Arresting Pore Formation of a Cholesterol-dependent Cytolysin by Disulfide Trapping Synchronizes the Insertion of the Transmembrane \( \beta \)-Sheet from a Prepore Intermediate

Eileen M. Hotze‡, Elizabeth M. Wilson-Kubalek§, Jamie Rossjohn¶, Michael W. Parker¶, Arthur E. Johnson**, and Rodney K. Tweten**

From the ‡Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190, the §Department of Cell Biology, Scripps Research Institute, La Jolla, California 92037, the ¶Biostatistical Biology Laboratory, St. Vincent’s Institute of Medical Research, Fitzroy, Victoria 3065, Australia, the **Departments of Medical Biochemistry and Genetics, of Chemistry, and of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843-1114, and the ¶Protein Crystallography Laboratory, St. Vincent's Institute of Medical Research, Fitzroy, Victoria 3065, Australia

Perfringolysin O (PFO), a member of the cholesterol-dependent cytolyis family of pore-forming toxins, forms large oligomeric complexes comprising up to 50 monomers. In the present study, a disulfide bridge was introduced between cysteine-substituted serine 190 of transmembrane hairpin 1 (TMH1) and cysteine-substituted glycine 57 of domain 2 of PFO. The resulting disulfide-trapped mutant (PFO\(^{C190-C57} \)) was devoid of hemolytic activity and could not insert either of its transmembrane \( \beta \)-hairpins (TMHs) into the membrane unless the disulfide was reduced. Both the size of the oligomer formed on the membrane and its rate of formation were unaffected by the oxidation state of the Cys190-Cys57 disulfide bond; thus, the disulfide-trapped PFO was assembled into a prepore complex on the membrane. The conversion of this prepore to the pore complex was achieved by reducing the C190-C57 disulfide bond. PFO\(^{C190-C57} \) that was allowed to form the prepore prior to the reduction of the disulfide exhibited a dramatic increase in the rate of PFO-dependent hemolysis and the membrane insertion of its TMHs when compared with toxin that had the disulfide reduced prior to the conversion of this prepore to the pore complex on the membrane. Therefore, the rate-limiting step in pore formation is prepore assembly, not TMH insertion. These data demonstrate that the prepore is a legitimate intermediate during the insertion of the large transmembrane \( \beta \)-sheet of the PFO oligomer. Finally, the PFO TMHs do not appear to insert independently, but instead their insertion is coupled.

Perfringolysin O (PFO)\(^{1} \) is a member of the large family (1) of cholesterol-dependent cytolyisins (CDCs). Two defining characteristics of CDC cytolysis are the formation of large homooligomeric structures (2, 3) on sterol-containing membranes, and the absolute dependence of pore formation on the presence of cholesterol, or closely related sterols, in the membrane (4, 5). These toxins are produced as soluble monomers that ultimately form the large homo-oligomeric pore-forming structure on sterol-containing membranes. The only crystal structure of a soluble, monomeric form of a CDC was recently solved by Rossjohn et al. (6) for perfringolysin O from Clostridium perfringens. The structure of the membrane-bound oligomeric structure of a CDC is not presently available; however, we have used multiple fluorescence techniques to identify the residues in domain 3 that form two \( \beta \)-hairpins that span the membrane in the inserted oligomeric PFO complex (7, 8). The corresponding residues were found to exist as six short \( \alpha \)-helices in the crystal structure of the soluble PFO monomer (6). The \( \alpha \)-helical to \( \beta \)-strand transition in the secondary structure together with the contribution of two \( \beta \)-hairpins per monomer to the transmembrane \( \beta \)-sheet are so far unique to PFO and perhaps the other CDCs.

