Membrane Biology:
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Membrane Damage by an α-Helical Pore-forming Protein, Equinatoxin II, Proceeds through a Succession of Ordered Steps

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Background: Actinoporins are pore-forming toxins that damage cellular membranes by α-helices.

Results: An engineered mutant of actinoporin equinatoxin II reveals sequential steps during pore formation.

Conclusion: Pore formation is composed of a succession of ordered steps: fast membrane binding followed by the N-terminal region association with the membrane and oligomerization.

Significance: Equinatoxin II pore formation does not require stable prepore intermediate as is often found in other pore-forming toxins.

Actinoporin equinatoxin II (EqtII) is an archetypal example of α-helical pore-forming toxins that porate cellular membranes by the use of α-helices. Previous studies proposed several steps in the pore formation: binding of monomeric protein onto the membrane, followed by oligomerization and insertion of the N-terminal α-helix into the lipid bilayer. We studied these separate steps with an EqtII triple cysteine mutant. The mutant was engineered to monitor the insertion of the N terminus into the lipid bilayer by labeling Cys-18 with a fluorescence probe and at the same time to control the flexibility of the N-terminal region by the disulfide bond formed between cysteines introduced at positions 8 and 69. The insertion of the N terminus into the membrane proceeded shortly after the toxin binding and was followed by oligomerization. The oxidized, non-lytic, form of the mutant was still able to bind to membranes and oligomerize at the same level as the wild-type or the reduced form. However, the kinetics of the N-terminal helix insertion, the release of calcine from erythrocyte ghosts, and hemolysis of erythrocytes was much slower when membrane-bound oxidized mutant was reduced by the addition of the reductant. Results show that the N-terminal region needs to be inserted in the lipid membrane before the oligomerization into the final pore and imply that there is no need for a stable prepore formation. This is different from β-pore-forming toxins that often form β-barrel pores via a stable prepore complex.

A large number of lipid membrane-active proteins and peptides have evolved to affect viability of targeted cells by compromising their membrane integrity. Understanding the kinetics and steps in structural changes upon interaction of these molecules with the lipid membrane is essential for understanding their role in the cell physiological processes. Pore-forming toxins (PFT) were found to be a useful model to study many of these processes and provided clues on structural features of membrane proteins, their folding, and protein-protein interactions in the membrane environment. PFT intoxicate cells by a multistep mechanism that involves binding to the cell plasma membrane, oligomerization in the plane of the membrane, and final pore formation (1–3). PFT are a large group of proteins, produced by all major groups of organisms. They are divided primarily by the structural element of the final pore (1). β-Sheet PFT form pores by transmembrane β-barrels (4), whereas α-helical PFT use clusters of amphipathic or hydrophobic helices (2). In general, the details of the pore-forming mechanism of α-PFT are not as well understood as those of the β-PFT. Apart from structural details of the pore formed by cytolsis A from Escherichia coli (5), molecular properties of α-PFT pores are poorly known (*i.e.* the stoichiometry of the pore, protein regions that participate in the final pore, etc.) (1).

Actinoporins are a unique group of PFT, expressed by sea anemones, and are very important to the understanding of how α-PFT function (6–8). The two most studied examples are equinatoxin II (EqtII) from the sea anemone Actinia equina and sticholysin II from Stichodactyla helianthus. Actinoporins are 20-kDa, mostly basic proteins. Structures of three homologues

The abbreviations used are: PFT, pore-forming toxin(s); S-NO-PC, 1-palmitoyl-2-stearoyl-(5-doxyl)-sn-glycero-3-phosphocholine; BBRC, bovine red blood cell(s); DOPC, 1,2-dioleoyl-sn-glycerophosphocholine; DSS, disuccinimidyl suberate; EqtII, equinatoxin II; IANBD, N-(2-(iodoacetoyl))ethyl-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole; NBD, 7-nitrobenz-2-oxa-1,3-diazole; L/T, lipid to toxin molar ratio; LUV, large unilamellar vesicle(s); NTSB, 2-nitro-5-thiosulfobenzoate; PLB, planar lipid bilayer(s); SM, sphingomyelin; A488, Alexa Fluor® 488 C5 maleimide; pS, picosiemens.
revealed a conserved fold composed of tightly folded β-sandwich core with two α-helices flanked on each side of the molecule (9–13) (Fig. 1A). Actinoporins form cation-selective pores with a diameter of ~2 nm, mostly on membranes containing sphingomyelin (SM) (14–16). The initial membrane attachment is achieved by an exposed aromatic cluster situated on the broad loop on the bottom of the molecule and the C-terminal α-helix and by a phosphorylcholine binding site (11, 17–21). The N-terminal region, residues 1–28, was shown to be the only part of the molecule that undergoes a conformational change, and its flexibility is essential for the formation of the final pore (19, 22–24). After membrane binding, the N terminus is detached from the protein core and translocated into the lipid-water interface (22, 25) and finally across the lipid membrane, where it participates in formation of the final pore (25, 26). The molecular details about the final transmembrane pore of actinoporins are still lacking. Based on the kinetic data and cross-linking experiments, three to four monomers were deduced to be part of the final pore (14, 15). The pore was proposed to contain also lipid molecules from the membrane in a toroidal arrangement (27, 28). However, this model of the final pore was recently challenged by the crystal structure of fragaceatoxin (FraC), an actinoporin from the sea anemone Actinia fragacea (12). FraC crystals contain a nonamer complex of molecules, and based on the cryo-electron microscopy data, this oligomeric structure was proposed to be the functional unit in lipid membranes. The FraC monomer structure in the nonamer arrangement was superimposed with EqtII very well, and the N-terminal region remained associated with the β-sandwich. It was proposed that the observed structure is the pre-pore of actinoporins. Prepores were observed in many β-PFT (4) and were proposed also for some α-PFT (5, 29).

