*) and surrounding pores with about 30 nm diameters. These permit flux of ions and macromolecules (*3*, *5*). SLO is increasingly used by cell biologists for the controlled permeabilization of cell membranes (*6*).

The collective results of various experimental approaches have provided some information on structure−function relationships in thiol-activated toxins. All of these are rendered inactive by chemical modification of a single cysteine residue that is located in a highly conserved region located close to the C-terminus (*1*). The inactivated proteins no longer associate with membranes (*7*, *8*), which proves a role of this sequence element for membrane binding. The latter conclusion has been corroborated by analysis of point mutations close to that cysteine (*9*). A C-terminal tryptic fragment of perfringolysin O could be obtained which binds to membranes and interferes with the oligomerization of wild-type toxin (*10*). A similar effect was elicited by two monoclonal antibodies, the epitopes of which have been mapped to the N-terminal half of the pneumolysin molecule (*11*). Thus, both N- and C-terminal segments of the toxin molecules appear to be involved in oligomerization.

Hydrodynamic and electron microscopic data on pneumolysin (another member of the family) have indicated that the molecule is elongated and divided into four subdomains (*12*). This has been confirmed by the recent crystal structure studies of one of the members (perfringolysin O) in its water-soluble, monomeric state (*13*) (Figure 1). In the latter report, it was proposed that only the C-terminally located domain 4 contacts the target membrane. This contention is in accord with all available functional data concerning the binding of the monomer. However, during oligomerization, a part of domain 3 (around residue 286) of the streptolysin O molecule most probably comes into contact with the target membrane. At the same time, residues 213 and 245 (which are part of domain 1) become buried inside the oligomer itself and probably make contacts to adjacent subunits within the oligomer (*14*). Residues 200−300 (belonging to domains 1 and 3) thus appear to contribute to both oligomerization and membrane penetration. There is another less obvious but equally important feature of toxin function which has not yet been assigned to structure. While streptolysin O oligomerizes readily in the picomolar range when bound to cells, the toxin monomer can be kept in solution at micromolar concentrations. The molecule thus does not prematurely oligomerize before binding to a target membrane. This strongly suggests that conformational differences exist between the free and the membrane-bound state of the

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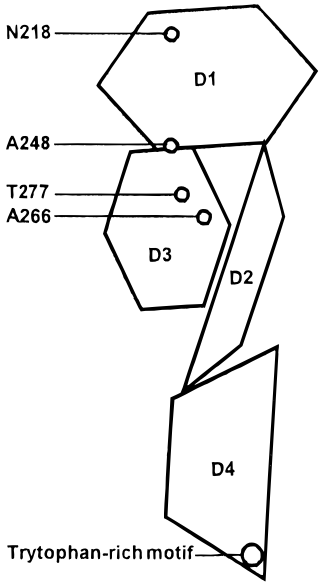

FIGURE 1: Scheme of the structure of monomeric streptolysin O [based on the crystal structure of the homologous molecule perfringolysin O (*13*)]. The molecule has an elongated shape and consists of four domains (D1−D4). The tryptophan-rich motif within domain 4 is involved in primary binding to membrane cholesterol. Also indicated are the approximate locations of the residues that were singly replaced by cysteine in this study (residues 218, 248, 266, and 277).

monomer. However, no such difference was evident in a previous screen of 19 cysteine mutants labeled with the polarity-sensitive fluorescent dye acrylodan (*14*). Focusing on the functionally important region spanning residues 200−300 that emerged from the latter study, we have produced additional cysteine mutants and now provide evidence that indeed the monomer undergoes a conformational change when binding to the membrane. The change directly affects sites that are essential for oligomerization. In conjunction with previous evidence, our data indicate that the interaction of streptolysin O with target membranes is allosteric in nature. We propose that allostery constitutes the functional link that must exist between the membrane binding site and sterically remote domains that are involved in oligomerization and membrane penetration.

