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Staphylococcal α-toxin: the role of the N-terminus in formation of the heptameric pore — a fluorescence study [1]

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Abstract

Staphylococcus aureus α-toxin forms heptameric pores on eukaryotic cell membranes. Assembly of the heptamer precedes formation of the transmembrane pore. The latter event depends on a conformational change that drives a centrally located stretch of 15 amino acid residues into the lipid bilayer. A second region of the molecule that has been implicated in the pre-pore to pore transition is the far N-terminus. Here, we used fluorescently labeled single cysteine replacement mutants to analyze the functional role of the far N-terminus of α-toxin. Pyrene attached to mutants S3C, I5C and I7C forms excimers within the toxin pore complex. This indicates that the distance of adjacent N-termini is less than 10–12 Angstrom. By labeling with the polarity-sensitive fluorophore acrylodan, pore formation is shown to cause distinct environmental changes in the N-terminus. Removal of membrane lipids from the labeled heptamers has no effect upon the acrylodan spectrum, indicating lack of direct contact of the N-terminus with the target membrane. The environmental alterations to the N-terminus are thus due to altered protein structure only. Both acrylodan emission shifts and pyrene excimers were shown to be absent in toxin heptamers that were arrested at the pre-pore stage. Therefore, while not being directly involved in membrane penetration, the N-termini of the α-toxin heptamer subunits move into immediate mutual proximity concomitantly with transmembrane pore formation.

Keywords: Heptameric pore; α-Toxin; N-terminus; (*Staphylococcus aureus*)

1. Introduction

Staphylococcus aureus α-toxin serves as a prototype for the study of pore formation by proteinaceous toxins in lipid bilayers [1–4]. The toxin is secreted as a hydrophilic molecule of 293 amino acids lacking cysteine [5], and binds in monomeric form to target membranes. Collision of bound monomers ultimately results in formation of tightly associated heptamers surrounding a central transmembrane pore. The heptamer is arrested at the stage of a non-lytic pre-pore at 4°C. Similar effects are observed after different amino acid substitutions [6,7] and with truncation mutants [8]. Exposure to the detergent deoxycholate has been shown to cause slow transformation of monomers to heptamers [9].

The toxin molecule has been scanned for functional domains by construction of 41 point mutants carrying single cysteine residues at various positions of the sequence. The cysteines were labeled with the

Abbreviations: cps, counts per second; DOC, sodium deoxycholate; S3C (I5C, I7C, H35R), α-toxin mutant with cysteine (arginine) instead of serine (isoleucine, histidine) at amino acid position 3 (5, 7, 35)

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environmentally sensitive fluorophore acrylodan, and environmental changes to the labeled residues accompanying pore-formation were detected by shifts of the acrylodan emission. This led to identification of residues 126–140 as the a domain that lines the transmembrane pore [10].

Within this membrane-inserting loop (at residue K131), there is a site of preferential tryptic cleavage of the toxin monomer. This cleavage site becomes inaccessible concomitantly with toxin pre-pore assembly [8]. A second site at residue K8 differs from the first one by remaining accessible in the pre-pore form of the heptamer, suggesting a role for the far N-terminus of the toxin molecule in the pre-pore to pore transition, possibly by direct interaction with the target lipid bilayer. By contrast, an engineered cysteine replacing amino acid S3 remains accessible to hydrophilic reagents in the pore, suggesting that it does not enter the lipid bilayer [11].

The present study was performed to gain more information on the structural and functional roles of the far N-terminus. To this end, three N-terminally located single cysteine mutants were constructed and derivatized with fluorescent probes detecting protein contact and environmental alterations. The collective data confirm that the N-terminus is involved in the pre-pore to pore transition. By contrast with the central loop, it does not enter the lipid bilayer. Rather, the N-terminus undergoes a profound conformational change that takes adjacent subunits of the heptamer into immediate mutual contact.

2. Materials and methods

2.1. Mutagenesis and cloning

The cysteine mutations were generated by PCR mutagenesis according to published procedures [12]. The mutant PCR products were cloned into the plasmid pDU 1212 [13] to allow for their expression in *S. aureus*.

2.2. Toxin isolation

Wild-type α-toxin and mutant toxins were isolated as described [14]. Briefly, the α-toxin negative *S. aureus* strain DU 1090 [13] was transfected with mutant derivatives of pDU1212 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 and the mutant toxins purified by ion exchange chromatography on S-Sepharose FF (Pharmacia, Freiburg, Germany). Proteins were stored with 5 mM dithiothreitol (DTT) at -70°C.