In the present work, distinct steps of EqtII pore formation, binding, insertion of the N-terminal α-helix into the membrane, and oligomerization were studied by the use of an engineered EqtII molecule. We show that the insertion of the N-terminal helix in the lipid membrane is dependent on the lipid composition of the membrane and that it immediately follows the initial binding of the toxin on the membranes containing SM. The inactive mutant, which has a membrane-penetrating region attached to the core of the molecule by an introduced disulfide bond, still binds to the lipid membrane and is able to oligomerize on the membrane surface. When such a membrane-bound molecule was treated with reductant dithiothreitol (DTT), the kinetics of the N-terminal insertion and pore forming ability was much slower as compared with the pre-reduced form. The results imply that the N-terminal region must be inserted into the membrane before the final oligomerization and pore formation occurs. Results imply that stable prepores, observed in almost all cases of β-PFT, are not needed in the pore formation of actinoporins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine-brain SM, 1,2-dioleoyl-sn-glycerophosphocholine (DOPC), 1,2-diphtytyl-sn-glycerophosphocholine and 1-palmitoyl-2-stearoyl-(5-doxyl)-sn-glycer-3-phosphocholine (5-NO-PC) were from Avanti Polar Lipids (Alabaster, AL). All other materials were from Sigma unless specified differently.

**Cloning, Expression, and Isolation of the Mutants**—The recombinant wild-type EqtII and mutants EqtII{eq}^{118C}\), EqtII{eq}^{119C}\), EqtII{eq}^{179C}\), EqtII{eq}^{118C,119C,K69C}\), and EqtII{eq}^{178C,K69C,A179C}\) were prepared as described (30). The expression vectors of the single and triple cysteine mutants were prepared by replacing corresponding EqtII residues with cysteine as described previously (23, 25). All proteins were expressed in an E. coli BL21 (DE3) strain and purified from the bacterial cytoplasm as described elsewhere (30). Wild-type EqtII and mutants were purified to homogeneity as observed on SDS-polyacrylamide gels.

**Protein Labeling**—For NBD labeling, EqtII{eq}^{118C,119C,K69C}\) was incubated in 20 mM phosphate buffer, pH 7.2, with the addition of oxidant (0.05 mM 1,10-phenantroline and 0.01 mM CuSO₄) at an ambient temperature to obtain oxidation and disulfide bond formation between amino acid residues Cys-8 and Cys-69. After 15 min, protein was separated from oxidant with a PD-10 desalting column (GE Healthcare). This step was omitted for EqtII{eq}^{118C}\). Thereafter, N-(2-iodoacetoxymethyl-N-methyl)-amino-7-nitrobenz-2-oxi-1,3-diazole (IABD) (Invitrogen) was added in an 8-fold molar excess in order to label Cys-18. The mixture was incubated on a magnetic stirrer for 1 h at ambient temperature. Labeled protein, EqtII{eq}^{118C,119C,K69C-NBD}\), was separated from the excess probe by using a 1-ml HiTrap SP HP ion exchange chromatography column (GE Healthcare). The percentage of labeled protein was determined spectrophotometrically from the absorbance at 280 nm (labeled and unlabeled protein) and 495 nm (labeled protein), using molar absorption coefficients of 43,850 M⁻¹ cm⁻¹ for the mutant protein and 25,000 M⁻¹ cm⁻¹ for IANBD (25). A correction factor of 0.95 was used for the absorbance of IANBD at 280 nm.

Similarly, EqtII{eq}^{179C}\) and EqtII{eq}^{178C,K69C,A179C}\) were labeled at the 179 position with the thiol-reactive dye Alexa Fluor® 488 C₅ maleimide (A488; Invitrogen) to obtain EqtII{eq}^{179C,488}\) and EqtII{eq}^{178C,K69C,A179C,488}\), respectively. The percentage of labeled protein was determined spectrophotometrically from the absorbance measured at 280 nm (labeled and unlabeled protein) and 478 nm (labeled protein), using molar absorption coefficients of 43,850 M⁻¹ cm⁻¹ for the mutant protein and 25,000 M⁻¹ cm⁻¹ for A488. A correction factor of 0.084 was used for the absorbance of A488 at 280 nm.

**Treatment of Mutants with Reductant or Oxidant**—The unlabeled or labeled triple cysteine mutants were treated either with 20 mM DTT or 0.5 mM 1,10-phenantroline and 0.1 mM CuSO₄ for 30 min at 37 °C to obtain a reduced (e.g., EqtII{eq}^{118C,119C,K69C-NBD-RED}\) or oxidized (EqtII{eq}^{118C,119C,K69C-NBD-OX}\) form of the mutant, respectively (23). These concentrations were sufficient to obtain fully oxidized or reduced sample according to SDS-PAGE. Samples were immediately used for measurements. The final concentration of the reductant did...
not exceed 2 mm and, therefore, had no effect on liposomes or bovine red blood cells (data not shown).

**Hemolytic Assay**—Hemolytic activity was measured turbidimetrically by the use of a microplate reader (MRX; Dynex Technologies) (18). Thoroughly washed bovine red blood cells (BRBC) were suspended in hemolysis buffer (130 mM NaCl, 20 mM Tris-HCl, pH 7.4) to a dilution of \( A_{630} = 0.5 \). One hundred \( \mu l \) of BRBC suspension were added to 100 \( \mu l \) of 2-fold serially diluted protein. Hemolysis was monitored by measuring the absorbance at 630 nm for 20 min at room temperature.

