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Molecular architecture of a toxin pore: a 15-residue sequence lines the transmembrane channel of staphylococcal α-toxin

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***Staphylococcus aureus* α-toxin is a hydrophilic polypeptide of 293 amino acids that produces heptameric transmembrane pores. During assembly, the formation of a pre-pore precedes membrane permeabilization; the latter is linked to a conformational change in the oligomer. Here, 41 single-cysteine replacement toxin mutants were thiol-specifically labelled with the polarity-sensitive fluorescent probe acrylodan. After oligomerization on membranes, only the mutants with acrylodan attached to residues in the sequence 118–140 exhibited a marked blue shift in the fluorescence emission maximum, indicative of movement of the fluorophore to a hydrophobic environment. Within this region, two functionally distinct parts could be identified. For mutants at positions 126–140, the shifts were partially reversed after membrane solubilization by detergents, indicating a direct interaction of the label with the membrane lipids. Membrane insertion of this sequence occurred together with the final pre-pore to pore transition of the heptamer. Thus residues 126–140 constitute a transmembrane sequence in the pore. With labelled residues 118–124, pre-pore assembly was the critical event to induce the spectral shifts, which persisted after the removal of membrane lipids and hence probably reflects protomer–protomer contacts within the heptamer. Finally, a derivative of the mutant N121C yielded occluded pores which could be opened by reductive reversal of the modification. Therefore this residue probably lines the lumen of the pore.**

Keywords: acrylodan/fluorescence emission shift/membrane insertion/protein oligomerization/single cysteine mutants

Introduction

Staphylococcus aureus α-toxin is serving as a prototype for the study of pore formation by proteinaceous toxins in lipid bilayers (Arbuthnott *et al.*, 1973; Fuessle *et al.*, 1981; Bhakdi and Tranum Jensen, 1991; Bayley, 1994). The toxin is secreted as a hydrophilic molecule of M_r 33 000 lacking cysteine (Gray and Kehoe, 1984), and binds in a monomeric form to target membranes. Collision of the membrane-bound protomers in the bilayer leads to the formation of tightly associated, ring-structured oligomers that form the transmembrane pores (Fuessle *et al.*, 1981; Bhakdi *et al.*, 1993). Counting the subunits of unnatural heteromers and preliminary X-ray crystallographic studies indicate that the oligomers are heptamers (Gouaux *et al.*, 1994). Using mutants defective at different stages of oligomerization (Walker *et al.*, 1992, 1993), it was shown that a non-lytic pre-pore complex yields the final transmembrane pore after a conformational transition. The aim of our study was to define the molecular regions which enter the membrane and to localize regions involved in protomer–protomer contacts and in the pre-pore to pore transition.

There is accumulating evidence that of the 293 amino acid residues contained in α-toxin, only a small number are involved in pore formation; this probably includes the central domain of the molecule. Early ultrastructural studies have indicated that most of the toxin mass remains outside the bilayer after pore formation (Fuessle *et al.*, 1981; Bhakdi and Tranum Jensen, 1991). In addition, the overall secondary structure of the toxin remains largely unaffected by membrane binding and pore formation (Tobkes *et al.*, 1985). Toxin molecules harbouring a cysteine at position 130 could be labelled with the polarity-sensitive dye 6-acryloyl-2-dimethyl-amino-naphthalene (acrylodan) with the preservation of functional activity. Following oligomerization, the emission spectrum of the acrylodan exhibited a marked blue shift, which was partially reversible after solubilization of the oligomers in detergent, indicative of movement of the reporter molecule into the lipid bilayer during pore formation (Valeva *et al.*, 1995). In heptamers on membranes, there was a strong fluorescence energy transfer from acrylodan at position 130 to an acceptor dye fixed on the inner surface of the membranes, indicating that acrylodan had moved into close proximity to the internal membrane surface (Ward *et al.*, 1994). In another study, it was shown that biotin attached to Cys130 was inaccessible to streptavidin–fluorescein offered to the membrane exterior (Palmer *et al.*, 1993a). Further, the proteolytic nicking of α-toxin in the central region altered the functional properties of the toxin without affecting its binding properties (Palmer *et al.*, 1993b). Moreover, toxin mutants engineered to yield overlaps, nicks and gaps in this region exhibited altered pore-forming activities (Walker *et al.*, 1993). Finally, toxin mutants carrying histidine residues in this region formed pores that could be closed by zinc added to the *cis* or *trans* sides of planar lipid membranes (Walker *et al.*, 1994). When combined, all findings point to a pivotal role for the central molecular domain in forming the pore. However, detailed information regarding the boundaries of the pore-forming regions and the contact sites between subunits in the oligomers is still required.

For this investigation we performed a detailed study using acrylodan (Prendergast *et al.*, 1983) attached to single-cysteine substitution mutants. We present data that corroborate and markedly extend our proposed model. Data indicate that (i) residues 126–140 interact with the lipid bilayer and participate in forming the pore, (ii) residues 118–124 in the α-toxin molecule are involved in protein–protein interactions and (iii) residue 121 lines the pore lumen so that its derivatization leads to pore occlusion.