MATERIALS AND METHODS

Mutagenesis and Cloning. Mutants were constructed according to published procedures (*15*) and confirmed by DNA sequencing. The mutant genes were cloned into the plasmid pMal c2 (New England Biolabs) and expressed in fusion to *Escherichia coli* maltose binding protein (*16*).

Purification of Mutant Proteins and Sulfhydryl-Selective Derivatization with Acrylodan. The mutant proteins were purified from *E. coli* TG1 by successive affinity chromatography, trypsin cleavage, and hydroxyapatite chromatography as described (*16*). Modification with acrylodan and determination of labeling yield were done as stated previously (*17*).

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 were incubated at 37 °C for 30 min before visual reading of the hemolytic titer.

Binding of Acrylodan-Labeled Streptolysin O Mutants to Rabbit Erythrocytes. To verify binding of a nonlytic labeled mutant (A248C, see Results) to membranes, the protein was dissolved at 1 μg/mL in PBS/0.1% BSA, and rabbit erythrocytes were added to 5%. The sample was placed on ice for 5 min 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), and a control sample to which no red cells had been added served as reference.

Preparation of Erythrocyte Membranes. Pelleted rabbit erythrocytes (500 μL) were washed three times in PBS and lysed osmotically in 5 mM Tris/HCl, pH 7.5; the membranes were then pelleted by centrifugation (5 min, 10000*g*). They were then repeatedly resuspended in the latter buffer and centrifuged until the supernatant remained clear. The membranes were finally resuspended in 500 μL of TBS/BSA (Tris, 10 mM; NaCl 150 mM; BSA, 1 mg/mL; pH 8.0).

Analysis of Membrane-Associated Acrylodan-Labeled Proteins. The samples were kept at less than 4 °C throughout. Labeled mutant protein (1−2 μg) was diluted with 50 μL of TBS/BSA. Erythrocyte membrane suspension (50 μL) was added, and the mixture was incubated for 5 min to allow for binding of the monomeric protein. The sample was diluted to 900 μL with TBS/BSA prior to fluorimetry. To confirm that oligomerization had not occurred under these conditions, a parallel sample was spiked with 1 ng of mutant N218C that had been labeled with ^{125}I using fluorescein maleimide as a carrier according to ref 18. The latter sample was then solubilized with sodium deoxycholate (5% final concentration) and analyzed by density gradient centrifugation as described (*3*, *8*). The nonradioactive sample was then supplemented with 10 μg of unlabeled mutant C530A and incubated for 15 min at 37 °C to induce oligomerization. It was then returned to the chilled sample chamber of the spectrofluorometer. Again, a parallel radioactive sample was analyzed by density gradient centrifugation to verify that oligomerization now had occurred.

Isolation of Oligomers of Acrylodan-Labeled Streptolysin O Mutant A248C. The labeled mutant (50 μg) was mixed with 200 μg of unlabeled mutant C530A and 100 μL of erythrocyte membranes. The samples were incubated for 15 min at 37 °C to induce oligomerization. The membranes were recovered by centrifugation (5 min, 12000*g*) and solubilized with 250 μL of 5% sodium deoxycholate. Labeled oligomers were isolated by density centrifugation and transferred into 0.2% sodium deoxycholate, 10 mM Tris, and 50 mM NaCl by passage through a Sepharose 6B (Pharmacia) column. The samples were then analyzed by spectrofluorimetry with and without addition of Nonidet P40 (Roth, Karlsruhe, Germany) to 1% final concentration.

Spectrofluorimetry. Emission spectra (400−600 nm) were recorded in a SPEX Fluoromax spectrofluorimeter (excitation wavelength, 365 nm; bandpasses: excitation, 8.5 nm; emission, 2.1 nm; scanning interval, 2 nm). All spectra were subtracted with the appropriate buffers or membrane suspensions. Where membrane suspensions were employed, a cuvette with narrow lumen (2 mm) was used to minimize spectral distortions due to scattering of emitted light.