**Planar Lipid Bilayer Experiments**—Solvent-free planar lipid bilayers (PLB) were made of 1,2-diphytanoyl-sn-glycero-phosphocholine and 20% SM (w/w) (31). EqtII\(^{V8C,I18C,K69C−RED}\) or EqtII\(^{V8C,I18C,K69C−OX}\) was added at 2 and 10 nm concentration, respectively, to stable preformed bilayers on the cis side only (the cis side is where electrical potential was applied, and the trans side was grounded). When using EqtII\(^{V8C,I18C,K69C−OX}\), 1 mM DTT was added after 30 min of incubation, first into the trans and later into the cis side. All experiments were started in symmetrical conditions, using 10 mM Tris-HCl, 100 mM KCl, pH 8.0, on both sides of the membrane. In addition, when experiments with EqtII\(^{V8C,I18C,K69C−RED}\) were performed, the buffer contained 0.5 mM DTT in order to keep the toxin fully applied transmembrane potential. A PC equipped with a PC and later into the transmembrane potential.

**Binding to Liposomes**—Binding of toxin to liposomes was determined by measuring the residual hemolytic activity of the unbound toxin. 50 nM EqtII\(^{V8C,I18C,K69C−RED}\) was incubated with liposomes at the desired molar lipid/toxin (L/T) ratio in a total volume of 100 \( \mu l \) for 10 min at a room temperature. Thereafter, the lipid/toxin mixture was 2-fold serially diluted. One hundred \( \mu l \) of BRBC suspension (\( A_{630} = 0.5 \)) was added, and hemolysis was measured as described above.

**Fluorescence Spectroscopy**—All fluorescence measurements were performed by using a Jasco FP-750 spectrofluorimeter (Jasco Corp.). The sample compartment was equipped with a Peltier thermostated single-cell holder. All experiments were done at 25 °C and at a protein concentration of 250 nm in the vesicle buffer. The excitation and emission slits were set to 5 nm. NBD fluorescence spectra were measured by excitation at 470 nm, and emission was scanned from 500 to 600 nm. Subsequently, the appropriate amount of liposomes, with or without 5-NO-PC, was added at the desired L/T ratio. The spectra were corrected for the dilution factor, and the background was subtracted using the appropriate blank with liposomes. The emission wavelength was set to 538 nm in measurements of the kinetics of NBD fluorescence. The experiment was started with the desired amount of the toxin in the vesicle buffer, and after 1 min, liposomes were added to reach the desired L/T ratio.

**Permeabilization of BRBC ghosts loaded with calcein was monitored in a 1.5-ml cuvette. The excitation and emission wavelengths were set to 485 and 520 nm, respectively; both slits were set to 5 nm. Ghosts at the desired concentration were stirred at 25 °C. 50 nm protein was added after 1 min, and the fluorescence was followed for 10–30 min. The maximal release was obtained by the addition of final 2 mM Triton X-100. The percentage of calcein release (i.e. the permeabilizing activity (P (%)) was calculated as follows,

\[
P(\%) = \left( \frac{F_{\text{Tox}} - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} \right) \times 100
\]
**EqtlII Pore Formation Kinetics Reveal No Prepore Requirement**

\[ F_{T_{ox}} \] is steady-state fluorescence intensity obtained after toxin addition.

**Stopped-flow Fluorescence Measurements**—Experiments were carried out in vesicle buffer at 20 °C. Temperature was controlled by an SC150/A10 bath circulator (Thermo Fisher Scientific). Changes of intrinsic tryptophan fluorescence and NBD or A488 fluorescence upon interaction with DOPC/SM (1:1) LUV were monitored by using a stopped-flow SX-20 apparatus (Applied Photophysics). The protein concentration was 100 nM, and the lipid concentration used was 10 μM, resulting in an L/T molar ratio of 100. The excitation wavelength for tryptophan was 295 nm to avoid tyrosine fluorescence, and a 357/44-nm band pass filter (Semrock) was used for emission. NBD was excited at 470 nm, and emission was monitored with a 495-nm cut-off filter (Schott). NBD fluorescence traces were corrected for NBD-labeled protein in solution alone. For A488 excitation in a single-quenching experiment, 480-nm light was used. A488 emission intensity was monitored by using a cut-off filter at 495 nm (Schott) in combination with a 410–510-nm band pass filter (Schott). The molar ratio of A488/unlabeled protein was 3:7 in order to avoid large signal amplitudes. Self-quenching kinetics was unaffected by the A488 dilution (not shown). Fourteen traces were removed by eye, and remaining traces were averaged to obtain a single fluorescent trace. The experimental data were fitted to a single- or double-exponential function,

\[ F = F_0 + A_1 \times (e^{-k_1 \times t}) + A_2 \times (e^{-k_2 \times t}) \]  

(Eq. 4)

where \( F \) and \( F_0 \) are fluorescence and fluorescence at time \( t = 0 \), \( A_1 \) and \( A_2 \) are the amplitudes of the increase, and \( k_1 \) and \( k_2 \) are the observed rate constants.