The timing of oligomer assembly and of the insertion of individual transmembrane domains is controversial. Rossjohn et al. (6) originally proposed that the CDCs form a prepore complex on the membrane prior to its insertion into the membrane, similar to other unrelated \( \beta \)-barrel pore-forming toxins. In the prepore model (9), individual membrane-spanning \( \beta \)-hairpins of each monomer are brought together to form an oligomeric prepore that coordinates the insertion of the pre-\( \beta \)-hairpins into the membrane as a single membrane-spanning \( \beta \)-barrel. This model has been shown to be used by toxins that form small oligomeric prepore complexes such as Aeromonas hydrophila aerolysin, Staphylococcus aureus \( \alpha \)-hemolysin, and Clostridium septicum \( \alpha \)-toxin (9–12). In support of this model for the CDCs, we have recently shown (13) that PFO oligomerization can be uncoupled from the membrane insertion of the transmembrane \( \beta \)-hairpins by lowering the temperature. Shepard et al. (13) also demonstrated that PFO insertion into planar bilayers resulted in the formation of discrete, large channels. Based on these findings, PFO was proposed to form a prepore example of the nomenclature for a derivative of PFO\(^{C459A} \) (the cysteine-less derivative of PFO) in which residues 190 and 57 have been substituted with cysteines that form a disulfide (the residue after the slash indicates a residue that has been replaced by a cysteine that is not in a disulfide and therefore has a free sulfhydryl group).

\(^{1} \) This work was supported by National Institutes of Health Grant AI37657 (to R. K. T.) and by the Robert A. Welch Foundation (to A. E. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^{2} \) To whom correspondence should be addressed: Dept. of Microbiology and Immunology, BMSB, Rm. 1053, 940 Stanton L. Young Blvd., University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190. Tel.: 405-271-2333; Fax: 405-271-3117; E-mail: rod-tweten@ouhsc.edu.

\(^{3} \) The abbreviations used are: PFO, perfringolysin O; TMH, transmembrane \( \beta \)-hairpin; IANBD, N,N'-dimethyl-N-(iodoacetyl)-N'(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; IATR, tetramethylrhodamine-5-and 6-iodoacetamide; FRET, fluorescence resonance energy transfer; AGE, agarose gel electrophoresis; DTT, dithiothreitol; NBD, N,N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; CDC, cholesterol-dependent cytolyisins; EM, electron microscopy; PFO\(^{C190-C57} \), an example of the nomenclature for a derivative of PFO\(^{C459A} \) (the cysteine-less derivative of PFO) in which residues 190 and 57 have been substituted with cysteines that form a disulfide (the residue after the slash indicates a residue that has been replaced by a cysteine that is not in a disulfide and therefore has a free sulfhydryl group).
complex prior to the insertion of its transmembrane domain.

We have now trapped the PFO oligomer in a prepore state by the introduction of a disulfide bridge into the structure of the soluble PFO monomer. In the present study, a disulfide bridge was introduced between cysteine-substituted serine 190 of transmembrane hairpin 1 (TMH1) and glycine 57 of domain 2. In the oxidized state, the same large oligomeric prepore complex described by Shepard et al. (13) was found to form on the membrane surface. Furthermore, the initial rate of PFO binding to the membrane, mediated by domain 4 (Fig. 1), was not altered by the presence or absence of the disulfide link in PFO (14). However, the disulfide-linked PFO oligomers were unable to insert their TMHs or form pores in the membrane. Thus, the disulfide-trapped PFO allowed us to convert the membrane-bound monomer into a prepore complex and then (at our discretion) to synchronize the conversion of the prepore to pore complex by the addition of reducing reagent. These studies provide the first direct evidence that the prepore facilitates the membrane insertion of the large transmembrane β-sheet of the CDCs into the bilayer.

**EXPERIMENTAL PROCEDURES**

**Design of the Disulfide in PFO**—Potential sites for cysteine residue pairs that might form disulfide bonds were selected using the method of Hase and Dijkstra (15). Briefly, pairs of cysteine residue pairs were initially selected on the basis of appropriate Cβ-Cβ distances. Sulfur positions were generated for these residues, and a check was made to determine whether certain steric criteria were obeyed. An important criterion was that the Cβ angles of a potential bridge should not deviate by more than 30° from observed preferences. Selected pairs were subjected to energy minimization, and energetically favorable conformational changes were chosen (less than 10 kcal/mol). This method will be successful if main-chain conformations are very similar between wild-type and mutant. Calculations were based on the crystal structure of PFO (6). The model had been refined to an R-factor of 0.211 (R<sub>free</sub> of 0.268) at 2.2 Å resolution with good stereochemistry. The best candidate for engineering a disulfide bridge between domain 2 and TMH1 was the residue pair of G57 and S190 (Fig. 1). The two residues were predicted to form a bond that complied with the stereochemical (Cβ-approximately -57.0°) and conformational (7.9 kcal/mol) criteria.