RESULTS

Construction and Activity of Unlabeled Mutants. From the cysteine-less active mutant C530A (*19*), the following single cysteine replacement mutants were derived: N218C, A248C, A266C, and T277C. Residue 218 is located within domain 1; residues A266C and T277C are part of domain 3, and residue 248 is at the interface between domains 1 and 3 (Figure 1). All mutants showed the same specific hemolytic activity as the parent mutant C530A (hemolytic titer of a 1 mg/mL solution, 1:40000), which in turn equals wild-type toxin in hemolytic capacity (*19*).

Properties of Acrylodan-Labeled Mutants. All mutants were labeled with acrylodan in a yield of >70%. Upon labeling, mutants N218C, A266C, and T277C displayed unaltered hemolytic activity, indicating preservation of their ability to bind to and oligomerize on membranes. In contrast, the hemolytic capability of A248C was reduced to <1% of the unlabeled protein, indicating that either monomer binding to the membrane or oligomerization of the bound toxin must be impaired. Upon incubation of labeled A248C with rabbit erythrocytes, 90% of the label fluorescence was absorbed from the supernatant (Figure 2A), which showed that binding to erythrocyte membranes was not affected. Oligomerization of membrane-bound streptolysin O can be detected by solubilization of the membranes with deoxycholic acid and subsequent density gradient centrifugation (3). When this method was applied to the acrylodan-labeled mutant A248C, no label fluorescence was detected in the bottom fractions of gradients, indicating that oligomerization had not occurred. However, oligomer incorporation of acrylodan-labeled A248C could be achieved by mixing it with an excess of the unlabeled active mutant C530A (*19*) before incubation with membranes (Figure 2B).

Selective Control of Membrane Binding and of Oligomerization. Streptolysin O can bind to membranes at low concentration and temperature. These conditions restrict oligomerization (1, 8), which allowed us to examine the acrylodan-labeled mutants as monomers in the membrane-bound state. Subsequent oligomerization of the bound labeled toxin could then be induced by adding unlabeled toxin in excess and transiently shifting the temperature to 37 °C. For the conditions chosen in the fluorimetric experiments below, this was verified by density gradient centrifugation of deoxycholate-solubilized membranes. While no oligomerization was detectable with the labeled toxin only at 4 °C, the bulk of the protein was recovered as oligomers after addition of unlabeled toxin and transient warming (Figure 3).

Binding of the Streptolysin O Monomer to Membranes Causes a Change in Conformation. In a previous report on single cysteine mutants of streptolysin O, oligomerization was found to impart major changes in environment and, by inference, conformation to several amino acid residues. In marked contrast, no such effects were observed during binding of the monomer (*14*). It thus appeared questionable if monomer binding would influence conformation at all. However, the present series of mutants now clearly demonstrates conformational effects of binding.

Figure 4A shows the emission spectra of acrylodan-labeled mutant N218C as monomer in solution, as membrane-bound monomer, and after oligomerization (induced by addition of the unlabeled mutant C530A in excess). Upon binding, the acrylodan emission maximum was red-shifted from 502 to 520 nm. This reflected an increase in environmental polarity, probably due to movement of the label to a more solvent-exposed position (*20*). Evidently, binding elicited a conformational change that involved the labeled residue. Following oligomerization, there was only a minor spectral change. The emission maximum remained at 516 nm, and the labeled residue in an aqueous environment.