**Cross-linking Experiments**—Cross-linking experiments were performed with BRBC. Proteins were incubated with 50 μl of BRBC suspension in 30 mM polyethylene glycol 3350 (\( A_{280} = 1.0 \)) at a desired concentration and L/T ratio for 10 min at room temperature. Disuccinimidyl suberate (Pierce), prepared freshly as a 2.5 mM stock solution in DMSO, was added to a ~100 μl final concentration and incubated at room temperature for 5 min. Samples were centrifuged for 10 min at 14,000 × g at room temperature. The supernatant was removed, and pellet was resuspended in 10 μl of SDS-PAGE buffer. Proteins were resolved by a PHAST system (GE Healthcare) on 8–25% gradient gels essentially as described by Anderluh et al. (17). Proteins were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and blotted with rabbit anti-EqtlII serum. Bands were stained with goat anti-rabbit antibodies conjugated with horseradish peroxidase using 4-chloro-1-naphthol/H₂O₂ as a substrate.

**Assaying the Accessibility of the Disulfide Bond**—The accessibility of the disulfide bond was assayed using 2-nitro-5-thiosulfobenzoate (NTSB) as described before (34). Briefly, EqtlII^V8C,I8C,K69C-OX or EqtlII^V8C,I8C,K69C-OX preincubated for 10 min at room temperature with DOPC/SM (1:1) LUV at an L/T ratio of 100 was assayed in 200 mM Tris, 100 mM Na₂SO₄, 3 mM EDTA, 0.325 mM NTSB, pH 8.5, at a final 5 μM protein concentration. The absorbance was followed at 412 nm for 600 s at room temperature.

**Statistics**—Student’s two-sample t test was performed for statistical analysis of data, with a confidence interval of 95% and significance level at 0.05 (\( p < 0.05 \)).

**RESULTS**

**Design of EqtlII Triple Cysteine Mutant That Allows Study of Different Steps in Pore Formation**—Native EqtlII does not possess any cysteine. We have shown previously that the disulfide formation between engineered cysteines at positions 8 and 69 abolished the hemolytic activity. The disulfide prevented the N-terminal region of the protein from transferring to the lipid membrane and participating in the formation of a functional pore (19, 23). Such an EqtlII variant might be useful in studying the molecular mechanism of EqtlII pore formation. Extrinsic fluorescent probes attached to different parts of the molecule may report the changes in the environment during different steps of the pore forming process, and the presence of the cleavable disulfide bond at the same time enables control over the helix transfer to the lipid membranes. We chose residues 18 and 179 as sites for the probe attachment (Fig. 1A). Labeling of position 18 could report membrane interactions of the N-terminal region. We have previously shown that labeling of the EqtlII^V8C was sufficiently good and that this residue was transferred to the lipid bilayer during the pore formation (25). The labeling at position 179 has been shown not to affect permeabilizing activity (35), and it is fully exposed to the solvent when the protein is in solution or bound to membranes (17, 35). The C terminus is located on the side opposite that occupied by regions of the molecule that participate in membrane binding (18, 19, 36) or formation of the final pore (23, 25), and any labeling does not interfere with various steps in pore formation. This position would thus be suitable to report oligomerization at the surface of the membrane, when labeled with convenient fluorescent probes.

The resulting triple cysteine mutants EqtlII^V8C,I8C,K69C and EqtlII^V8C,K69C,A179C were expressed in E. coli and purified in amounts comparable with the wild-type protein (30). EqtlII^V8C,I8C,K69C was readily labeled with IANBD at position 18 (to yield EqtlII^V8C,I8C,K69C-NBD) (Fig. 1B). The degree of the oxidation was checked by SDS-polycrylamide gels and measurement of hemolytic activity (Fig. 1, B and C). The oxidized mutant, EqtlII^V8C,I8C,K69C-NBD-OX, traveled farther on the SDS-polycrylamide gels. There were no double bands visible, indicating that the protein prepared in this way was fully oxidized. The pretreatment with DTT fully reduced the disulfide bond formed between residues 8 and 69 (EqtlII^V8C,I8C,K69C-NBD-RED; Fig. 1B) and shifted the band to the position of the wild type. The hemolytic activity was evaluated on bovine erythrocytes (Fig. 1C). EqtlII^V8C,I8C,K69C-RED and EqtlII^V8C,I8C,K69C-NBD-RED were active at a level comparable with that of the wild-type EqtlII on BRBC, showing that the mutations and labeling with IANBD did not change hemolytic activity (supplemental Table 1) (\( \phi_{\text{EqtlII}} = 0.19 \pm 0.03 \mu \text{g/ml}, \phi_{\text{EqtlII}^V8C,I8C,K69C-NBD-RED} = 0.18 \pm 0.04 \mu \text{g/ml}, \phi = 0.39 \)) (Fig. 1C). EqtlII^V8C,I8C,K69C-OX and EqtlII^V8C,I8C,K69C-NBD-OX were not hemolytic up to the highest protein concentration tested of 4 μg/ml. Similar results were obtained also with
mutants at position 179, which was labeled with A488 (data not shown).

