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Correct oligomerization is a prerequisite for insertion of the central molecular domain of staphylococcal α-toxin into the lipid bilayer

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Abstract

Staphylococcal α-toxin is a primarily hydrophilic molecule that binds as a monomer to target membranes and then aggregates to form amphiphilic oligomers that represent water-filled transmembrane channels. Current evidence indicates that a region located in the center of the molecule inserts deeply into the bilayer. In the present study, we sought to determine whether membrane insertion was triggered by the oligomerization process, and whether insertion correlated with pore formation. Double mutants of α-toxin were prepared in which His-35 was replaced by Arg, and cysteine residues were introduced at positions 69, 130 and 186. Substitution of His-35 with Arg rendered the toxin molecules incapable of proper oligomerization, so that they remained in nonlytic form after binding to membranes. The sulfhydryl groups were labelled with the polarity-sensitive fluorescent dye acrylodan. Functionally intact, single mutant toxins containing only the cysteine residues were utilized as controls. Measurements of the fluorescence emission spectrum of acrylodan were performed for the active and inactive α-toxin mutants in free solution and in membrane-bound form. The collective results demonstrate that proper oligomerization is required for membrane insertion of the central region in the α-toxin molecule, and that lack of insertion correlates with absence of pore formation.

Keywords: Pore-forming toxin; Membrane insertion; Oligomerization; α-Toxin; Pore formation; (*Staphylococcus*)

1. Introduction

Staphylococcal α-toxin was the first pore-forming bacterial toxin to be described [1,2] and, with aerolysin, today represents the best-studied prototype of an oligomerizing, membrane-damaging cytolysin. The toxin is secreted by *S. aureus* as a water-soluble monomer of M_r 34 000 containing no cysteine residues [3]. Binding to target membranes can occur via a high affinity interaction with an unidentified receptor, or via low affinity, non-specific absorption to the lipid bilayer [4–6]. In both cases, toxin molecules diffuse laterally in the membrane plane, ultimately colliding to form oligomeric ring structures. These were previously estimated to consist of six subunits [2], but a recent work indicates that they may in fact be heptamers [7]. These oligomers are stable in SDS at low temperature but can be dissociated by boiling in this detergent [2]. That correct oligomerization is required for pore formation is supported by two main lines of evidence. First, the process of membrane binding can be dissociated from oligomer formation at low temperature, and absence of oligomers correlates with absence of hemolytic activity [6]. Second, proper oligomerization can be abrogated by replacement of His-35 with Arg (or with other amino acids). Although such mutants still bind normally to target membranes, they are devoid of pore-forming capacity [8]. Aberrant oligomers are visible by they are detergent-sensitive and can no longer be detected by SDS-PAGE.

To identify the membrane-inserting regions in α-toxin, we are analyzing mutant toxins containing cysteine residues at defined positions to which the polarity-sensitive dye acrylodan can be attached. The fluorescence emission

Abbreviations: S69C, α-toxin mutant with cysteine instead of serine at position 69. Analogous designations were used for the other mutants as well; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; EM, electron microscopy; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EYPC, egg yolk phosphatidylcholine; EYPG, egg yolk phosphatidylglycerol; DOC, sodium deoxycholate; Tris, trishydroxymethylaminomethane; CD, circular dichroism.

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spectrum of acrylodan reveals whether the dye is in a hydrophilic or hydrophobic environment [9–11]. We recently obtained evidence that Cys-130, located quite precisely in the center of the toxin molecule, inserts deeply into the lipid bilayer during oligomer formation [12]. That this molecular region is directly involved in the channel-forming activity of α-toxin has been demonstrated by replacement of amino acids 130 through 134 with histidine which rendered the toxin channels sensitive to blocking with zinc ions [13]. Consistent with the view that this region lines the inner surface of the channel is the accessibility of an engineered cysteine in position 129 to sulfhydryl specific reagents [14].

In contrast, acrylodan attached to S69C exhibited no change in fluorescence emission spectrum upon oligomerization, and this region hence would be expected not to penetrate into the membrane. Both of these findings were corroborated by measurements of fluorescence energy transfer to labelled membrane phospholipids [12]. Moreover, biotin attached to S69C and S186C in membrane bound oligomers was accessible to fluorescently-labelled streptavidin [15].