Results

Functionality of acrylodan-labelled α-toxin mutant proteins

The following mutant proteins were constructed and isolated: D13C, K21C, D29C, E31C, K37C, K46C, R66C, S69C, K75C, D92C, G94C, D108C, S114C, Y118C, F120C, N121C, G122C, V124C, G126C, D127C, D128C, T129C, G130C, K131C, G134C, G137C, A138C, V140C, S141C, G143C, H144C, D152C, K154C, K164C, S186C, D208C, K240C, R251C, H259C, K266C and K283C. In the haemolytic assay employed, the specific activity of all underivatized mutants equalled that of wild-type toxin. Mutants with cysteines located in the range of residues 118–124 and V140C showed an increased tendency to oligomerize in solution at room temperature (in fact, when purifying these proteins, yields were reduced by ~90%). The extensive formation of heptamers (which at room temperature resist dissociation by SDS) was evident on SDS–PAGE in samples from culture fluid. Accordingly, monomers of these proteins had to be handled at 4°C throughout.

Each cysteine mutant was labelled with acrylodan, and unreacted label was removed by passing the protein over the moderately hydrophobic chromatography material methyl-Sepharose. The extent of labelling ranged from 60 to 95%, as judged by measuring both the tryptophan and acrylodan absorbance of labelled proteins. With the exception of R66C (which was already deficient in binding; Walker and Bayley, 1995), all labelled mutants efficiently formed heptamers on liposomes, as judged by SDS–PAGE. After labelling, only A138C displayed a reduced haemolytic activity (without impaired oligomerization). With all other labelled proteins, no differences were detected in the haemolytic activity compared with that of wild-type toxin.

Oligomerization in lipid bilayers of labelled mutants 118C–140C is associated with a large blue shift in the acrylodan emission spectrum

Emission spectra were obtained for each acrylodan-labelled toxin mutant as monomers in solution and as oligomers on liposome membranes. Upon oligomerization, 15 mutants with acrylodan attached to Cys residues between positions 118 and 140 exhibited a marked blue shift, indicating movement of the polarity-sensitive probe to a more hydrophobic environment. In contrast, comparable blue shifts were not noted for mutants labelled at any residue outside this central domain (Figure 1). To ascertain that the observed blue shifts were not caused by mutual interaction of the acrylodan molecules attached to neighbouring toxin monomers within the oligomers, the labelled

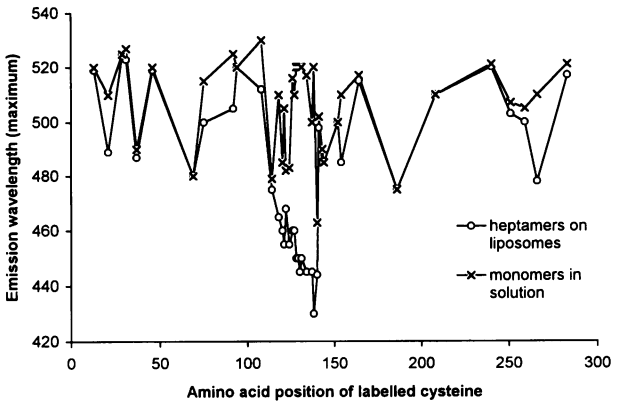


Fig. 1. Effect of oligomerization upon fluorescence emission of acrylodan-labelled α-toxin cysteine mutants. Emission spectra of acrylodan attached to cysteine mutants were recorded both with monomeric protein in solution and with heptamers on liposomes. The emission maxima of both monomers and oligomers were plotted against the amino acid position of the engineered cysteines. (×) Monomeric labelled toxins in solution; (○) heptameric labelled toxins on membranes. An emission blue shift indicates that the label has moved to a more hydrophobic environment. The most pronounced blue shifts are clustered in the central region.

mutants were admixed with a 10-fold excess of (unlabelled) wild-type toxin prior to oligomerization on the membranes. The spectra of these samples were indistinguishable from those obtained with labelled toxins only.

It appeared plausible that residues 118–140 constituted a membrane-inserting domain of α-toxin. To test if the observed blue shifts were caused by a direct interaction of the label with membrane lipids, the latter were removed by solubilization with detergent and gel filtration. Figure 2A exemplifies the results obtained with mutant G126C. The blue shift of the membrane-bound oligomers was reversed to varying extents with different detergents, with deoxycholate exhibiting the most pronounced effect. The residual deviation from the monomer spectrum was probably caused by interaction of the label with the detergent, possibly with a contribution from the altered protein conformation within the oligomer. Nevertheless, a direct role of the membrane lipids in the hydrophobic environment of acrylodan is evident.

In contrast, with mutant N121C no reversal of the initial blue shift was effected by solubilization with the same detergents (Figure 2B). We interpret this to indicate that acrylodan in this case became embedded not in the bilayer, but in a hydrophobic protein environment that may arise from contact with the neighbouring toxin protomers.

Figure 2C summarizes the results obtained within the central molecular region, including the effects obtained by solubilization with deoxycholate. It is seen that two different parts can be distinguished: all residues from positions 126–140 essentially displayed the same behaviour as detailed above for mutant G126C. Thus we assume that this part constitutes a membrane-inserting domain of the toxin molecule. On the other hand, the effects in the range of residues 118–124 uniformly equalled those of mutant N121C. Therefore we conclude that this region is involved in protein–protein contacts rather than direct interaction with the membrane.