**Preparation of the PFO Derivatives and Their Modification with Fluorescent Probes**—The cysteine-less gene for PFO encoding PFOC<sub>459</sub>A was that cloned in the expression vector pTRchxA (InvivoGen, Carlsbad, CA) (8) and used as the template in PCR mutagenesis to create the double mutant PFO<sub>190-C57</sub>. Four additional mutants of PFO<sub>190-C57</sub> were generated for these residues, and a check was made to determine whether certain stereochemical criteria were obeyed. An important criterion was that the Cβ angles of a potential bridge should not deviate by more than 30° from observed preferences. Selected pairs were subjected to energy minimization, and energetically favorable conformational changes were chosen (less than 10 kcal/mol). This method will be successful if main-chain conformations are very similar between wild-type and mutant. Calculations were based on the crystal structure of PFO (6).

**Fluorescence Measurements**—All fluorescence measurements were performed in a SLM-8100 photon counting spectrophotometer (SLM Instruments) as described previously (8). An excitation wavelength of 470 nm was used for NBD, and the emission intensity was measured between 500 and 600 nm. The bandpass was 4 nm for all experiments. Emission scans of NBD-labeled residues in TMH1 (Cys<sub>215</sub> or Cys<sub>202</sub>) or TMH2 (Cys<sub>268</sub> or Cys<sub>382</sub>) were recorded in both the presence and absence of 10 mM DTT in 2 ml of buffer A (50 mM HEPES (pH 7.5), 100 mM NaCl) at 37 °C. Excess liposomes were added to monomeric NBD-labeled PFO<sub>190-C57</sub> derivatives (185 nM) and allowed to incubate for 30 min at 37 °C to ensure that oligomerization and insertion into the membrane were complete before the intensity measurements were made.

**Fluorescence resonance energy transfer (FRET)-based kinetic experiments** were carried out with only the following changes to our previous procedures (17). The donor dye was NBD, and the acceptor dye was tetrathymethylboradime (final PFO concentration was 190 nM). The donor-labeled PFO<sub>190-C57</sub> was mixed at a 1:1 molar ratio with acceptor-labeled PFO<sub>190-C57</sub> in 2 ml of buffer A in the presence or absence of 10 mM DTT at 37 °C. The excitation wavelength was set at 470 nm, and the donor emission intensity was measured every 20 s at 450 nm. After 30 s, excess liposomes were injected into the mixture and the emission intensity was monitored for an additional 1170 s. The reversible-labeled liposomes had been titrated as described previously to ensure that an excess of liposomes was used to quantitatively bind and insert the PFO (8).

**Electron Microscopy**—Denaturing agarose gel electrophoresis was performed as described previously (13). Briefly, in all samples in which PFO was incubated with liposomes, the liposomes (45 mol% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 55 mol% cholesterol) were incubated with PFO for 30 min at 37 °C. Oligomeric complexes were solubilized with SDS sample buffer and separated on 1.5% SeaPlaque agarose (FMC, Rockland, ME) in SDS gel reservoir buffer (18). Samples

---

2 J. Rossjohn, M. W. Parker, and R. K. Tweten, unpublished data.
were loaded and the gel was run at 100 V for 3 h. Gels were fixed in 10% (v/v) acetic acid, 30% (v/v) methanol overnight, then dried in an Easy Blot gel dryer ( Hoefer, San Francisco, CA). The dried gel was stained with Coomassie Brilliant Blue R and then destained to visualize the protein bands.