2.3. Labeling of sulfhydryl groups

The mutant proteins were thiol-specifically labeled with either N-3′-pyrenyl-maleimide or 6-acryloyl-2-dimethyl-amino-naphthalene (acrylodan; both from Molecular Probes, Eugene, OR) as described [10]. To remove excess label, the protein was applied to a column of hydroxyapatite (Biogel HTP, Bio-Rad), rinsed for 1 h with 20 mM sodium phosphate, pH 7.2, and eluted with 75 mM sodium phosphate. The stoichiometry of labeling was assessed spectrophotometrically (see [10] for details). Wild type α-toxin treated in parallel with the mutants was modified neither by acrylodan nor N-3′-pyrenyl-maleimide. The activity of the labeled protein was verified by hemolytic titration carried out in duplicate according to published procedures [2] with wild-type toxin serving as standard.

2.4. Formation of α-toxin heptamers on liposome membranes

Liposomes consisting of egg yolk phosphatidylcholine, egg yolk phosphatidylglycerol and cholesterol (molar ratio 5:1:4) were prepared by sonication as described previously [15]. Labeled toxins (10 μg of labeled toxin, if not stated otherwise) were incubated with liposomes at a toxin: lipid ratio of 1:10 by weight.

Where formation of pre-pores was desired, the incubation was done on ice. Gel filtration (see below) was carried out to verify formation of heptamers. These were dissociated to monomers on SDS-PAGE at room temperature, which is typical of pre-pores as opposed to pores [10]. By contrast, incubation of the toxins with liposomes at room temperature yielded SDS-resistant pore heptamers.

The acrylodan-labeled mutants were also co-oligomerized with unlabeled H35R or wild type toxin

to yield either pre-pores (H35R) or pores (wild-type). Here, the unlabeled species was admixed in tenfold excess over the labeled one prior to addition of liposomes.

2.5. Preparation of delipidated acrylodan-labeled α-toxin oligomers

Liposomes carrying heptamerized toxin mutants (100 μg) were solubilized with 125 mM sodium deoxycholate (DOC) and applied to a Sephacryl S-300 column equilibrated with 6.25 mM DOC, 150 mM NaCl, 20 mM trishydroxymethyl-aminomethane (Tris), pH 8.3, to separate oligomers from lipids and residual monomers [9]. Fractions containing the oligomers were collected, checked for purity on SDS gels and used for fluorimetry.

2.6. Spectrofluorimetry

Emission spectra were recorded in a SPEX Fluoromax spectrofluorimeter (excitation wavelength: 365 nm for acrylodan, 339 nm for pyrene; excitation bandpass 4 nm, emission bandpass 2 nm). The appropriate buffers and liposome preparations were checked for the absence of significant fluorescence and used as blanks.

3. Results

3.1. Construction, labeling and functionality of α-toxin mutant proteins

The following mutant proteins were constructed and isolated: S3C, I5C, and I7C. Labeling yields ranged from 70 to 95%. The pore-forming effect (as assessed by specific hemolytic activity) of labeled mutants was identical to that of wild-type toxin (hemolytic titer of a 1 mg/ml solution, 1:20 000).

3.2. The subunits of the toxin pore are in close proximity at their N-termini

Fig. 1 shows the emission spectra of mutant S3C, labeled with pyrene maleimide, in monomeric and liposome-associated heptameric form. In the oligomer, the emission of monomeric pyrene is strongly suppressed, and a broad peak around 470 nm emerges which is characteristic of pyrene excimers. This indicates that the labeled residues of adjacent subunits of the heptameric pore are within a distance of less than about 10–12 Angstrom [16]. Equivalent results were also observed with I5C and I7C (data not shown).

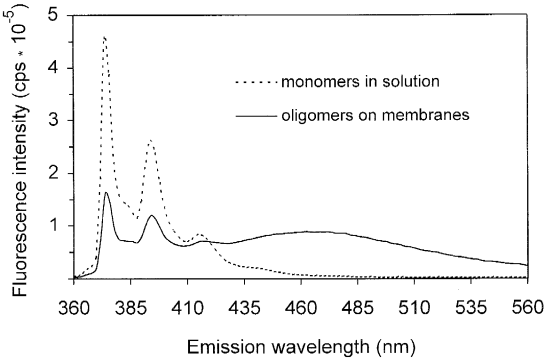

Fig. 1. Pyrene excimer formation indicates contact among N-termini within the α-toxin pore. Mutant S3C was labeled with pyrene maleimide. Both prior to and after toxin oligomerization on liposome membranes, the fluorescence emission of pyrene was recorded. In both experiments, 10 μg of labeled toxin were used. After pore assembly on membranes, the emission of pyrene monomers is decreased, and a broad peak around 470 nm emerges. This peak is characteristic of pyrene excited dimers (excimers).