With labelled mutants Y118C–V124C, their tendency

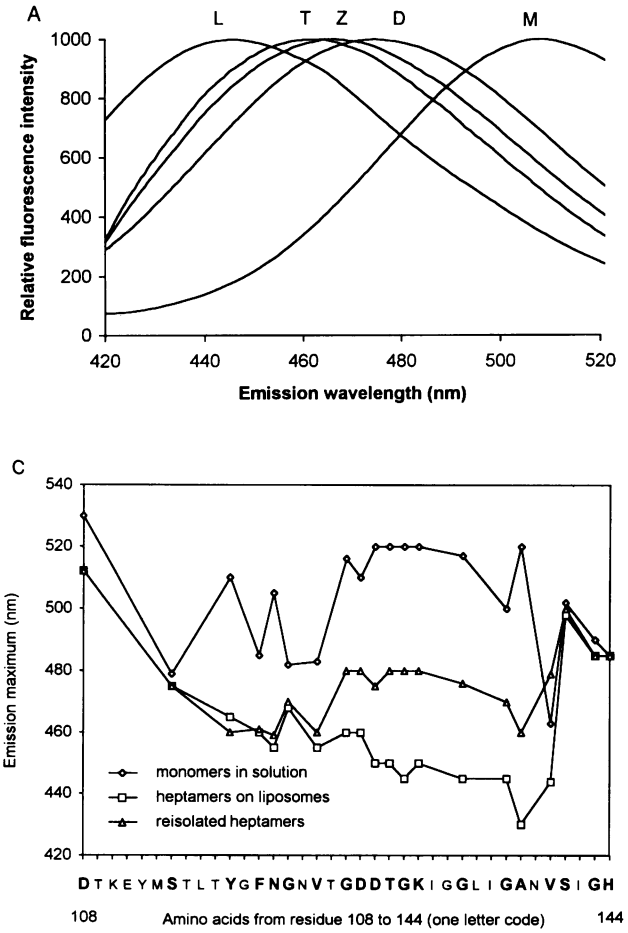


Fig. 2. A stretch of 15 amino acids within the central molecular domain interacts with the membrane. Acrylodan emission spectra were recorded for labelled mutants in the range 118–140. The spectra displayed represent toxin monomers in solution (M), heptamers bound to liposomes (L) and heptamers solubilized with Triton X-100 (T), Zwittergent 3-14 (Z) or deoxycholate (D; – – – in B). (**A**) Mutant G126C. The blue shift evident upon oligomerization on membranes was partially reversed by the removal of membrane lipids, proving the direct contribution of the latter to the hydrophobic environment of the label. (**B**) Mutant N121C. In this instance, solubilization with deoxycholate (as well as other detergents; results not shown) effected no reversal of the blue shift, indicating a lack of direct contact of residue 121 with the membrane interior. (**C**) Acrylodan emission maxima of labelled toxin mutants were plotted against the sequence of the central domain (bold characters represent residues replaced by cysteines). Emission maxima of membrane-bound and deoxycholate-solubilized heptamers differed for residues 126–140, proving the direct interaction of the probe with the membrane. In contrast, no such interaction was detected for residues 118–124.

to spontaneously oligomerize also allowed emission blue shifts of the same magnitude to be observed in the absence of both membrane lipids and detergents (data not shown). This constitutes additional evidence that acrylodan at positions 118–124 indeed entered a hydrophobic protein environment afforded within the oligomer.

The following mutants located outside the central region also displayed distinct emission blue shifts upon oligomerization on the membranes: K21C, D92C, D108C, K154C and K266C. After solubilization with deoxycholate, the spectra were identical to those of membrane-bound heptamers, and hence provided no evidence suggesting membrane insertion of any domain outside the central region.

It may be worth noting that the results of the deoxycholate solubilization experiments could be anticipated by the inspection of UV-irradiated SDS gels. Here, heptamers emitting maximally below about 470 nm in deoxycholate exhibited blue fluorescence, whereas those fluorescing strongest at higher wavelengths appeared green (data not shown).

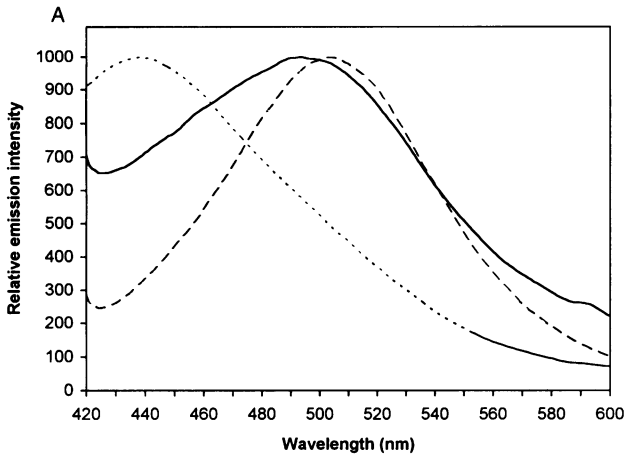
The two parts of the central domain are involved in different steps of toxin action

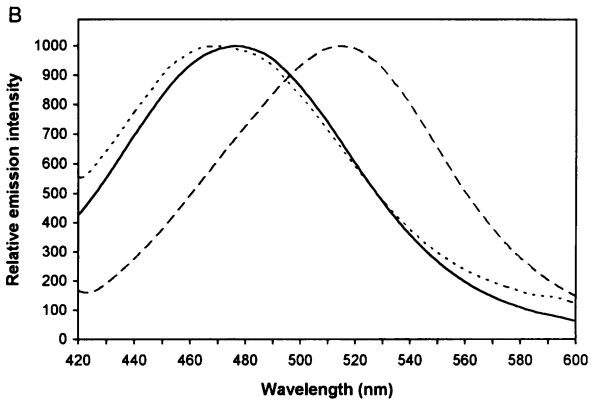
The above results relate to the disposition of the central molecular domain only in the first and the last stage of toxin action, namely the monomer in solution and the final pore. Besides those, two intermediate forms exist: the membrane-bound monomer and the heptameric pre-pore complex that has not yet undergone the conformational change leading to membrane permeabilization. Therefore, experiments were conducted to correlate the spectral effects within the central domain to individual steps of pore formation.