RESULTS

Hemolytic Activity of the Disulfide-trapped PFO—The substitution of cysteines for amino acid residues Ser\textsuperscript{190} and Gly\textsuperscript{57}, and the subsequent formation of the disulfide bridge between TMH1 and domain 2, rendered PFO hemolytically inactive (Table I). If the disulfide bridge alone were responsible for the lack of activity of the double mutant, then the activity would be restored when the disulfide bond was reduced. As predicted, once reducing agent was added to the sample, the hemolytic activity was restored to nearly wild-type levels (Table I). Similarly, the NBD-labeled and unlabeled derivatives of PFO\textsuperscript{C190-C57} (PFO\textsuperscript{C190-C57/C215}, PFO\textsuperscript{C190-C57/C202}, PFO\textsuperscript{C190-C57/C288}, and PFO\textsuperscript{C190-C57/C303}) exhibited less than 6% of the hemolytic activity of the parent toxin PFO\textsuperscript{C459A} in a nonreducing environment (Table I).

We have previously determined the kinetics of erythrocyte hemolysis by PFO by monitoring the decrease in right-angle light scattering that accompanies hemolysis (17). In their studies, Harris et al. (17) showed that PFO-dependent hemolysis exhibited a lag period prior to the onset of hemolysis. When pre-reduced PFO\textsuperscript{C190-C57} was injected into a stirred suspension of erythrocytes, the same hemolysis kinetics were observed. Hemolysis began after about 100 s and reached completion by \(\sim 370\) s after the injection of the toxin (Fig. 2). In contrast, no hemolysis was detected when the disulfide-trapped mutant PFO\textsuperscript{C190-C57} was examined by this method in the absence of DTT. However, when DTT was added to the disulfide-trapped mutant \(370\) s after it had been mixed with the erythrocytes, hemolysis began almost immediately and was complete within 60 s. The lysis of the erythrocytes was nearly 7 times faster than when the disulfide-trapped mutant was reduced with dithiothreitol prior to adding the toxin to the erythrocytes. Therefore, even though PFO\textsuperscript{C190-C57} was hemolytically inactive before reduction of the disulfide, it was clear that the cytolytic mechanism had progressed to a stage that largely eliminated the typical lag period. The rate-limiting step in hemolysis therefore must occur prior to the stage at which the oxidized PFO\textsuperscript{C190-C57} derivative is trapped. We therefore characterized the oligomeric state of the disulfide-trapped mutant and examined the environment of the two transmembrane domains in the disulfide-trapped state.

Characterization of the Prepro Complex by Agarose Gel Electrophoresis and Electron Microscopy—As shown in Fig. 2, di-sulfide lacking TMH1 to domain 2 of PFO trapped the PFO in a structural state that did not allow pore formation to occur, but did prime the system for hemolysis. Although the structural nature of this trapped state was initially unclear, we suspected that it was trapped in a prepore complex. Therefore, we employed a SDS-agarose gel electrophoresis (SDS-AGE) system, previously described by Shepard et al. (13), to directly examine the distribution of PFO oligomers under nonreducing and reducing conditions. As expected, in the absence of liposomes, PFO and PFO\textsuperscript{C190-C57} (unreduced or reduced) migrate as a monomer in the SDS-agarose gel system (Fig. 3, lanes 1–3). In the presence of cholesterol-containing liposomes under non-reducing conditions, PFO\textsuperscript{C190-C57} (Fig. 3, lane 5) formed large oligomeric complexes comparable to the parent toxin PFO\textsuperscript{C459A} (lanes 4 and 6) and to those formed under conditions in which the disulfide of PFO\textsuperscript{C190-C57} was reduced (lane 7). Although oxidized PFO\textsuperscript{C190-C57} formed the same-sized oligomer as the cysteine-less parent toxin PFO\textsuperscript{C459A}, it was more susceptible to dissociation by SDS than if it was reduced and allowed to insert (compare lane 5 with lane 7). If a chemical cross-linker is first used to stabilize this complex, we do not observe the dissociation of the oxidized PFO\textsuperscript{C190-C57} oligomer into monomers (data not shown). The oligomer of the reduced form of PFO\textsuperscript{C190-C57} appeared to be stable to SDS as the cysteine-less parent toxin PFO\textsuperscript{C459A}. These data suggest that the formation of the transmembrane \(\beta\)-barrel additionally stabilizes the oligomer to dissociation by SDS.