In the present study, we conducted experiments with single and double mutants of α-toxin with several questions in mind. First, does the shift in emission spectrum of acrylodan attached to Cys-130 truly reflect interaction with lipid? Second, is proper oligomerization a prerequisite for the insertion of Cys-130 into the bilayer? Related to this is the decisive question of whether insertion of Cys-130 correlates with pore formation. The affirmative results obtained in this study support the hypothesis that the central molecular domain of α-toxin is directly involved in forming the wall of the transmembrane pore.

2. Materials and methods

2.1. Plasmid constructions

A derivative of plasmid pDU1212 [16] carrying the mutation H35R [8] was cut with KpnI and the digest retransfected into *Escherichia coli* TG1, which resulted in deletion of an internal 660 bp fragment from the α toxin gene. This fragment was replaced by the corresponding ones carrying the cysteine codons introduced earlier [15].

2.2. Toxin isolation

Wild-type α-toxin and mutant toxins were isolated as described [15]. Briefly, the α toxin-negative *S. aureus* strain DU 1090 [17] was transfected with the plasmids and grown in 2 × TY broth. The culture supernatants were concentrated by membrane filtration and the mutant toxins purified by ion exchange chromatography on S-Sepharose HR (Pharmacia). Proteins were stored with 5 mM DTT at −70° C.

2.3. Labelling of sulfhydryl groups

Labelling with biotin maleimide (Sigma) or acrylodan (Molecular Probes, Eugene, USA): proteins were transferred into degassed PBS with 1 mM EDTA (PBS/EDTA) using PD10 columns (Pharmacia), and a 5-fold molar excess of reagent was immediately added. After 15 min (biotin) or 2 h (acrylodan), DTT was added to 5 mM and unbound label removed by gel filtration. Acrylodan labelling was evident on UV-transilluminated SDS-gels. H35R α-toxin (which contains no cysteine) was not detectably labelled.

2.4. Protection against hemolysis by mutant toxins

Mutant toxins in PBS/EDTA were pre-incubated with several SH-reagents at room temperature for 15 min and serial 2-fold dilutions were prepared in PBS containing 0.1% BSA. Rabbit erythrocytes were added to $2 \cdot 10^8$ cells/ml. After incubation for 60 min at room temperature, wild-type α-toxin was added to 5 μg/ml. Following a second incubation for 60 min, the lowest concentration of mutant toxin that completely inhibited hemolysis was determined by visual reading. H35R toxin and unlabelled double mutants were used for comparison [8].

2.5. Liposome preparation

EYPC, EYPG and cholesterol (FLUKA; molar ratio 5:1:4) were dried down from chloroform/methanol (2:1, v/v) with N_2 and resuspended in PBS. Liposomes were prepared by ultrasonication (10 min with a Branson probe sonifier 250, output scale set to 40) and used directly.

2.6. Binding of α-toxin to liposomes

Acrylodan-labelled toxins were incubated with liposomes at room temperature. After 45 min, liposomes were collected in a Beckman airfuge (100 000 × *g*, 10 min). They were resuspended in PBS and used for fluorimetry, and also analyzed by SDS-PAGE with visualization of labelled proteins by an UV-lamp.

2.7. Preparation of delipidated acrylodan-labelled 130C-α toxin oligomers

Liposomes were incubated with labelled 130C-α-toxin to generate membrane-bound oligomers. The liposomes were solubilized with 125 mM DOC and applied to a Sephacryl S-300 column equilibrated with 6.25 mM DOC, 150 mM NaCl, 20 mM Tris, pH 8.3 to separate oligomers from lipids and residual monomers. Fractions containing the oligomers were collected, checked for purity on SDS gels and used for fluorimetry.

2.8. Spectroscopy

Acrylodan emission spectra were recorded in a Hitachi 2001F fluorometer (excitation wavelength 336 nm; excitation and emission bandpass 5 nm). All buffers and liposome preparations were checked for absence of significant fluorescence.

3. Results

3.1. Characterization of double α-toxin mutants

The single mutant H35R has previously been shown to bind normally to membranes, but not to form SDS-stable oligomers [8]. This mutant is also totally devoid of hemolytic activity. We produced three double mutants, H35RG130C, H35RS69C, and H35RS186C, and first ascertained that these mutant toxins were endowed with similar properties as the single mutant H35R. All three mutants were found to be entirely devoid of hemolytic activity. To confirm that binding properties were intact, protection experiments were performed in which rabbit erythrocytes were first incubated with the toxin mutants and then exposed to 5 μg/ml wild-type toxin. The underlying rationale was that total blockade of the high affinity binding sites should occur at approx. 10 μg/ml, provided that the binding affinity of the mutant protein remained unaltered. This contention was verified in a previous study by the use of radio-immunoassays [8].