In order to assess the effects of monomer binding to the membrane, the labelled toxins were applied to liposomes at protein/lipid ratios sufficiently low to prevent oligomerization. The latter could subsequently be triggered by raising the concentration of membrane-bound toxin through the addition of wild-type toxin. Figure 3A depicts the results obtained with G126C, which was characteristic of all toxins tested (Y118C–V140C).

That initial binding of the toxin was exclusively in monomer form was confirmed by SDS–PAGE (not shown). Under these conditions, there was also no shift in the acrylodan fluorescence emission spectrum. Upon addition of unlabelled wild-type toxin, the blue shift occurred concomitantly with oligomerization (Figure 3A). These experiments confirmed that, for both parts of the central domain, the blue shift was not due to lipid contacts or conformational changes of the bound monomer, but reflected a change linked to the oligomerization process.

Then, the alterations within the central domain could arise either from assembly of the pre-pore or from its transition into the pore state. To distinguish between these possibilities, an unlabelled H35R mutant was employed. This mutant binds normally to membranes and form heptamers. These are, however, arrested in a pre-pore state (Jursch *et al.*, 1994; Panchal and Bayley, 1995). Again, the labelled mutants to be tested were first applied to





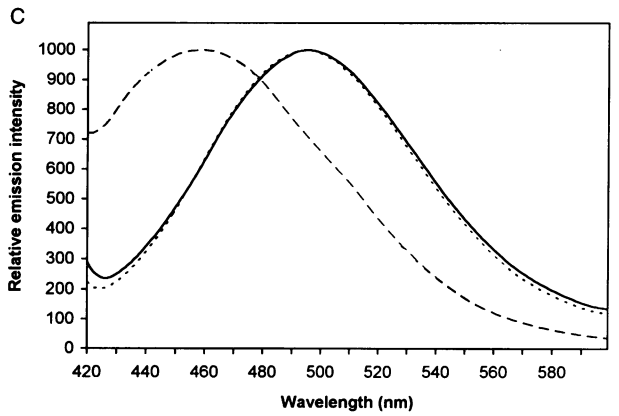


Fig. 3. Correlating the spectral effects within the central region with different stages of toxin action. (**A**) Effect of binding. Emission spectra of acrylodan-labelled mutant G126C were recorded for monomeric toxin both in solution (– – – –) and when bound to membranes (———). To prevent oligomerization, the labelled toxin was applied to liposomes at a low protein:lipid ratio (0.25 μg toxin:100 μg lipid). On binding alone, the emission spectrum was largely unaltered. The blue shift ensued only after oligomerization, which was induced by adding a 20-fold excess of unlabelled wild-type toxin (-----). Mutants Y118C–V140C yielded analogous results. (**B** and **C**) Effect of pre-pore assembly. Oligomers of the α-toxin mutant H35R are arrested in the pre-pore state. Mixed oligomers of labelled cysteine mutants and H35R toxin were generated to analyse the behaviour of the central region in pre-pore complexes. (B) Mutant N121C. Monomers bound to liposomes (– – – –) resembled those in solution (see Figure 2B). After the addition of a 10-fold excess of H35R α-toxin, a blue-shifted spectrum appeared (———), indistinguishable from that of functionally intact homogeneous N121C heptamers (-----). Labelled residues 118–124 behaved alike, proving that with this region pre-pore assembly suffices to induce the environmental change. (C) Mutant G126C. (– – – –) Homogeneous G126C heptamers on liposomes. Hybrid oligomers (———) were generated by admixing H35R prior to incubation with the liposomes. Their spectrum closely resembled that of monomeric toxin (-----). Thus, within the pre-pore complexes residue 126 was prevented from entering the membrane, as were residues 127–140.

liposomes at low protein/lipid ratio. Their incorporation into pre-pore complexes was then induced by adding a tenfold excess of H35R toxin. Figure 3B gives the results obtained with mutant N121C. Upon co-oligomerization with H35R toxin, a blue shift ensued that was identical to that effected by wild-type toxin. The same was found with all mutants in the range 118–124. Thus, formation of the pre-pore is the critical event imposing the change of environment to this sequence.

In contrast, with the mutants G126C–G137C and V140C, the addition of H35R toxin effected no blue shift. To rule out the lack of formation of hybrid oligomers in these cases, the experiments were modified as described for mutant G126C (Figure 3C). It was ascertained that labelled G126C toxin displayed the expected blue shift when added to liposomes at protein:lipid ratios of both 1:10 and 1:100. The same amount of the labelled mutant was then admixed with a 10-fold excess of H35R toxin prior to incubation with liposomes (total protein:lipid ratio, 1:10). The total loss of the spectral shift indicated that the co-oligomerization of H35R and labelled G126C had prevented the latter from penetrating the membrane. The same behaviour was confirmed for D127C–G137C and V140C.

With G130C, the reaction was scaled up and the sample was solubilized with deoxycholate (see Materials and methods). Gel filtration yielded the same elution profile as with heptamerized wild-type toxin (data not shown). The heptamers displayed the same emission maximum as the respective monomers and they were not stable in SDS–PAGE, as is typical of H35R oligomers (Jursch *et al.*, 1994).