The oligomeric complexes formed by PFO\textsuperscript{C190-C57} were also examined by electron microscopy (EM) after their formation on lipid monolayers in the presence (Fig. 4A) or absence (Fig. 4B) of DTT. Primarily rings and some arcs were observed by EM and are typical of the structures typically seen by EM when membranes are treated with PFO (e.g. Ref. 13). Furthermore, a comparison of the structures present in the presence or absence of DTT reveals that the oxidation state of the disulfide of PFO\textsuperscript{C190-C57} has little effect on the overall topography of the PFO oligomers as seen by EM.

Oligomerization Rates of Reduced and Unreduced PFO\textsuperscript{C190-C57/C215} Determined by FRET—The extent of FRET between a donor dye and an acceptor dye depends upon, among

| Mutant          | DTT   | Hemolytic activity |
|-----------------|-------|--------------------|
| Pre-reduced     |       |                    |
| PFO\textsuperscript{C190-C57} <1  | NA    |
| PFO\textsuperscript{C190-C57} 92  | NA    |
| PFO\textsuperscript{C190-C57/C215} 6  | 0     |
| PFO\textsuperscript{C190-C57/C202} 80  | 65    |
| PFO\textsuperscript{C190-C57/C202} 2  | 0     |
| PFO\textsuperscript{C190-C57/C288} 67  | 67    |
| PFO\textsuperscript{C190-C57/C288} 2  | <1    |
| PFO\textsuperscript{C190-C57/C288} 81  | 54    |
| PFO\textsuperscript{C190-C57/C303} 2  | <1    |
| PFO\textsuperscript{C190-C57/C303} 75  | 65    |
other things, the distance between the dyes. FRET is therefore an excellent technique for detecting the proximity of two proteins if one polypeptide is labeled with a donor dye and the other is labeled with an appropriate acceptor dye. We have previously used this approach to monitor the oligomerization of PFO monomers (17). Furthermore, since fluorescence is a non-destructive technique, one can monitor the signal continuously and hence determine the kinetics of association.

The rate of oligomerization of the disulfide-trapped PFO was examined by FRET between donor NBD-labeled PFO<sup>C190-C57/C215</sup> and acceptor tetramethylrhodamine-labeled PFO<sup>C190-C57/C215</sup> when cholesterol-containing liposomes were added to a 1:1 mixture of these proteins. The existence of FRET, and hence the close approach of donor- and acceptor-labeled subunits, can be detected by the reduction in donor emission intensity as excitation energy is transferred to the acceptor. As shown in Fig. 5, the introduction of liposomes to a mixture of donor- and acceptor-labeled PFO subunits causes a decrease in NBD intensity, indicating that the subunits have associated. The decrease in NBD intensity is due to FRET because no decrease in NBD intensity was observed when the sample lacked acceptor dyes (Fig. 5). Most important, the rate and extent of FRET were virtually identical whether or not the disulfide was reduced. Hence, the introduction of the disulfide did not appreciably affect the rate of PFO oligomerization, thereby demonstrating that the rate-limiting step in the process that leads to oligomer formation is not significantly altered by the presence of the disulfide bond.

Membrane Insertion of TMH1 and TMH2 in the Disulfide-trapped Complex—Since hemolysis was prevented (Table I, Fig. 2), but the formation of the oligomer was unaffected by the introduction of the C190-C57 disulfide (Fig. 5), we presumed that the disulfide bond prevented the insertion of at least TMH1 because the disulfide bond covalently links residues 190 of TMH1 and 57 of domain 2 (Fig. 1). Although it was easy to envision that the disulfide bridge could prevent TMH1 from assuming the conformation necessary for proper insertion into the bilayer, it was not clear whether inhibiting the movement of TMH1 would also prevent TMH2 from moving into the membrane. In contrast to TMH1, which is partially buried in the core of the PFO molecule, TMH2 is largely exposed to the solvent (6) and hence this domain, unrestricted by a disulfide, might partially or fully insert into the membrane independent of TMH1.