As shown in Table 1, all three double mutants indeed mimicked the single H35R mutant in their protective capacity. Furthermore, this property was not affected when the sulfhydryl group in the 130C mutant was derivatized with biotin, fluorescein or acrylodan, and only partially affected when the other two double mutants were derivatized similarly. Thus, all the hemolytically inactive toxin mutants were still able to bind to the membrane.

Table 1
Concentrations of single and double α-toxin mutants conferring protection against hemolysis by wild-type toxin on rabbit erythrocytes

Toxin	Modification	Toxin concentration (μg/ml)
H35R		10
H35RS69C		10
H35RS69C	biotin-maleimide	20
H35RS69C	acrylodan	20
H35RS69C	fluorescein-maleimide	40
H35RG130C		10
H35RG130C	biotin-maleimide	10
H35RG130C	acrylodan	10
H35RG130C	fluorescein-maleimide	10
H35RS186C		10
H35RS186C	biotin-maleimide	20
H35RS186C	acrylodan	20
H35RS186C	fluorescein-maleimide	20

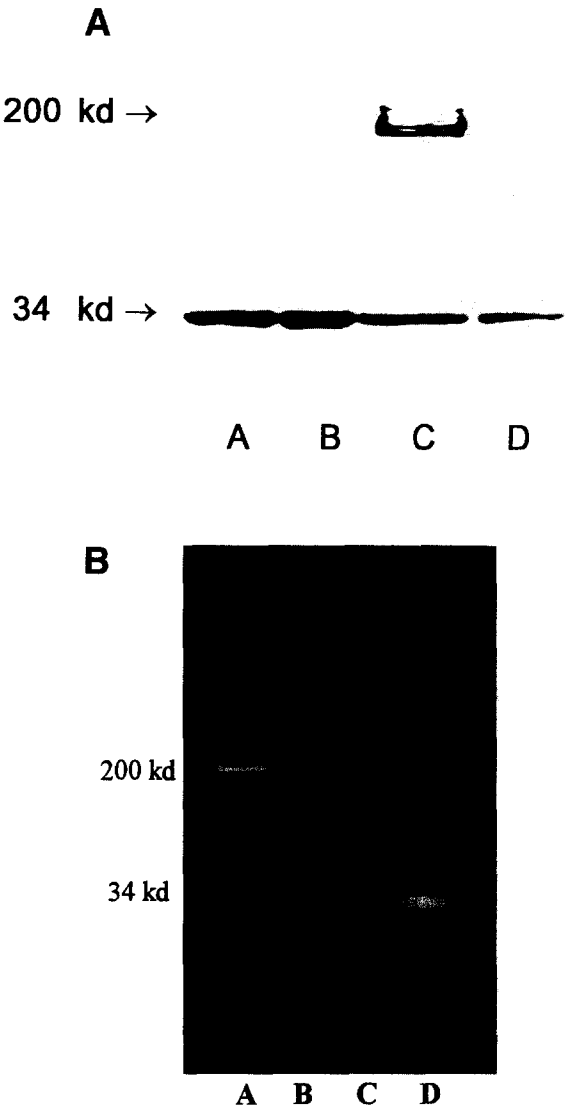

Fig. 1. (A) SDS-PAGE (Coomassie-stained) of active, single G130C mutant α-toxin (A, C) and inactive H35R G130C double mutant (B, D), all labelled with acrylodan. (A,B) Purified toxins in buffer; (C,D) toxins bound to liposomes showing formation of the 200 kDa oligomer only with the active single G130C mutant toxin. (B) SDS-PAGE (UV-transilluminated) of α-toxin single mutants S69C (A) and S186C (C) as well as double mutants H35R S69C (B) and H35R S186C (D), all labelled with acrylodan and bound to liposomes. Only single mutants form SDS-resistant oligomers.

Fig. 1A shows the results of SDS-PAGE analyses of active G130C and inactive H35RG130C toxins in solution (A, B) and after binding to liposomes (C, D), which were separated from unbound toxin by centrifugation. It is evident that formation of SDS-stable oligomers occurred only with the active single 130C mutant, as described previously [12,15]. Fig. 1B demonstrates the same behaviour for acrylodan-labelled 69 and 186 single and double mutants.