Within the pre-pore, mutants G126C–G137C and V140C thus failed to insert into the lipid bilayer. Therefore, this domain within the central region does not penetrate the membrane until the ultimate step of pore formation occurs. The only mutant within this membrane-inserting region to display a blue shift upon pre-pore assembly was A138C. The magnitude of this shift did not approach that observed upon co-oligomerization with wild-type toxin, but rather equalled that of deoxycholate-solubilized oligomers. Thus this residue displayed properties of both functional subdomains within the central region and may be involved in pre-pore assembly and membrane penetration.

Evidence that residue 121 lines the transmembrane pore

The N121C mutant displayed remarkable properties which led us to propose that this residue directly lines the transmembrane pore.

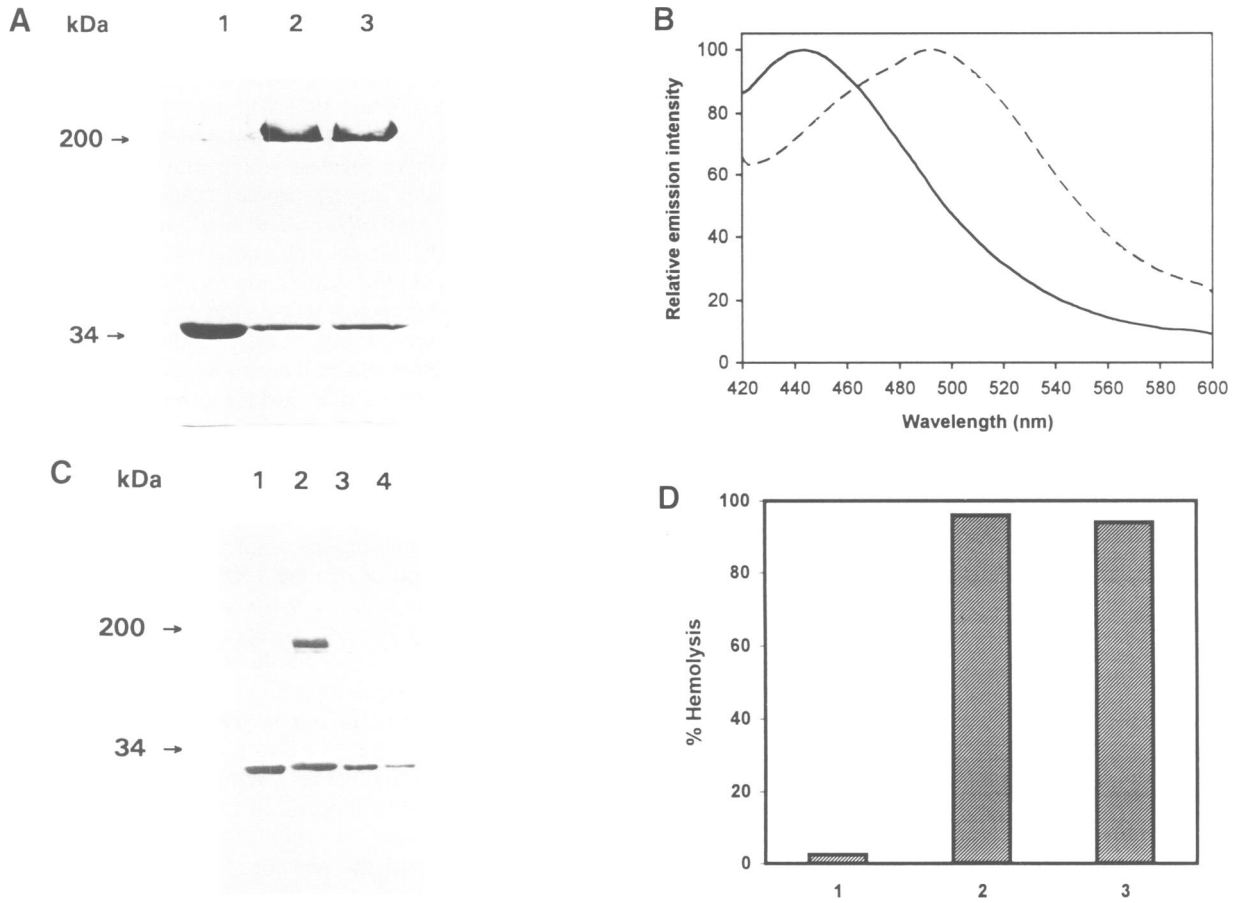


Fig. 4. Residue 121 is located inside the pore complex and can be used to switch its activity. (**A**) Biotinylated α-toxin N121C was incubated with liposomes and subjected to SDS–PAGE. Lane 1, monomeric biotinylated protein; lane 2, biotinylated protein after oligomerization; lane 3, unmodified N121C after oligomerization. Modification of the thiol group did not prevent the formation of SDS-resistant heptamers. (**B**) Acrylodan-labelled α-toxin G126C was bound to liposomes at a low protein:lipid ratio. The lack of an emission blue shift indicated the absence of oligomer formation (– – – –). Adding an excess of biotinylated N121C (———) effected the blue shift, resembling that obtained with wild-type toxin (Figure 3A), proving the formation of properly membrane-inserted oligomers. (**C**) Biotinylated N121C and G126C. Both monomers and oligomers were run on SDS gels, blotted and exposed to streptavidin peroxidase. Lane 1, monomeric G126C. Lane 2, oligomeric G126C. Oligomerization does not alter the accessibility for streptavidin. Lane 3, monomeric N121C. Lane 4, oligomeric N121C. Biotin is no longer detectable with streptavidin, which points to an occluded location inside the heptamer. (**D**) N121C was modified with the disulfide-forming reagent APDP. Rabbit erythrocytes were laden with the modified protein and washed to remove unbound toxin. Haemolysis was quantified photometrically. Lane 1, without reduction with DTT: red cells remained intact. Lane 2, after reductively releasing the thiol group with 2 mM DTT, total haemolysis occurred as with unmodified N121C (lane 3).