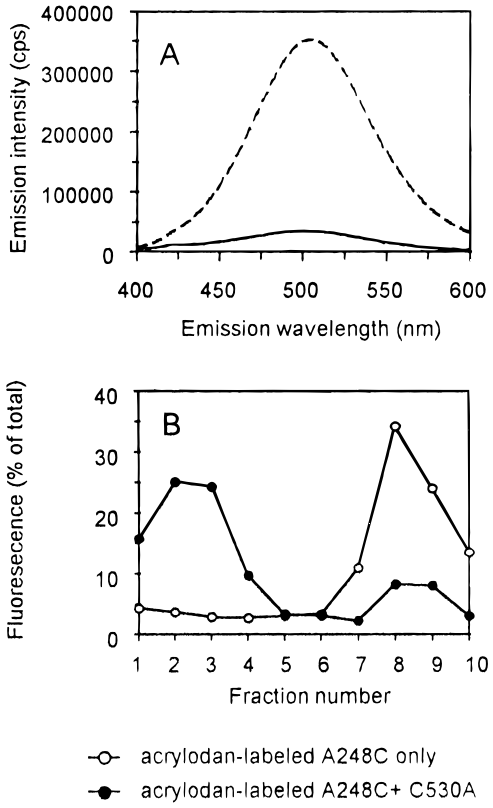

FIGURE 2: Deficient oligomerization of the nonlytic acrylodan-labeled mutant A248C. Pore formation by streptolysin O requires monomer binding to the target membrane and subsequent oligomerization. The two steps were separately analyzed for mutant A248C that loses hemolytic activity upon thiol-specific labeling with acrylodan. (A) Labeled A248C does bind to rabbit erythrocytes. The protein was incubated at 1 μg/mL with 5% (v/v) rabbit erythrocytes. After 5 min, the cells were removed by centrifugation, and the supernatant assayed for unbound protein by measurement of acrylodan fluorescence (—). Comparison to a control sample without erythrocytes (- - -) indicated that 90% of the toxin had bound to the cells. (B) Analysis of oligomerization by density gradient centrifugation. Acrylodan-labeled A248C (50 μg) with or without unlabeled active streptolysin O was incubated with erythrocyte membranes at 37 °C. After 15 min, the sample was solubilized with sodium deoxycholate and subjected to sucrose density gradient centrifugation. Gradient fractions were assayed for A248C streptolysin O by measurement of acrylodan fluorescence. With the mixed sample, the label was found mainly in the bottom fractions (1−5), indicating that the labeled protein had become incorporated into large oligomers. With pure A248C, the labeled protein remained as a monomer in the top fractions. The nonlytic phenotype of the protein is thus explained by its inability to oligomerize on its own.

Figure 4B−D shows the results obtained under the same conditions with mutants A248C, A266C, and T277C. With A248C, membrane binding and oligomerization each caused a definite blue-shift of the acrylodan emission, and the environment of the label became more hydrophobic upon membrane binding and even more so upon oligomerization.

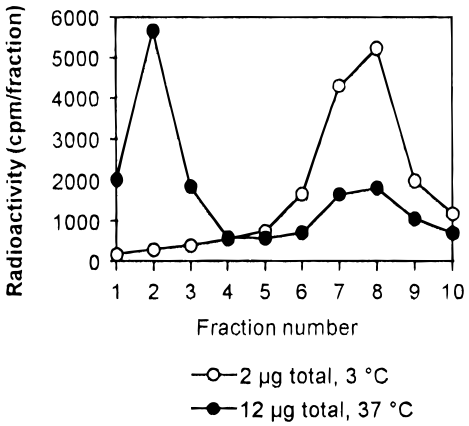

FIGURE 3: Streptolysin O mutants can be examined on membranes in their monomeric and oligomeric forms. The mutant N218C is fully active upon labeling, which implies that it is able to bind and oligomerize. When 2 μg of acrylodan-labeled protein (spiked with 1 ng of radiolabeled protein) was added to erythrocyte membranes at 3 °C, no oligomers were detected in the bottom fractions of sucrose density gradients, indicating that the toxin had remained on the membranes in monomeric form. Upon addition of unlabeled toxin in excess (10 μg) and incubation at 37 °C for 15 min. the major part of the label shifted to the bottom fractions. The same conditions were also used for fluorimetric analyses of membrane-associated monomeric and oligomeric acrylodan-labeled mutants.

Residue 266 displayed quite the opposite behavior (two successive emission red-shifts), apparently becoming more solvent-exposed at each step, while residue 277 experienced a slight blue-shift upon binding but a distinct red-shift during oligomerization.