3.3. Direct interaction of adjacent acrylodan molecules results in fluorescence quenching

The mutant proteins were also labeled with the polarity-sensitive dye acrylodan. The labeled toxins were transformed to heptamers by incubation with liposomes. Matching samples were prepared by using identical amounts of labeled toxin admixed with (unlabeled) wild-type toxin in excess. For mutant S3C, Fig. 2 gives the spectra of both homogeneous and hybrid heptamers. The fluorescence intensity of acrylodan is strongly quenched in the homogeneous oligomers. In addition, the emission maximum is blue-shifted, probably indicating a mutual hydrophobic interaction of adjacent acrylodan molecules. Similar results were obtained with I5C and I7C (not shown). To eliminate these extrinsic environmental changes to the probe, all further heptamer acrylodan

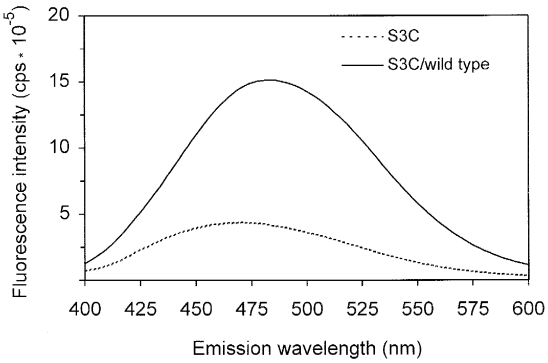

Fig. 2. The fluorescence of N-terminally attached acrylodan is self-quenched in the heptameric pore. Mutant S3C was labeled with the polarity-sensitive fluorescent dye acrylodan. 5 μg of the labeled protein (S3C) was oligomerized on membranes. A parallel sample containing the same amount of labelled toxin was supplemented with unlabeled wild type toxin in excess to effect spatial separation of acrylodan molecules in the heptamer (S3C/wild type). The emission spectra of both types of heptamers were recorded. Immediate contact among acrylodan molecules results in a pronounced decrease of fluorescence intensity. In addition, the emission maximum is blue-shifted, indicating a mutual influence upon environmental polarity of adjacent acrylodan molecules.

spectra were acquired with samples containing unlabeled toxin in excess.

3.4. Environmental effects of pore-formation to the N-terminus of α-toxin

Fig. 3A displays the acrylodan emission spectra of S3C monomers and membrane-bound as well as deoxycholate-solubilized heptamers. Oligomerization on membranes is accompanied by a distinct blue shift in acrylodan emission, which indicates a more hydrophobic environment of the label in the heptamer. The heptamer spectrum is not affected by removal of membrane lipids by detergent, which indicates lack of direct interaction between label and lipid bilayer. Residue S3C thus does not penetrate the target membrane during pore formation. Therefore, the environmental difference between monomers and heptamers is due to the contact of subunits in the oligomer and, possibly, altered conformation of the individual subunit. Less pronounced but entirely consistent effects were observed with mutant I5C (not shown). By contrast to the above two mutants, mutant I7C displays a distinct emission red shift upon pore formation (Fig. 3B), which is not sensitive to deoxycholate solubilization either.

3.5. The spectral effects to the N-terminus occur with the pre-pore to pore transition

The above results relate only to the first and the last stage of pore assembly, i.e. to the monomer and the heptameric pore. To examine the N-terminus within the pre-pore, the acrylodan-labeled cysteine mutants were co-oligomerized with the non-lytic toxin