Derivatization of the sulfhydryl group with a larger group than acrylodan, e.g. with biotin maleimide, totally inactivated the toxin. When cells or liposomes were treated with the biotinylated and inactive derivative, SDS–PAGE revealed the presence of SDS-stable oligomers (Figure 4A). To discern whether the lack of functionality was caused by the absence of membrane insertion of the central domain, the following experiment was performed. Liposomes were incubated with a low concentration of the acrylodan-labelled G126C mutant, such that monomer binding without oligomerization took place. As expected, no blue shift occurred. The biotinylated N121C toxin was then added. As shown (Figure 4B), the blue shift in G126C occurred immediately. Hence, the non-functionality of biotinylated N121C was not caused by a lack of membrane insertion of the central molecular domain. Next we probed the location and accessibility of biotin in heptamers after extraction from membranes. Liposomes were laden with either biotinylated N121C or G126C. They were pelleted, subjected to SDS–PAGE and blotted. Incubation with streptavidin peroxidase showed that biotin attached to G126C was accessible to streptavidin peroxidase in both the heptamer and the monomer (generated by boiling of the sample prior to SDS–PAGE). In contrast, biotin contained within the G121C heptamer was entirely inaccessible for the reaction with streptavidin peroxidase, but it became accessible after dissociation to the monomer (Figure 4C). This suggested that the biotin might lie within the lumen of the pore, which is too narrow to permit the access of streptavidin peroxidase. Were this the case, then the reductive removal of a reversibly bound sulfhydryl reagent might open the preformed pore.

To test this, N121C was modified with *N*-[4-(*p*-azidosalicylamido)butyl]-3′-[2′-pyridyldithio]-propionamide (APDP), a reagent with a molecular weight similar to that of biotin maleimide and which forms a disulfide bond with sulfhydryl groups. Again, this modification caused total loss of the haemolytic activity, with the retention of

oligomerizing properties. Rabbit erythrocytes were laden with this derivatized mutant for 20 min. The intact cells were washed to remove unbound toxin and resuspended in saline. No lysis occurred. Upon treatment with dithiothreitol (DTT), total lysis occurred within 5 min (Figure 4C). This experiment indicated that the removal of a blocking moiety from the –SH group of N121C indeed opened the preformed pore, and further supported the contention that this residue lines the pore.

Discussion

Probing structure–function relationships in oligomerizing pore-forming toxins poses a number of difficulties. Accumulating evidence indicates that these toxins cannot be dissected into linear functional domains. For example, the deletion of short sequences at either the N- or C-terminus of α-toxin leads to its inactivation (Walker *et al.*, 1992). Proteolytic dissection of the assembled pore is also problematic because of the high resistance of oligomers towards degradation (Fuessle *et al.*, 1981; Tobkes *et al.*, 1985; Walker *et al.*, 1993). Thus application of the latter approach does not permit the identification of membrane-inserting regions in α-toxin. An additional difficulty lies in the probability that only small regions in the molecules enter the membrane, and their identification may thus prove to be generally difficult. For these reasons, approaches based on site-directed mutagenesis are a logical choice (Jursch *et al.*, 1994; Krishnasastry *et al.*, 1994).

Engineered single cysteines offer a convenient way of site-specific labelling. Cysteine replacement mutants of colicins A and E1 have been studied using environmentally sensitive fluorescent (Lakey *et al.*, 1991a,b) and electron-spin resonance (Shin *et al.*, 1993) probes. We decided to use acrylodan, which offers a remarkable sensitivity towards changes of polarity, e.g. Stokes shifts up to 80 nm were observed in our study. However, it is sometimes difficult to achieve complete modification of the protein to be labelled. The question may then arise as to whether the functional activity of the labelled protein preparations is really caused by labelled molecules or by residual unlabelled ones. Here, all mutants were labelled to an extent of at least 60%. The labelled proteins readily formed heptamers which were resistant towards dissociation by SDS. With one exception (A138C), there was no reduction in haemolytic titre because of labelling. However, the latter mutant showed some clear spectral effects of oligomerization, which confirm the participation of the labelled molecules in pore assembly. The small functional effects of acrylodan labelling stand in contrast to the more extensive perturbations observed with several of these mutants and the sulfhydryl-specific reagent 4-acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonate (Walker and Bayley, 1995), which is larger than acrylodan and carries two negative charges.

An examination of α-toxin mutants derivatized with acrylodan led us to identify the sequence encompassing residues 126–140 as a major domain that forms the transmembrane pore. The arguments for this contention can be summarized as follows.

Amphiphilic proteins like the α-toxin oligomer (Fuessle *et al.*, 1981) make hydrophobic contacts with the apolar interior of membranes. Upon the removal of membrane lipids with detergents, these may cover the membrane-inserted regions of the protein (Helenius and Simons, 1975). Still, detergents of different structure may provide the membrane contact sites with environments of varying polarity. Such variations can be detected by environmentally sensitive probes, which may therefore be used to pinpoint sites in the protein that contact the apolar interior of the lipid bilayer. The acrylodan-labelled mutants G126C–V140C all showed partial reversal of the large bilayer-induced blue shifts upon membrane solubilization. Among the detergents tested, the largest effects of this kind were observed with deoxycholate. It is possible that the rigid structure of the latter molecule prevents its close adaptation to the apolar surface offered by the protein. However, clear reversals of the blue shifts were also observed with flexible molecules such as *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulfonate (Zwittergent 3-14), albeit to a lesser extent.