To determine whether either TMH in a disulfide-trapped PFO molecule could insert into the membrane, four derivatives of PFO<sup>C190-C57</sup> were generated in which a cysteine was substituted for amino acid residues located either in TMH1 at amino acids Val<sup>202</sup> or Ala<sup>215</sup> or in TMH2 at amino acids Lys<sup>288</sup> or Ile<sup>303</sup>. All four residues have been previously shown to face the membrane in TMH1 or TMH2 (7, 8) and hence are excellent indicators of the transmembrane insertion of the two TMHs. These mutants were modified with NBD, an environmentally sensitive fluorescent dye that we have used previously to map the orientation of these residues in the membrane (7, 8). Since the emission of NBD is strongly quenched by water, the fluorescence intensity increases significantly when the dye at these places interacts with a polar environment such as the hydrophilic lipid headgroups.
locations in the TMHs moves from an aqueous to a nonaqueous environment (7, 8, 13).

The fluorescence emission spectrum of each mutant was determined spectrophotometrically before and after incubation with liposomes in the absence or presence of reducing reagent (Fig. 6). In three cases, the fluorescence intensities of NBD in the PFO190-C57 mutants that had been incubated with liposomes in the absence of DTT were nearly the same as those of the respective soluble monomers (Fig. 6). For the V202C mutant, which has the dye located at the tip of TMH1 near the \( \beta \)-turn of the hairpin, the NBD appeared to enter a somewhat more nonpolar environment, but not the environment observed when the protein is allowed to fully insert. It therefore appears that a probe at the other end of TMH1 from the disulfide bond may interact to some extent with the bilayer, but cannot insert properly into the membrane. However, upon reduction of the disulfide, the probe at residue 202, as well as the other residues, fully entered the membrane because the fluorescence intensities of the NBD dyes located at these four positions increased to approximately the same levels as reported previously for these residues as they enter the membrane (7, 8).

Thus, neither TMH1 nor TMH2 were able to interact normally with the membrane in oxidized PFO190-C57 because neither TMH1 nor TMH2 inserted into the membrane in the disulfide-trapped PFO prior to reduction of the Cys190-Cys57 disulfide bridge. These data also suggest that the insertion of both TMHs may proceed in a concerted fashion and that TMH2 apparently cannot insert independent of TMH1.

Insertion Kinetics of TMHs—Pore formation by PFO requires its binding to the membrane, oligomerization, and TMH insertion into the bilayer. The rate-limiting step in this process is unknown, but could occur in any of these three stages of pore formation. Since pore formation is blocked in the oxidized PFO190-C57 protein (Fig. 2, Table I) without significantly altering either pre-pore complex assembly (Figs. 3 and 4) or the interaction of domain 4 with the membrane (14), binding and oligomerization can be examined in the absence of insertion. Thus, the kinetics of binding and oligomerization can be measured both with and without insertion to determine whether insertion slows the process, i.e. is the rate-limiting step in the process. The rate of domain 4 exposure to the membrane is the same for reduced and oxidized PFO190-C57 (14), and the rate of PFO oligomerization is also unaffected by the presence or absence of the disulfide bond (Fig. 5). These results suggest that insertion is not rate-limiting.

To determine directly whether TMH insertion or a previous step is rate-limiting, we have taken advantage of the disulfide bond to arrest the pore formation process prior to insertion. As noted above, oxidized PFO190-C57 will form a prepore complex, but will not insert its TMHs. Thus, by allowing the disulfide-trapped PFO to bind and assemble into prepore complexes on the membrane, we can then measure the intrinsic rate of TMH insertion in these arrested complexes simply by adding DTT. This approach allows us to synchronize TMH insertion in the sample since most or all of the TMHs will already be in prepore complexes and will be poised to insert into the bilayer.

Membrane insertion of the TMHs was monitored by the increase in fluorescence intensity over time of NBD located at the membrane-facing residues C215 in TMH1 (PFO190-C57/C288) and C288 located within TMH2 (PFO190-C57/C288) (Fig. 7). In these experiments, liposomes were injected after 30 s into a stirred solution of the NBD-labeled PFO in the presence or absence of reducing agent. For samples that were not pre-reduced prior to the addition of liposomes, DTT was injected into the unreduced samples at either 120 or 370 s after the addition of the liposomes. Those samples that had not been pre-reduced were therefore able to form prepore complexes prior to the addition of DTT, but were not able to form pores.