We also checked the pore forming activity of EqtII V18C,K69C by measuring current through pores by the PLB approach. This is considered to be the most suitable approach to directly study permeabilizing activity of proteins (31). EqtII V18C,K69C-RED readily formed pores in planar lipid membranes with conductances lower than that of the wild-type pores (G EqtII = 308 ± 85 pS, G EqtII V18C,K69C-RED = 125 ± 65 pS, p = 0.0006) but with the same conductance as the single cysteine mutant EqtII V18C (G EqtII V18C = 145 ± 72 pS, p = 0.705). The current-voltage characteristics of the wild-type EqtII and EqtII V18C,K69C-RED were similar (data not shown). Cation selectivity, expressed as the ratio of permeabilities of cations over anions, P+ / P−, was slightly higher for the cysteine mutant (P+/P − EqtII = 9 ± 1, P+/P − EqtII V18C,K69C-RED = 12 ± 1, p = 0.009). The slightly higher cation permeability of the reduced mutant could be ascribed to the removal of the positive Lys-69 at the pore entrance and to the partially charged Cys-8 at this pH, because this cysteine residue is present in the lumen or close to the lumen of the pore and thus affects the selectivity (25). EqtII V18C,K69C-RED did not form pores in PLB, even after prolonged, 60-min incubation (data not shown) or when the reductant (final 1 mM DTT) was added into the trans chamber (Fig. 1D). The pores formed only when DTT was added into the cis chamber (Fig. 1D). The conductance of these pores (G EqtII V18C,K69C-RED = 138 ± 43 pS, p = 0.158), the cation permeability (P+/P − EqtII V18C,K69C-RED = 12 ± 2, p = 0.883, reduced versus oxidized), and the current/voltage characteristics were similar to those of the pores formed by EqtII V18C,K69C pretreated with the reductant (see above). On the other hand, the time needed to form pores was significantly longer for EqtII V18C,K69C-RED after reductant addition (3–5 min) in comparison with the wild-type EqtII or EqtII V18C,K69C-RED addition to planar lipid membranes (5–30 s).

We also checked whether labeling affects aggregation of EqtII V18C,K69C in solution. To this end, we performed gel filtration chromatography. EqtII (20 kDa) shows aberrant traveling through gel filtration column because the elution volume was significantly larger as compared with the marker lysozyme (14 kDa). However, both labeled variants,
EqtII\textsuperscript{V8C,I18C,K69C-NBD-RED} and EqtII\textsuperscript{V8C,I18C,K69C-NBD-OX}, traveled to a similar position as the wild-type protein, and there were no higher molecular weight aggregates visible (Fig. 1E).

According to the results, we concluded that protein prepared in this way has extrinsic fluorescent probe attached at position 18 and a cleavable disulfide bond that can be manipulated by the addition of reductant. Such a protein variant has properties closely resembling those of the previously characterized double cysteine mutant EqtII\textsuperscript{V8C,I18C} (19, 23) (i.e. there was no activity in the oxidized form, and the reduced form possessed a hemolytic and pore-forming ability closely resembling those of the wild-type EqtII). We then used labeled protein engineered in this way to study the association with the membranes during different steps of pore formation.

The Insertion of the N-Terminal Helix into the Lipid Environment—The fluorescence properties of NBD depend on the environment (25). Therefore, we next checked the fluorescence of EqtII\textsuperscript{V8C,I18C,K69C-NBD} for both reduced and oxidized states in solution and in the presence of LUV at an L/T molar ratio of 100 (Fig. 2 and Table 1). By measuring the residual hemolytic activity, we estimated that 85% of the protein was associated with liposomes (average of two independent experiments). We also ensured, by using SDS-PAGE, that the reduced and oxidized versions of the labeled triple mutant bind to membrane lipids in comparable degrees (see below). This behavior is in agreement with that of the double cysteine mutant EqtII\textsuperscript{V8C,K69C}, which in reduced or oxidized form was still able to bind to tethered lipid membrane in comparable nanomolar affinities to the wild-type protein as shown by surface plasmon resonance or by Western blotting using BRBC (19).

The EqtII\textsuperscript{V8C,I18C,K69C-NBD-RED} in solution had a fluorescence emission maximum at 539 nm (Fig. 2 and Table 1), consistent with previous work (25). The fluorescence intensity of EqtII\textsuperscript{V8C,I18C,K69C-NBD-OX} in solution was increased and blue-shifted to 536 nm in comparison with the reduced form, indicating that the probe was positioned in a slightly more hydrophobic environment. The increase of NBD is not due to aggregation of EqtII\textsuperscript{V8C,I18C,K69C-NBD-OX} in solution, which could result in probe located in a more hydrophobic environment. The protein was found to be monomeric in solution according to SDS-PAGE and gel chromatography (Fig. 1). Residue 18 is located near the \beta-sandwich in the solution structure (Fig. 1A), so it is likely that the probe is located in a more hydrophobic environment induced by the disulfide formation in the oxidized state (i.e. between the helix (Cys-8) and the sandwich (Cys-69)). In agreement, the reduction of EqtII\textsuperscript{V8C,I18C,K69C-NBD-OX} in solution caused a drop in fluorescence and maximum emission shift to values similar to those for the reduced form of the mutant (Table 1), further indicating that the difference in the initial fluorescence intensity between the oxidized and reduced form of the mutant was due to the orientation of the probe toward the hydrophobic core of the protein.

The fluorescence of EqtII\textsuperscript{V8C,I18C,K69C-NBD-RED} was increased and blue-shifted when DOPC/SM LUV or red blood cell ghosts were added (Fig. 2). This indicates that the probe was placed in a more hydrophobic lipid membrane environment. Apart from insertion into the lipid bilayer, the changes in fluorescence intensity could also be the result of protein structural rearrangements induced by the lipid membrane that would locate the probe in the more hydrophobic protein environment. The probe could also be located at the interfaces between different toxin monomers in the final pore. A lipophilic collisional quencher (5-NO-PC), which has a nitroxide quenching group located in the region of membrane acyl chains, was used to ascertain that the N-terminal helix indeed inserts into the lipid bilayer. The increase in the fluorescence intensity was considerably lower (~50%), when 5-NO-PC was incorporated into the LUV (DOPC/5-NO-PC/SM = 3:2:5 molar ratio) (Fig. 2 and Table 1), indicating insertion of the N-terminal helix with the probe into the membrane. EqtII shows poor permeabilizing activity toward DOPC LUV (36), and accordingly, no changes in fluorescence properties of NBD were visible when these vesicle preparations were used.