In a previous communication, it was shown that membrane penetration of residue 130 is confined to the actual pore conformation of the α-toxin heptamer (Valeva *et al.*, 1995). Based on the results reported here, this contention can be extended to the whole sequence 126–140, which thus appears to be a continuous pore-forming domain of α-toxin. Moreover, in our study hybrid oligomers were employed, consisting of both active and functionally defective toxin molecules. Within such oligomers, the inactive monomers suppressed the membrane penetration of the active ones *in trans*. This is evidence that the functionally critical pre-pore to pore transition is cooperative in nature.

Several mutants outside this region also showed emission blue shifts upon oligomerization. However, these proved insensitive towards membrane solubilization with detergents and hence were most probably caused by hydrophobic interactions of acrylodan within the protein complex. The most pronounced effects of this type cluster directly adjacent to the membrane-inserting region, i.e. in the amino acid sequence 118–124. Shielded from direct contact with lipid molecules, this stretch may nevertheless assume a position beneath the external membrane plane. Even then, a functional distinction from residues 126–140 lies in the fact that the environmental effects observed with residues 118–124 coincide with assembly of the pre-pore rather than pore formation itself. The blue shifts in this region are likely to arise from intersubunit contacts in the pre-pore, although an intrasubunit interaction cannot be ruled out.

The behaviour of N121C was singular. The attachment of biotin maleimide or APDP caused the total loss of pore-forming activity, despite the fact that SDS-stable, membrane-inserted oligomers were formed. Biotin attached to this protein was not exposed in isolated oligomers. Thus, biotin and APDP probably assume a position inside the pore. Remarkably, the pores could be opened by the reductive removal of APDP from preformed oligomers on intact erythrocytes. Therefore, we conclude that residue 121 lines the pore. A series of triggers and switches has been engineered into the α-toxin pore (Bayley, 1995), and the present experiments are another demonstration of how a triggerable pore can be constructed which may be of use in cell biology.

While acrylodan attached to residue 121 in the oligo-

meric state detects a fairly hydrophobic environment, the action of DTT upon APDP-modified N121C toxin proves the accessibility of this residue to small hydrophilic compounds. This need not be contradictory: while an only occasional aqueous exposure of a given amino acid residue should afford it with chemical reactivity, the fluorescence of the attached labels might give a representation of its 'average' behaviour. However, there are several hydrophilic amino acid residues within the membrane-inserting region which are likely to face the pore lumen. This should then also hold for the corresponding cysteine replacement mutants; indeed one of these (T129C) has been shown to be accessible to hydrophilic reagents in the oligomeric state (Krishnasastry *et al.*, 1994). Nevertheless, when derivatized with acrylodan prior to oligomerization, the label apparently faced the outer surface of the pore wall. This apparent discrepancy cannot simply be ascribed to the hydrophobic character of acrylodan, because in two instances (D127C and T129C) the same result was found for the charged probe IAEDANS (data not shown). Indeed, the lack of periodicity of the acrylodan environment in the putative pore-forming sequence remains a puzzle for which we have no satisfactory explanation. However, this does not invalidate the basic conclusion that the central stretch of amino acids in the α-toxin molecule penetrates into the lipid bilayer.

In summary, we propose that the central molecular region of α-toxin is divided into two functional domains: residues 118–124 participate in assembly of the pre-pore, while residues 126–140 insert into the lipid bilayer and become part of the pore wall. Residue 121 probably lines the pore lumen near the external membrane surface. If 15–18 amino acids were to insert into the hydrophobic membrane interior, they would theoretically be able to traverse the membrane bilayer twice in the form of antiparallel β-strands. The penetration of seven peptide loops into the bilayer would be compatible with the formation of a β-barrel with a 1–2 nm diameter pore running through the centre of the toxin oligomer.

This identification of an extended segment of a pore-forming protein that enters the bilayer is the first of its kind. Ultimately, the validity of our model may be confirmed or refuted by a crystallographic analysis of the oligomers, a major prerequisite being that the structure of the oligomer formed in detergent is identical to that of its membrane-inserted counterpart.

Materials and methods

Mutagenesis and cloning

Most of the cysteine mutations had been introduced previously into a pT7-derived *Escherichia coli* expression vector (Walker and Bayley, 1995). These were recloned using appropriate restriction sites into the plasmid pDU 1212 (Fairweather *et al.*, 1983) to allow for their expression in *Staphylococcus aureus*. Additional mutants were generated by PCR mutagenesis according to published procedures (Cormack, 1987), with cloning of the mutant PCR products directly into pDU 1212.

Toxin isolation

Wild-type α-toxin and mutant toxins were isolated as described previously (Palmer *et al.*, 1993a). Briefly, the α-toxin-negative *S.aureus* strain DU 1090 (Fairweather *et al.*, 1983) was transfected with mutant derivatives of pDU 1212 and grown in 2 l of 2× TY broth. Protein from culture supernatants was concentrated and transferred into 20 mM ammonium acetate, pH 5.8, by membrane filtration. The mutant toxins were purified by ion-exchange chromatography on S-Sepharose FF (Pharmacia, Freiburg, Germany). Proteins were stored with 5 mM DTT at −70°C.