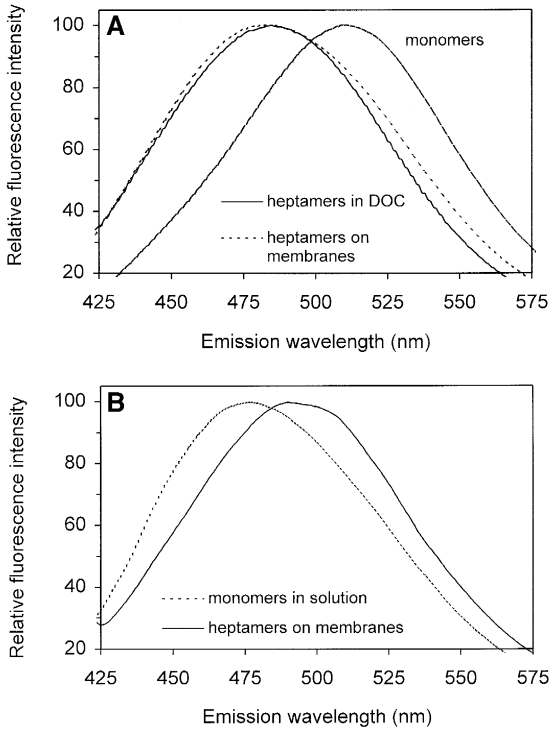

Fig. 3. Environmental effects to the N-terminus accompanying pore-formation. A. The far N-terminus is not involved in contact with the lipid bilayer. Acrylodan-labeled S3C toxin was co-oligomerized with unlabeled toxin in excess (cf. Fig. 2). The emission maximum of the heptamers on membranes is blue-shifted with respect to the monomer in solution, indicating a more hydrophobic environment in the heptamer. The heptamer emission is not affected by removal of the membrane lipids with deoxycholate (DOC), indicating lack of direct contact between label and membrane. The hydrophobic environment of the label is therefore afforded by the heptamer itself. B. The emission of acrylodan-labeled mutant I7C is red-shifted when the monomer forms heptamers on membranes. This emission red shift indicates a more hydrophilic environment of residue 7 in the heptamer. (Spectra are normalized to the same maximum intensity).

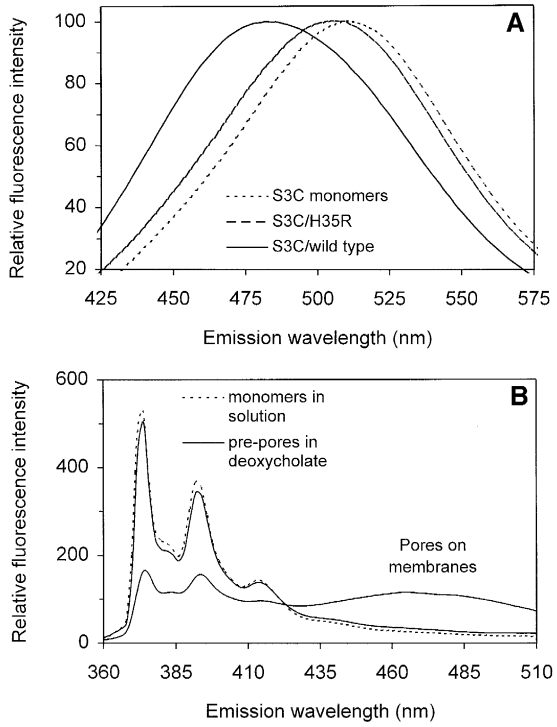

Fig. 4. The N-terminus is involved in the pre-pore to pore transition. A: Acrylodan emission spectra of S3C monomers, pore heptamers (S3C/wild type), and pre-pore heptamers (S3C/H35R). The emission blue-shift characteristic of pore-formation is largely absent from the pre-pore, indicating that the environmental change to the N-terminus has not yet occurred. (Spectra are normalized to the same maximum intensity). B: Pyrene spectra of labeled I7C monomers, pore heptamers, and pre-pore heptamers. No pyrene excimers are formed in the pre-pore, indicating lack of direct contact among adjacent N-termini within the pre-pore. (Spectra are normalized to the same average intensity).

mutant H35R in excess. This has been found to result in hybrid heptamers which are arrested in the pre-pore stage [10]. Fig. 4A gives the spectrum of S3C/H35R pre-pore oligomers. The emission maximum assumes the same position as that of the monomer, indicating that the environmental alteration has not yet occurred at this stage. Again, equivalent data were obtained with I5C and I7C.

Pre-pores were also produced of pyrene-labeled mutant proteins by incubation with liposomes on ice for 30 min. Oligomerization was verified by gel filtration on Sephacryl S300; the oligomers were dissociated by SDS at room temperature as is typical of pre-pores [8]. Fig. 4B gives the pyrene spectrum of I7C pre-pore heptamers isolated by gel permeation chromatography. There is virtually no emission around 470 nm, which means that the N-termini of the heptamer subunits are not in sufficient proximity to permit pyrene excimer formation.

4. Discussion

Fluorescent dyes attached to specific sites of proteins are powerful probes that allow for monitoring the proteins in action. Pyrene maleimide [16] and acrylodan [17] are established probes of spatial proximity or polarity of environment, respectively. When attached N-terminally to the subunits of the α-toxin heptamer, they consistently indicated that changes of both environment and mutual distance accompany the pre-pore to pore transition. No such changes were apparent during assembly of the pre-pore, which therefore does not involve the N-terminus of the toxin molecule. The same conclusion had previously been reached by limited proteolysis [8].