![Image](http://www.jbc.org/)
icles were used as a negative control (Fig. 2 and Table 1). The fluorescence of EqtII\textsuperscript{V8C,I18C,K69C-NBD-RED} was not changed when any of the tested liposomes or ghosts were added at any L/T ratio. This lack of change in the NBD environment indicates that the N-terminal helix remained fixed on the molecule and was not involved in protein oligomerization at the membrane surface or membrane insertion.

**Table 1**

Properties of NBD steady-state fluorescence spectra presented in Fig. 2

<table>
<thead>
<tr>
<th></th>
<th>EqtII\textsuperscript{V8C,I18C,K69C-NBD-RED}</th>
<th>EqtII\textsuperscript{V8C,I18C,K69C-NBD-OX}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{\text{max}}$</td>
<td>$F_L/F_S$</td>
</tr>
<tr>
<td>Solution</td>
<td>539 ± 1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>DOPC</td>
<td>539 ± 2</td>
<td>3.20 ± 0.04</td>
</tr>
<tr>
<td>DOPC/SM (1:1)</td>
<td>530 ± 1</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Ghosts</td>
<td>531 ± 1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>DOPC/5-NO-PC/SM (3:2:5)</td>
<td>534 ± 1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

$^a$ This value reports the ratio between the fluorescence of oxidized and reduced version of the protein in solution.

**Figure 3.** Comparison of binding, insertion of the N-terminal region, and oligomerization by stopped-flow fluorescence measurements. A, a pore-forming mechanism of EqtII and different steps that were studied. Approximate positions of residues at positions 18 and 179 are presented by blue and red labels, respectively. B, fluorescence traces of different probes as denoted. The proteins that were used in each experiment are noted. The concentration of lipids was 10 \textmu{}M. DOPC/SM (1:1) LUV in 140 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 8.5, were used. Protein concentration was 100 nM to achieve an L/T ratio of 100. A minimum of four single injections were averaged to obtain a single fluorescent trace (gray). Residuals of the measured data to an exponential fit are shown below the traces. The red trace shows fit to a mono- or biexponential equation (as reported under “Results”).

**Kinetics of Different Steps in the Pore-forming Mechanism** — To obtain better insight into the kinetics of different steps in pore formation induced by actinoporins, we used stopped-flow fluorescence kinetics measurements at similar conditions as in the experiments reported in Fig. 2 (L/T ratio of 100) (Fig. 3A). The EqtII binding can be conveniently monitored by intrinsic tryptophan fluorescence due to the increase in the fluorescence of exposed tryptophans from the aromatic cluster that participate in the membrane association (18, 19, 37). We compared the kinetics of membrane binding with the kinetics of the N-terminal helix transfer to the membrane by monitoring NBD fluorescence. The oligomerization of protein monomers at the surface of the mem-
brane can be monitored by self-quenching of the A488 dye (38, 39) (Fig. 3A).

The toxin binding was extremely fast. The increase in tryptophan fluorescence could be fitted to a monoexponential curve and was finished in less than 1 s (Fig. 3B). The increase in NBD fluorescence was much slower and could reliably be fitted to a biexponential equation. One of the processes was approxi-
mately 10 times slower than the binding, with the observed rate of −1 s⁻¹. The other process was much slower (observed rate of −0.04 s⁻¹) (Table 2). As expected, EqII V8C,I18C,K69C-NBD showed similar kinetics to EqII H18C-NBD, because a reduced disulfide bond enables an N-terminal conformational change in a fashion similar to that of EqII H18C-NBD (Table 2). In the oxidized state of the mutant, there was no significant change in the NBD fluorescence, in agreement with the steady state data presented in Fig. 2. Fluorescence traces of A488, which report self-quenching, could be fitted to a monoexponential equation in the case of the wild-type EqII. The observed rate was similar to the slowest process of the NBD fluorescence (quenching observed rate 0.034 s⁻¹). We also checked self-quenching of the reduced and oxidized triple mutant and found it comparable with that of the wild-type at both conditions. However, in these cases, there were deviations from the monoeXponential fit. We also noted that the amplitude of the signal change was larger in the case of the oxidized version in comparison with the wild type and the reduced form. This could indicate that monomers of the oxidized form can come closer to each other at the surface of the lipid membrane.

Membrane-induced Aggregation—The stopped-flow results are particularly interesting for the oxidized version of the triple mutant because it did not show any changes in NBD fluorescence but obviously has the capacity to oligomerize at the surface of liposomes. To independently assess oligomerization at the plane of the membrane, we employed cross-linking of the protein bound to the membrane. It was previously shown that it is possible to cross-link monomeric EqII at the surface of LUV (14) to provide qualitative data on its membrane binding and oligomerization. We therefore used disuccinimidyl suberate (DSS) to cross-link EqII or EqII V8C,I18C,K69C via amino groups after binding to bovine erythrocytes. All proteins bound to membranes at comparable levels (Fig. 4). A minor amount of dimers was visible together with predominate monomeric form when proteins were incubated with DSS in the absence of membranes. However, in the presence of the membranes and DSS, the monomeric form of all of the proteins was largely shifted to large molecular weight forms. Trimmers and sometimes even tetramers were enriched in comparison with the monomeric form. Some of the protein was found as large high molecular weight aggregates at the site of the application of samples; however, the amounts were very low in comparison with other forms (denoted as Agg in Fig. 4). In these tests, the oxidized version of EqII V8C,I18C,K69C behaved identically as the wild-type protein or the reduced version, indicating that membrane-induced aggregation is not prevented if movements of the N-terminal region are restricted by a disulfide formation.