Labelling of sulfhydryl groups

The mutant proteins were selectively derivatized on the single sulfhydryl groups with the following reagents: acrylodan (Molecular Probes, Eugene, OR), IAEDANS (Fluka, Buchs, Switzerland), APDP (Pierce, Rockford, IL) or biotin maleimide (Sigma, München, Germany). Prior to labelling, proteins were transferred into degassed PBS with 1 mM ethylenediaminetetraacetic acid (EDTA) (PBS/EDTA) using PD10 columns (Pharmacia). A 5-fold molar excess of reagent was then added and the mixture incubated at room temperature for up to 2 h. Except in the case of APDP, DTT was then added to 5 mM to stop the labelling reaction. Acrylodan that had not reacted with protein was removed by passing the reaction mixture over methyl-Sepharose (Bio-Rad). The other unbound reagents were removed by gel filtration. Successful removal of the unbound fluorescent molecules was verified by the absence of fluorescence at the dye-front of UV-transilluminated SDS gels. Wild-type α-toxin treated in parallel with the mutants was not labelled by the fluorescent reagents.

The extent of labelling was determined by measuring the absorbance in 6 M guanidine hydrochloride (pH 6.5) at 280 and 365 nm. The ε_{280} of α-toxin was calculated from sequence data according to Gill and von Hippel (1989); ε_{365} of acrylodan in guanidine was determined to be 1.57×10^4 $M^{-1}cm^{-1}$ with the 2-mercaptoethanol adduct (based on the ε_{391} of the latter in methanol of 2×10^4 $M^{-1}cm^{-1}$, as given by the supplier). The relative absorbance of acrylodan in guanidine at 280 nm was found to be 61% of that at 365 nm. This value was used to correct the total absorbance at 280 nm of the labelled proteins for calculating the protein concentration.

Liposome preparation

Egg yolk phosphatidylcholine, egg yolk phosphatidylglycerol and cholesterol (Fluka; molar ratio 5:1:4) were dried down from chloroform/methanol (2:1 v/v) with N_2 and resuspended to 5 mg/ml total lipid in PBS. Liposomes were prepared by ultrasonication (10 min with a Branson probe sonifier 250, output scale set to 30), centrifuged briefly to remove titanium particles and used directly.

Binding of α-toxin to liposomes and heptamer formation

Labelled toxins were incubated with liposomes at room temperature. In each case, the final volume was brought to 150 μl with PBS. Where complete heptamerization of the labelled protein was desired, 5 μg toxin were added to 50 μg lipid. To avoid heptamerization of the bound labelled toxin, 0.25 μg toxin were incubated with 100 μg lipid. Toxins G126C–G137C and V140C were also applied at 2 μg to 20 and 200 μg lipid. After 45 min, liposomes were collected in a Beckman airfuge (100 000 *g*, 30 min). They were resuspended in 1 ml PBS and analysed by spectrofluorimetry, SDS–PAGE or blotting. Samples containing monomeric toxin bound to liposomes were then incubated with 5 μg wild type or H35R α-toxin, incubated for another 45 min and re-examined.

Preparation of delipidated acrylodan-labelled α-toxin oligomers

Liposomes carrying heptamerized acrylodan-labelled toxin mutants were solubilized with detergent [either 125 mM sodium deoxycholate, 2% (v/v) Triton X-100 or 25 mM *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulfonate (Zwittergent 3-14; Calbiochem)]. To separate oligomers from lipids and residual monomers, the samples were then subjected to gel filtration (Bhakdi *et al.*, 1981) on a Sephacryl S-300 column, which was equilibrated with 150 mM NaCl, 20 mM Tris, pH 8.3, containing the same detergent in lower concentrations [6.25 mM sodium deoxycholate, 0.1% (v/v) Triton X-100 or 2.5 mM *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulfonate]. Fractions containing the oligomers were collected, checked for heptamer integrity on SDS gels and used for fluorimetry.

Spectrofluorimetry

Emission spectra were recorded in a SPEX Fluoromax spectrofluorimeter (excitation wavelength 365 nm for acrylodan, 340 nm for IAEDANS; excitation and emission bandpasses 1.2 nm). All buffers and liposome preparations were checked for the absence of significant fluorescence.

Haemolytic titration

Serial 2-fold dilutions of α-toxin mutants were prepared in a microtitre plate with PBS/0.1% bovine serum albumin (BSA)/1 mM DTT. Rabbit

erythrocytes were added to 1.25% and plates incubated at 25°C for 60 min before visual reading of the haemolytic titre.

Blotting

To judge the accessibility to streptavidin of biotin bound to monomeric and oligomeric α-toxin mutants, both forms were subjected to SDS–PAGE and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), as described previously (Bhakdi *et al.*, 1984). Membranes were saturated with 1% BSA in Tris-buffered saline (TBS) for 15 min, washed twice with TBS/0.1% Tween 20 (Serva, Heidelberg, Germany) and incubated with streptavidin peroxidase (Amersham, Braunschweig, Germany) for 2 h. After three washes with TBS/Tween, the blots were developed with diaminobenzidine/H_2O_2.

Reductive opening of α-toxin pores

APDP-labelled α-toxin N121C (0.7 mg) was incubated with 1 ml rabbit erythrocytes (2.5% w/v) for 45 min at room temperature. The cells were washed three times and resuspended with PBS. DTT was added to 2 mM and the mixture incubated for 20 min at room temperature. The sample was then centrifuged and the haemolysis quantified by absorption of the supernatant at 412 nm (osmotically lysed red cells serving as a standard).

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