Residue 248 Becomes Buried inside the Streptolysin O Oligomer. The relatively polar environments of labeled residues 218, 266, and 277 in the membrane-associated oligomer suggests that they are all superficially exposed and in contact with water. By contrast, residue 248 moved to a more hydrophobic environment during oligomerization. This suggested that residue 248 becomes either buried inside the oligomer or immersed in the lipid bilayer. To test for the latter possibility, acrylodan-labeled A248C oligomers were isolated, and the emission spectra of the label were recorded in the presence of different detergents. The underlying rationale is that, after removal of membrane lipids, the detergent molecules will cover the membrane-inserted sites of the oligomer (*21*). Labels in contact with different detergents should then experience variations in environmental polarity and hence yield different emission spectra (*14*). Figure 5 shows that the spectra of A248C oligomers in sodium deoxycholate and in Nonidet P40 were nearly congruent. The label was therefore not directly exposed to the detergent molecules and probably buried inside the oligomeric protein itself.

DISCUSSION

Since the initial characterization of staphylococcal α-hemolysin as a pore-forming, oligomerizing protein (*22*), this class of exotoxins has been joined by numerous new members from the entire bacterial kingdom. Despite their wide variation in size of both the individual molecules and the oligomeric pores, these toxins share important common functional features. Binding to the target membrane usually precedes oligomerization. Once formed, the oligomers are very stable, sometimes even in the presence of SDS. Oligomerization therefore releases energy, which is probably consumed in concomitant (or subsequent) membrane insertion and disruption. This exergonic nature of oligomerization raises the problem of limiting it to the membrane-bound state of the toxin monomer. Premature oligomerization in the absence of a target membrane would result in toxin inactivation, as it occurs with complement proteins in the well-known complement fixation reaction. Indeed, slow spontaneous oligomerization and inactivation tends to occur with highly concentrated toxin solutions (*23*, *24*). In the case of staphylococcal α-toxin, this slow tick-over is markedly accelerated with some point mutants (*17*). Thus, subtle changes to the structure of a pore-forming toxin may greatly enhance its ability to oligomerize, and its stability as a monomer in solution may not at all be taken for granted.

The different tendency toward to oligomerization of membrane-bound as opposed to unbound monomers suggests that they differ in conformation. Such differences, however, have not yet been directly demonstrated. Where crystallography has been applied to pore-forming toxins, it has been successful so far with either the free monomer or the oligomer, but not both (*13*, *25*, *26*). An exception with some reservations is the protective antigen component of anthrax toxin, the oligomer structure of which has been modeled on the basis of low resolution diffraction data (*27*). Of course, the membrane-bound monomer of any pore-forming toxin is entirely inaccessible to this approach.

A complementary source of structural information consists in the use of environmentally sensitive probes site-specifically attached to single amino acid residues, usually cysteines. A particularly well-suited probe is acrylodan (*20*). The emission maximum of this fluorophore is exceedingly dependent on environmental polarity; representative values are 540 nm in water and 430 nm in dioxane. Its use has enabled the pore-forming domain of α-toxin to be identified (*17*, *28*), a result that has been confirmed by the crystal structure of the toxin pore (*26*). This excellent validation should encourage application of this fluorimetric approach to other pore-forming molecules.

The large and heterogeneous oligomers of the thiol-activated toxins are virtually inaccessible to crystallography in their oligomeric form. By labeling single cysteine mutants with acrylodan, several amino acid residues were identified within domains 1 and 3 of the monomer (*13*) that move into the membrane or into a hydrophobic proteinaceous environment during oligomer assembly (*14*). As shown here, the same method also renders it possible to pinpoint conformational transitions that accompany binding of monomeric toxin to the membrane.