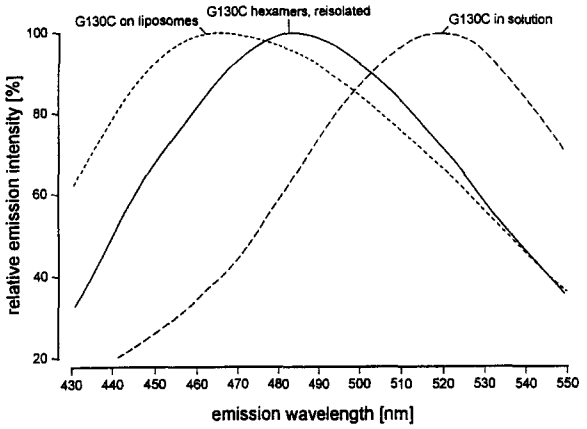

Fig. 2. Emission spectra of acrylodan-labelled protein with a λ_{ex} of 336 nm. Curves are G130C in solution, G130C bound to liposomes, and G130C oligomers after their delipidation in deoxycholate solution. Note partial reversibility of the blue shift in emission spectrum of the re-isolated oligomers.

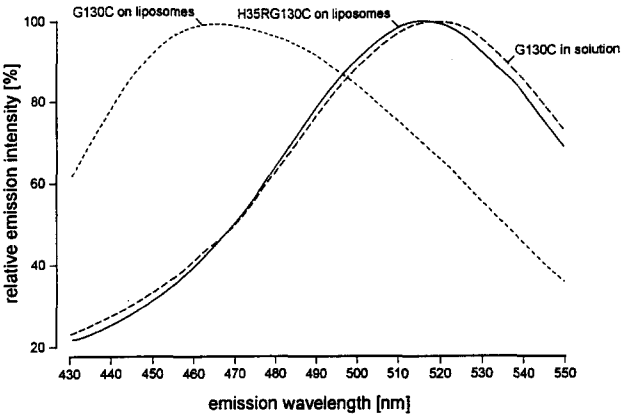

Fig. 3. Emission spectra of acrylodan-labelled protein with a λ_{ex} of 336 nm. Curves are G130C in solution, G130C bound to liposomes, H35R G130C bound to liposomes; the double mutant exhibited the same emission spectrum in solution. Note entire absence of a blue shift in emission spectrum of the inactive double mutant.

These results confirm that all inactive double mutants behaved like the H35R mutant which was previously demonstrated not to form SDS-stable oligomers [8].

3.2. Fluorescence emission shift of acrylodan at 130C reflects interaction with membrane lipid

These experiments confirmed and extended the previous report [12]. G130C was labelled with acrylodan and oligomers were isolated by gel filtration from DOC-solubilized liposomes. The emission spectrum of acrylodan was measured in comparison to membrane-bound oligomers and to monomeric toxin.

As is evident from Fig. 2, the blue shift which was associated with oligomerization on membranes was partially reversed when membrane lipids were removed. The difference to unbound toxin was probably due to locally altered protein conformation and/or to binding of detergent [18]. We interpreted partial reversibility of the blue shift to imply that lipids indeed directly contribute to the hydrophobic environment of acrylodan in membrane-bound oligomers.

3.3. Absence of oligomer formation correlates with lack of membrane insertion of 130C

Acrylodan attached to different positions of the polypeptide chain yielded varying emission maxima, probably reflecting influences of protein surface hydrophobicity peculiar to each microenvironment [12]. The results of experiments conducted with the labelled mutant toxins are summarized in Table 2, where the emission maxima of the different spectra are given. As reported previously [12], no change in the emission spectrum of the active S69C mutant was observed. In this study, a similar finding was made with the active S186C mutant. Hence, we infer that residues 69 and 186 do not gain close contact with the hydrophobic lipid environment. This contrasts with the behavior of G130C where a strong blue shift in emission spectrum of the acrylodan occurs [12]. When the membrane-bound double mutants were analyzed, it was found that all three exhibited unaltered emission spectra after binding to the membrane. Fig. 3 shows the spectra recorded for the important double mutant H35R G130C. This finding indicated that lack of, or faulty oligomerization prevented the conformational change to occur that was required for insertion of residue 130 into the bilayer.