The rate of insertion of the TMHs that were trapped on the membrane in the prepore state for either 120 or 370 s after the addition of liposomes proceeded much faster than that of the pre-reduced sample (Fig. 7). Therefore, the extent of the rapid insertion of the TMHs appears to reflect the extent of prepore formation because the rate of formation of the prepore oligomer by oxidized PFO190-C57 is nearly identical to that by reduced PFO190-C57. In fact, the observed rate of insertion, although rapid, includes the rate of mixing of the DTT and the rate at which the disulfide is reduced. Therefore, the actual rate of insertion of the two TMHs from the prepore complex is even faster than the observed rate. Not surprisingly, after the initial rapid rate of insertion of the \( \beta \)-sheet of the prepore complex at
The concept of a prepore as an intermediate state for pore-forming toxins was first proposed by Walker et al. (19) for the pore-forming toxin α-hemolysin from Staphylococcus aureus. The prepore presumably facilitates the insertion of the membrane spanning β-barrel, although this has not been directly demonstrated. By quantitatively interrupting the pore formation process and synchronizing PFO in its prepore state, we have been able to determine that the assembly of the prepore complex is the rate-limiting step in cytolyis and that, once the prepore is formed, the insertion of the transmembrane β-sheet is rapid. This observation is not only interesting in terms of defining a role of the prepore, it is fascinating that it occurs in the CDCs, which form channels that are substantially larger than most other pore-forming toxins and may be composed of up to 50 monomers (reviewed in Ref. 20). The central cavity of the CDC pore is ~25 nm in diameter, sufficiently large to permit the passage of large macromolecules such as proteins (reviewed in Refs. 21 and 22) and even DNA (23).

The large size of the assembled CDC pore has led to speculation as to the mechanism by which the CDC polypeptides form their large transmembrane β-sheet (6, 13, 24). Two models have been proposed to explain the insertion of the CDC β-sheet, one based on the prepore mechanism that has been shown to mediate the formation of small pores by several toxins (9–12) and the other on the gradual enlargement of a small oligomer and pore into a large oligomer and pore by the sequential addition of monomers to the inserted complex (24). However, recent studies by Shepard et al. (13) showed that the processes of PFO oligomerization and the insertion of the transmembrane β-sheet could be uncoupled, suggesting that a prepore mechanism might be the modus operandi for the CDCs. The results of the current studies strongly support the formation of a prepore as the penultimate step in the insertion of the transmembrane β-sheet of PFO (see Fig. 8).

By introducing a disulfide bridge between residues 190 and 57 to impede the movement of TMH1, we found that we could abrogate PFO-dependent hemolysis. Yet, if the disulfide bridge was reduced after the PFO had been incubated with erythrocytes, hemolysis proceeded at a significantly faster rate than if the PFO had been reduced prior to its addition to the erythrocytes. The typical lag period that we observed when the disulfide of this mutant was reduced prior to the addition of the erythrocytes, and which had been previously observed for PFO-dependent hemolysis (17), was nearly abolished when the disulfide-trapped PFO was preincubated with the erythrocytes prior to reduction of the disulfide bridge. This observation suggested that the disulfide trapped PFO had accumulated at a step that primed it for insertion and pore formation on the erythrocytes, presumably a prepore complex.

Analysis of the disulfide-trapped mutant by electron microscopy and by SDS-AGE revealed that PFOC190-C57 formed the same oligomers on liposome membranes whether or not the disulfide was oxidized (hemolytically inactive) or reduced (fully hemolytic). The oxidized and reduced oligomer exhibited similar mobilities in SDS-AGE gels (Fig. 3), although the disulfide-trapped prepore appeared to be somewhat less stable to the SDS. Therefore, the interaction of the transmembrane β-hairpins in the pore complex helps stabilize the oligomer since the inserted form of PFOC190-C57 exhibited the same stability as the cysteine-less parent toxin PFOC459A. The stability of the oligomeric complex of various pore-forming toxins to dissociation by SDS has been well documented for the heptameric pore complexes of S. aureus α-hemolysin, C. septicum α-toxin, and A. hydrophila aerolysin (9–12). Additionally, the rate of oligomerization, as determined by FRET, of the oxidized and reduced forms of PFOC190-C57 was unaffected by the presence of the disulfide bond. Therefore, changes in the oligomerization rate or the extent of oligomer formation could not account for the cytolytically inactive phenotype exhibited by oxidized PFOC190-C57.