It is probably not too surprising that the environmental alterations accompanying this conformational change are less pronounced than those that occur during insertion into a lipid bilayer (*14*, *17*). With respect to the individual labeled amino acid, the implication of an emission shift is purely qualitative, i.e., a shift means a change; we cannot imagine a general quantitative relation between the amplitude of the shift and the magnitude of the conformational change in question. A quantitative judgment might rather be attempted by relating the number of mutants that do show a change (i.e., the four described here) to the total number of mutants examined so far (the four above and additional nineteen in ref 14). It

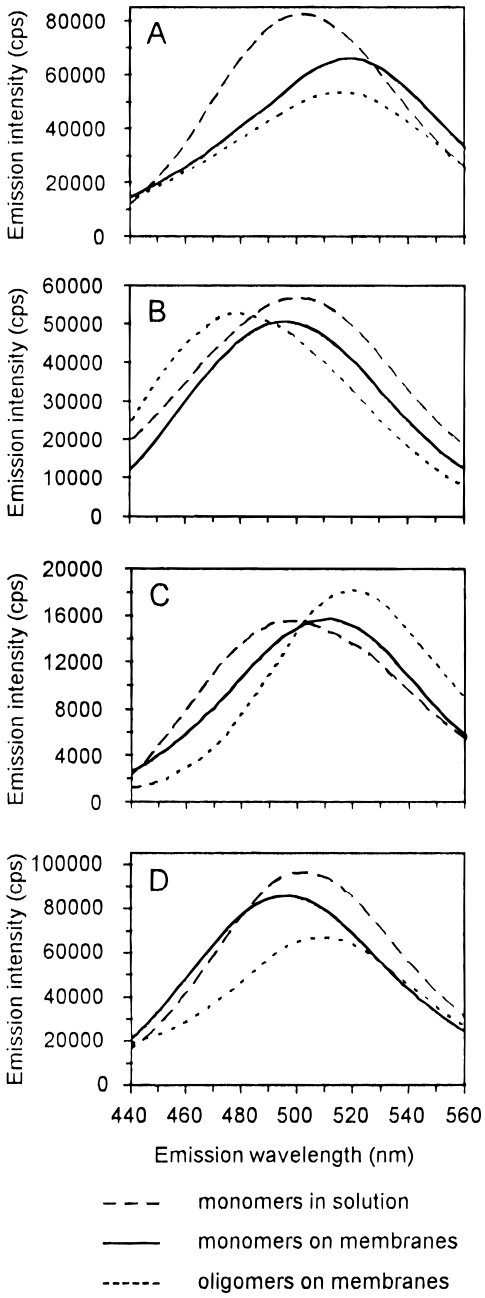

FIGURE 4: Effects of membrane binding and of oligomerization on the environment of single amino acid residues of streptolysin O. Mutant proteins carrying single cysteine residues at different positions were thiol-specifically derivatized with the environmentally sensitive fluorophore acrylodan. Emission spectra of the labeled proteins were recorded as monomers in solution, as monomers on membranes, and as oligomers on membranes (formed by addition of the unlabeled mutant C530A). (A) Mutant N218C. Upon binding to the membrane, the acrylodan spectrum of the monomeric protein was shifted to the right. The corresponding increase in environmental polarity must be due to a local change of conformation. Oligomerization effected another slight change in environment that is evident by a slight shift to the left and decrease in intensity. (B) Mutant A248C. Here, two successive emission blue-shifts were observed, a slight one upon binding and a pronounced one upon oligomerization. The hydrophobic environment of the label in the oligomer is suggestive of a buried location either within the oligomer or the membrane. (C) Mutant A266C. Two successive emission red-shifts indicated that the labeled residue became more solvent-exposed during binding and oligomerization. (D) Mutant T277C. A slight blue-shift occurred upon binding which was exceeded by a red-shift upon oligomerization. Again, both steps locally alter the conformation.