Table 2
Emission spectrum maxima of acrylodan-labelled proteins (λ_{ex}: 336 nm) in solution and after binding to liposomes

Protein	Unbound	On membrane (oligomers)	On membrane (monomers or irregular oligomers)
S69C	480 nm	480 nm	
H35RS69C	480 nm		480 nm
G130C	520 nm	460 nm	
H35RG130C	520 nm		520 nm
S186C	475 nm	475 nm	
H35RS186C	475 nm		475 nm

4. Discussion

Formation of pores in target membranes is very widespread in biology [19]. However, progress in understanding the molecular events underlying this phenomenon has been slow because of the intrinsic difficulties in obtaining detailed structural data of these molecules in their native and membrane-bound state. To date, only the crystal structure of native aerolysin has been solved, and a working hypothesis for pore formation by this toxin based on insertion of amphiphilic β-sheets from toxin oligomers into the bilayer has been advanced [20,21].

Staphylococcal α-toxin is another useful model, the study of which is generating concepts on the mechanism of pore formation by an oligomerizing toxin [1,2,5,8,12,22–24]. Binding of the monomers probably does not involve the regions around residues 69, 130 and 186, so that cysteines placed in these locations can be derivatized with fluorescent markers or biotin without marked loss of activity [15]. H35 is also not involved in membrane-binding, but may be located in a domain that is critical for correct oligomerization to occur [8]. Work from this [2] and other laboratories [22] clearly indicates that proper oligomerization is a prerequisite to pore formation, and recent evidence indicated that the central region in α-toxin becomes lipid-inserted [12]. The possibility that this central region might in fact be directly involved in pore formation, e.g., by forming at least part of the membrane-embedded channel wall, is supported by functional studies. Thus, biochemical modifications in this region, including proteolytic nicking, or creation of gaps or overlaps, all cause alterations in functional pore characteristics [24,25]. Introduction of histidine residues 130 through 134 creates channels that are sensitive to micromolar concentrations of zinc ions, which implies that those histidines are located close to each other at a functionally critical site of the pore, most probably its inner surface. In the present study, we sought to directly determine whether oligomerization triggered the insertion process, and whether insertion of 130C correlated with pore formation. Since replacement of H35 with arginine abrogated both oligomerization and lytic activity, we produced double H35R mutants containing cysteines at positions 69, 130 and 186. As expected, all three double mutants lacked oligomerization and lytic activity [8], but they retained their binding capacity even after attachment of labels at their sulfhydryl groups. We extended our previous observation with 130C toxin by showing that the blue shift exhibited by this active mutant after interaction with lipid was reversible following the removal of the lipids. This lent credence to the contention that the shift in emission spectrum indeed reflected a movement of Cys-130 into the hydrophobic membrane interior. The experiments with the double mutant yielded clear results, and no changes in fluorescence emission spectrum were observed between the membrane-bound and free toxin. The interpretation with regard to 130C is thus straightforward and our data indicate that proper oligomerization triggers insertion of the central region of the molecule into the bilayer. Moreover, lack of insertion parallels absence of pore formation, so that this domain indeed may form part if not all of the membrane-embedded pore. As discussed previously, only a very small part of the α-toxin molecule probably actually enters the membrane [2,26]. Ultrastructural measurements show that most of the oligomer is located outside the bilayer [2]. As a correlate, the sulfhydryl groups of 69C and 186C toxin remain accessible at the extracellular surface of osmotically protected cells [15]. This previous finding is in line with the present contention that the lack of a blue-shift truly indicates lack of membrane-insertion. It is notable that CD measurements indicate no significant changes in overall structure in monomeric and oligomeric toxin [22,27], again in accord with the model that predicts participation of only small and discrete domains in pore-formation. Insertion of just 1–2 amphiphilic regions from each of the 6 toxin molecules contained in a pore-forming ring structure would theoretically suffice to build the walls of a small channel approximately 1 nm in diameter. The central, glycine-rich region is now emerging as a prime candidate for such a membrane-spanning domain, whereby one unexpected point is noteworthy. It could be anticipated that a domain predestined to enter the bilayer should be hidden in the native molecule, and might spontaneously associate with lipid once the monomer has gained close contact with the membrane. However, the region around Cys-130 is accessible to proteinases, and Cys-130 can be quantitatively reacted with sulfhydryl reagents in the hydrophilic monomer. Even after primary binding to the membrane the domain remains in the hydrophilic environment. Thus, oligomerization represents the decisive event leading to membrane insertion. This subtle alteration cannot be detected by global spectroscopic methods such as measurements of circular dichroism, and the fluorimetric assays utilizing polarity sensitive dyes are much more useful in this regard. Detailed analyses of the entire domain surrounding residue 130 in the α-toxin molecule may eventually render it possible to construct a working model on the molecular architecture of the membrane embedded pore.

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