Helix Must Insert into the Membrane Prior to Oligomerization for Efficient Pore Formation—Finally, we tried to activate oxidized triple mutant with the addition of reductant after membrane binding. We therefore added 2 mM DTT to EqII V8C,I18C,K69C-NBD-OX after 5 min, when most of the mutant was bound to the membrane (Fig. 5, blue line). The kinetics of the N-terminal insertion was in this case considerably slower and was not finished even after 20 min (Fig. 5A). The differences in fluorescence properties between the reduced and oxidized forms of EqII V8C,I18C,K69C-NBD-OX enabled us also to monitor the kinetics of disulfide reduction in the buffer at the same experimental conditions. The mutant is fully reduced in less than 3 min upon the addition of DTT because the NBD fluorescence decreased to the level of EqII V8C,I18C,K69C-NBD-RED, much faster than the helix inser-

### Table 2

| Parameters obtained from fitting the stopped-flow data presented in Fig. 3 |
|-----------------------------|-----------------------------|-----------------------------|
|                            | $k_1$ | $A_1$                  | $k_1$ | $A_1$                  |
| Tryptophan fluorescence    |       |                        |       |                        |
| EqII                       | 19.3 ± 3.4 | 0.13 ± 0.04          |       |                        |
| NBD fluorescence           |       |                        |       |                        |
| EqII V8C,I18C,K69C-NBD-RED | 0.70 ± 0.05 | 0.14 ± 0.04          | 0.040 ± 0.005 | 0.36 ± 0.07          |
| EqII V8C,I18C,K69C-NBD     | 0.66 ± 0.06 | 0.26 ± 0.04          | 0.060 ± 0.002 | 0.74 ± 0.08          |
| A488 self-quenching        |       |                        |       |                        |
| EqII V8C,I18C,A179C-A488   | 0.034 ± 0.012 | −0.45 ± 0.21        |       |                        |
| EqII V8C,K69C,A179C-A488-RED | 0.049 ± 0.004 | −0.58 ± 0.13        |       |                        |
| EqII V8C,K69C,A179C-A488-OX | 0.074 ± 0.005 | −0.85 ± 0.12        |       |                        |

**FIGURE 4. Oligomerization at the surface of the erythrocytes.** 0.25 nmol of protein was incubated with BRBC suspension. After a 10-min incubation and the addition of DSS, BRBC with bound cross-linked toxin were pelleted. Electrophoresis buffer was added to the pelleted erythrocytes, and proteins were resolved by SDS-PAGE and blotted with rabbit anti-EqtII serum. Mon, monomer; Dim, dimer; Tri, trimer; Tet, tetramer; Agg, high molecular weight aggregates; wt, wild-type EqII; Red, EqII V8C,I18C,K69C-RED; Ox, EqII V8C,I18C,A179C-A488-RED. The left panel shows EqII cross-linked in solution in the absence of BRBC. The gel was stained with Coomassie Blue.
In this paper, we studied the separate steps in the pore-forming mechanism of EqtII by employing an engineered mutant protein (Fig. 7). The results allow us to refine a current model of the actinoporin pore-forming mechanism (6–8) by providing evidence that the rate of disulfide bond reduction is not the limiting factor in helix insertion of membrane-bound activated EqtIIV8C,I18C,K69C-NBD-OX. Similar results were obtained when using BRBC ghosts (Fig. 5B).

We also assessed the permeabilizing activity of the triple mutant in different oxidation states by assaying hemolytic activity and calcein release from ghosts (Fig. 6). The time needed to reduce absorbance of BRBC suspension by 50% (t50) was less than 1 min for the wild-type (t50 < 45 ± 5 s, n = 6) and around 2 min for EqtIIV8C,I18C,K69C-NBD-RED (t50 = 124 ± 9 s, n = 6). EqtIIV8C,I18C,K69C-NBD-OX was not active in this assay (green trace in Fig. 6A). The activity was restored after the reductant was added to the suspension of BRBC. However, the kinetics of hemolysis was much slower when compared with prereduced mutant (Fig. 6A; t50 = 39 min (t50 < 45 ± 5 s, n = 19)). In addition, we checked the permeabilizing activity of EqtIIV8C,I18C,K69C-NBD-OX on ghosts loaded with calcein (Fig. 6B). EqtIIV8C,I18C,K69C-NBD-OX did not show permeabilizing activity on ghosts (Fig. 6B). Its activity was restored when 2 mM final DTT was added to the ghost-bound protein (Fig. 6B). The final values of permeabilization were similar in both cases (P(EqtIIV8C,I18C,K69C-NBD-RED) = 46.6 ± 2.6% (n = 3), P(EqtIIV8C,I18C,K69C-NBD-OX + DTT) = 37.1 ± 0.3% (n = 3), p = 0.3). However, the kinetics was considerably slower in the case of the DTT addition (Fig. 6B). To summarize, efficient pore formation is linked to movement of the N-terminal region of the protein to the lipid membranes, and this can proceed only if this region of the protein is free. Membrane-bound oxidized oligomerized mutant could be activated with the addition of the reductant; however, the kinetics of the N-terminal insertion and pore formation was considerably slower.