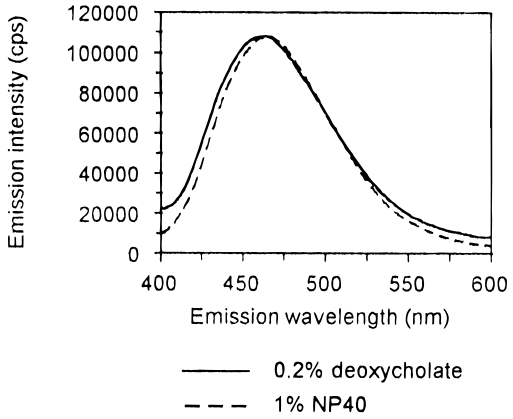

FIGURE 5: Residue 248 is buried inside the oligomer. Hybrid oligomers of acrylodan-labeled mutant A248C and the unlabeled active mutant C530A were isolated by density gradient centrifugation (cf. Figure 2) and supplemented with the detergents deoxycholic acid or Nonidet P40, respectively. The emission spectra were nearly congruent, indicating lack of interaction between detergent and label. By analogy, it may be concluded that the labeled amino acid residue does not come into contact with membrane lipids but rather is in a hydrophobic pocket inside the protein oligomer.

then appears that the conformational change during binding is of limited extent. Nevertheless, we think that the existing concepts on the molecular features of the thiol-activated toxins support the view that it is functionally important.

A salient feature in the interpretation of the present results is the spatial relation between the amino acid residues that are affected by the conformational change and the membrane binding site of the monomer. As to the latter, all available data indicate that it is located within the C-terminally located domain 4 (*7*, *8*, *10*). This is reflected in a molecular model of the membrane-associated monomer (*13*) (cf. Figure 1). In that model, only domain 4 is in touch with the lipid bilayer. Domain 3 remains a considerable distance from the membrane, while domain 1 forms the far end of the molecule. We will adopt this scheme in the discussion.

A movement across the hinge between domains 1 and 3 (Figure 1) has been proposed to occur during pore formation by perfringolysin O (*13*). The behavior of residue 248 (that is located at the hinge) ties up with this expectation, since both monomer binding and oligomerization alter the conformation around this residue. This is compatible with a change in the relative positions of domains 1 and 3. However, clear-cut environmental effects are observed at residues 218, 266, and 277 that are located within domain 1 or 3, but remote from their interface. It follows that membrane binding of the monomer rearranges both the relative positions and the internal structure of two domains that are not detectably in touch with the membrane.

Apart from its conformational effects upon sterically remote parts of the molecule, binding of monomeric streptolysin O is characterized by free reversibility (*8*) and a well-documented specificity for cholesterol (*29*). Streptolysin O (or the thiol-activated toxins in general) therefore meets the key criteria of an allosteric molecule. We propose that the allosteric transition acts as a trigger of oligomerization, since domain 1 becomes involved in contact among adjacent subunits of the oligomer (*11*, *14*). Similarly, binding of the monomer affects its structure within a part of domain 3 (residues 274−305) that, in contrast to the hypothetical

oligomer structure in (*13*), we believe gains contact with the target membrane during oligomerization and pore formation (*14*). It thus appears that membrane binding of the monomer sets the stage not only for oligomerization but also for the accompanying membrane-penetration step.

Finally, after binding to the membrane, the membrane-bound monomer undergoes another profound conformational transition during oligomerization. This leaves room for the above idea that energy stored in the conformation of the monomer is released during oligomerization and utilized in membrane permeabilization. It also tallies with the kinetics of streptolysin O self-assembly, which is governed by the mutual interaction of membrane-associated monomers as opposed to their individual interaction with the target membrane (8). The idea that a reaction of higher molecularity with respect to the bound monomer controls oligomerization is supported by the behavior of the acrylodan-labeled mutant A248C. The latter protein is incapable of oligomerization, but the defect can be complemented by wild-type streptolysin O as indicated by the formation of hybrid oligomers. Thus, labeled A248C is unable to initiate the first rate-limiting step, possibly dimerization, but it can participate in the subsequent reaction and become incorporated into the growing oligomer.

Beyond the family of thiol-activated toxins, the concept that membrane binding of monomers triggers a conformational transition, which in turn enables oligomerization, may be relevant to other pore formers. Even if there is no stereospecific interaction with a particular membrane lipid [as appears to be the case with staphylococcal α-toxin, (*30*)], a conformational change may be brought about by interaction of the bound monomers with charged headgroups of the lipid bilayer and/or with its apolar core beneath.

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