Despite the functional association of the N-terminus with membrane permeabilization, it does not enter the lipid bilayer itself. The pre-pore and the pore are thus conformationally distinct at sites outside the membrane. These different conformations will persist after removal of membrane lipids, which explains why both forms are differingly susceptible to dissociation by SDS.

With a larger series of acrylodan-labeled cysteine mutants of α toxin [10] and streptolysin O [18], emission shifts associated with oligomerization have been found to occur mainly toward shorter wavelengths. Where this is not due to immersion of acrylodan in the lipid membrane, the label may be provided with additional hydrophobic surfaces by adjacent oligomer subunits. One exception to this rule was observed here with I7C. Here, the label is expelled from a hydrophobic environment into a more hydrophilic one, suggesting lack of contact with adjacent protein molecules.

A few experimental details deserve short comment: with the differently labeled toxin heptamers, the formation of pyrene excited dimers is matched by quenching of acrylodan fluorescence. Most probably, the latter also arises from dimer formation. This is a common mechanism of fluorescence quenching [19], although we are not aware of previous reports on

dimers of acrylodan or prodan (of which acrylodan is a derivative). The shift in acrylodan emission that was associated with quenching points to a possible pitfall when employing environmentally sensitive probes with oligomerizing proteins.

In sum, besides the central membrane-inserting loop of α-toxin, the N-terminus constitutes the second part of the toxin molecule to display activity after pre-pore assembly. This is in line with the functional interconnection between these two regions which has been highlighted by truncation mutagenesis [8] and complementation of point mutations [20]. Data are not yet available to decide if this complementation takes place within one molecule or among adjacent subunits of the heptamer. Experiments are under way to address this question.

Acknowledgements

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References

[1] Arbuthnott, J.P., Freer, J.H. and Billcliffe, B. (1973) J. Gen. Microbiol. 75, 309–319.
[2] Fuessle, R., Bhakdi, S., Sziegoleit, A., Tranum Jensen, J., Kranz, T. and Wellensiek, H.J. (1981) J. Cell Biol. 91, 83–94.
[3] Bhakdi, S. and Tranum Jensen, J. (1991) Microbiol. Rev. 55, 733–751.
[4] Bayley, H. (1994) J. Cell Biochem. 56, 177–182.
[5] Gray, G.S. and Kehoe, M. (1984) Infect. Immun. 46, 615–618.
[6] Jursch, R., Hildebrand, A., Hobom, G., Tranum Jensen, J., Ward, R., Kehoe, M. and Bhakdi, S. (1994) Infect. Immun. 62, 2249–2256.
[7] Walker, B., Braha, O., Cheley, S. and Bayley, H. (1995) Chem. and Biol. 2, 99–105.
[8] Walker, B., Krishnasastry, M., Zorn, L. and Bayley, H. (1992) J. Biol. Chem. 267, 21782–21786.
[9] Bhakdi, S., Fuessle, R. and Tranum Jensen, J. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 5475–5479.
[10] Valeva, A., Weisser, A., Walker, B., Kehoe, M., Bayley, H., Bhakdi, S. and Palmer, M. (1996) EMBO J. 15, 1857–1864.
[11] Krishnasastry, M., Walker, B., Braha, O. and Bayley, H. (1994) FEBS Lett. 356, 66–71.
[12] Cormack, B. (1987) in Current Protocols in Molecular Biology (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.M., Seidman, J.G., Smith, J.A. and Struhl, K. eds.), Wiley and Sons,
[13] Fairweather, N., Kennedy, S., Foster, T.J., Kehoe, M. and Dougan, G. (1983) Infect. Immun. 41, 1112–1117.
[14] Palmer, M., Jursch, R., Weller, U., Valeva, A., Hilgert, K., Kehoe, M. and Bhakdi, S. (1993) J. Biol. Chem. 268, 11959–11962.
[15] Valeva, A., Palmer, M., Hilgert, K., Kehoe, M. and Bhakdi, S. (1995) Biochim. Biophys. Acta 1236, 213–218.
[16] Betcher Lange, S.L. and Lehrer, S.S. (1978) J. Biol. Chem. 253, 3757–3760.
[17] Prendergast, F.G., Meyer, M., Carlson, C.L., Iida, S. and Potter, J.D. (1983) J. Biol. Chem. 258, 7541–7544.
[18] Palmer, M., Saweljew, P., Vulicevic, I., Valeva, A., Kehoe, M. and Bhakdi, S. (1996) J. Biol. Chem. 271, 26664–26667.
[19] Chen, R.F. and Knutson, J.R. (1988) Anal. Biochem. 172, 61–77.
[20] Panchal, R.G. and Bayley, H. (1995) J. Biol. Chem. 270, 23072–23076.