**DISCUSSION**

In this paper, we studied the separate steps in the pore-forming mechanism of EqtII by employing an engineered mutant protein (Fig. 7). The results allow us to refine a current model of the actinoporin pore-forming mechanism (6–8) by providing...
two novel important insights. First, we have shown that the N-terminal region insertion in the membrane is relatively fast in comparison with oligomerization, and second, the N-terminal helical region needs to be inserted into the membrane prior to oligomerization in order to allow fast and effective pore formation.

The engineered fluorescently labeled EqtII mutant allowed us to compare the kinetics of three different steps in the pore-forming mechanism. Results presented in Fig. 3 showed that initial membrane association mediated by exposed aromatic amino acids is rapid (less than 1 s in our system) and followed by oligomerization to a final pore (Fig. 5A). B, mutant in which cysteines at positions 8 and 69 locked N-terminal to the body of the molecule could bind to the membrane and oligomerize but could not form the pore.

According to the increase in NBD fluorescence intensity, the N-terminal membrane insertion of bound EqtII(V8C,I18C,K69C-NBD-RED) occurred only when the reductant was added (Fig. 5). However, in this case, the kinetics of the N-terminal membrane insertion was significantly slower. This agrees with hemolytic assays and calcein release from red blood cell ghosts (Fig. 6), where activity of initially bound and subsequently reduced EqtII(V8C,I18C,K69C-NBD-RED) was compared with the activity of EqtII(V8C,I18C,K69C-NBD-RED). Although the kinetics of the pore formation is much slower once the toxin is attached to the membrane, the final pores have the same characteristics, as shown by planar lipid bilayer experiments (Fig. 1D), and proceed to the same final release of calcein in the BRBC ghost assay (Fig. 6B). The kinetics of EqtII(V8C,I18C,K69C-NBD-RED) disulfide bond reduction in solution is not the limiting factor for the observed slow kinetics because the protein is fully reduced in less than 3 min (Fig. 5A). The DTT may act on a toxin not associated with the lipid membrane. However, as shown in Fig. 4, and consistent with (19, 23), comparable amounts of reduced and oxidized forms are bound to the membrane. The accessibility of the disulfide bond should be the same for the protein in solution or bound to the membrane unless (i) EqtII tilts upon membrane binding, posing the helix more parallel to the membrane, or (ii) EqtII rapidly oligomerizes on the membrane, thus preventing the N-terminal region from dissociating from the body of the molecule due to steric reasons. The former possibility would imply a form of the membrane-bound EqtII not yet anticipated (i.e. none of the mutants from that side of the β-sandwich, that were used in a cysteine-scanning mutagenesis, were shown to interact with the lipid membrane) (17). Furthermore, in the current model of actinoporin pore formation, the membrane binding site is exactly opposite the site of the disulfide bond between residues 8 and 69 (Fig. 1A, dashed line) (6–8, 20, 21). This implies that the disulfide remains accessible on the membrane-bound form, which we confirmed by using an NTsB assay. In the case of tilting, EqtII(V8C,I18C,K69C-NBD-RED) should also move position 18 closer to the membrane, causing an increase in NBD signal, but this is not the case (Figs. 2 and 3).

Finally, the reason for the slow kinetics may be the aggregation of the oxidized version of the toxin on the membrane plane similar to the wild type or the reduced form, possibly to form a porelike state by a weak association of monomers. In this case, the helices could not be transferred to the lipid-water interface after the disulfide reduction due to steric reasons. Disassembly of the molecules in the pore would allow insertion and consequent formation of functional pores but slowed down the insertion of the N-terminal region to the lipid membrane and consequently hemolysis or calcein release. Consistent with this proposition, cross-linking experiments (Fig. 4) confirmed the ability of the oxidized form of EqtII(V8C,I18C,K69C-NBD-RED) to oligomerize in the presence of erythrocytes. One conclusion from the cross-linking study is also that the region covered by the N-terminal part of the toxin does not participate in protein-protein interactions because the aggregation pattern of the oxidized form was no different from the wild-type EqtII or the reduced form of the mutant.

In summary, the results in this study hint at a fundamental difference in the pore-forming process of actinoporins from that of PFT, which form pores by a prepore intermediate. Clearly, the major conformational change in the molecule...
occurs immediately after the binding in the case of actinoporins and not after oligomerization, as is the case in some other examples of PFT. The N-terminal region of EqtII must be transferred to the membrane surface before the oligomerization to the final pore occurs because there is not enough space to coordinate transfer four helices from the membrane surface across the membrane to the other side through the lumen of a pore of 2 nm in diameter (11, 16). The proposed mechanism for actinoporin pore formation (Fig. 7) is different from the one proposed by Mechaly et al. (12), where the N-terminal insertion occurs only after oligomerization in a prepore composed of nine monomers. Our observation is also in contrast to β-PFT that often form non-lytic prepore complexes. Prepores are stable associations of toxin subunits on the lipid membrane, which allow the formation of hydrogen bonds between the adjacent β-strands and a coordinate conformational change that brings them across the membrane as a β-barrel to form the final pores (4, 42–44). The resulting β-barrel structure does not leave any free non-hydrogen-bonded edge exposed to the hydrophobic milieu of the lipid membrane. In contrast, the actinoporin α-helix that will cross the lipid bilayer can exist at the lipid-water interface and in the lipid membrane core as single units because the stable hydrogen bond network is formed within the helix itself only, and therefore, there is no requirement for a stable prepore (45).

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