PFO is novel in that it contributes two transmembrane β-hairpins per monomer to the formation of the membrane-spanning β-sheet (7, 8), whereas other pore-forming toxins such as S. aureus α-hemolysin and anthrax protective antigen utilize a single TMH per monomer (11, 25). PFO is also unique in that each membrane-spanning β-hairpin in the PFO pore complex exists originally as three short α-helices in domain 3 of

**DISCUSSION**

![Fig. 7. Insertion kinetics of the PFOC190-C57 transmembrane β-sheet.](Image 81x447 to 266x730)

The rates of insertion of the transmembrane β-sheets of the disulfide-trapped PFO were followed by monitoring the increase in fluorescence intensity of NBD covalently attached to residue 215 in TMH1 (PFOC190-C57/C215-NBD) (upper panel) or in TMH2 at residue 288 (PFOC190-C57/C288-NBD) (lower panel). Residues 215 and 288 both face the membrane in the assembled pore (7, 8) and are excellent indicators of the insertion of TMH1 and TMH2, respectively, into the bilayer. Liposomes were injected at 30 s into a stirred mixture at 37 °C that contained nonreduced PFO derivative (dashed or dotted line) or PFO derivative that had been previously reduced with 10 mM DTT (solid line). The unreduced samples were then reduced by the addition of DTT at either 130 s (dashed line) or 370 s (dotted line) after the addition of liposomes.

120 or 370 s, the rate of insertion then decreased to a rate comparable to that of the pre-reduced sample. Therefore, the fraction of the PFO that had been converted to the prepore complex rapidly inserted its TMHs into the membrane, while the fraction that had not yet formed the prepore was much slower to insert its TMHs because it had not yet fully oligomerized into the insertion-competent prepore stage. These data demonstrate that TMH insertion occurs more rapidly than prepore complex assembly, and hence that the rate of prepore formation dictates the observed rate of pore formation. These results are therefore consistent both with the hemolysis kinetics (Fig. 2) and with the faster rate of pore formation observed when disulfide-arrested prepore complexes are released by DTT (14).
the soluble monomer (7, 8). In the soluble monomer, the TMH1 α-helices have a large percentage of their structure buried against domain 2 and the core β-sheet of domain 3, whereas the α-helices that comprise TMH2 are mostly solvent-exposed (6). Therefore, it was possible that TMH2 could have partially or wholly inserted into the bilayer even though the movement of TMH1 was restricted by the disulfide bond between Cys\(^{190}\) and Cys\(^{57}\) in oxidized PFO\(^{C190-C57}\). We had previously proposed that the domain 3 core β-sheet must both move away from its domain 2 and domain 3 contacts and twist for both TMHs to un-ravel from their α-helical structure in the monomer and form the extended transmembrane β-hairpins (7). By restricting the movement of TMH1 with a disulfide bond, the required movement of the domain 3 core β-sheet may also have been limited. NBD probes located on membrane-facing residues either near the predicted β-turn in TMH2 or within one of its membrane-spanning β-strands demonstrated that TMH2 also did not enter the membrane. Therefore, the disulfide bridge between TMH1 and domain 2 prevented the insertion of both TMH1 and TMH2 into the membrane. These observations indicate that the PFO TMHs do not insert independently into the membrane and that their insertion is likely coupled by hydrogen bonding between the hairpins and one or more prerequisite conformational changes in domain 3.

Since the rate and extent of oligomer formation were largely unaffected by the oxidation state of PFO\(^{C190-C57}\) and the inabiliy of TMHs to insert into the membrane, PFO\(^{C190-C57}\) was effectively trapped on the membrane surface in a fully oligomerized, uninserted state that, by definition, corresponded to a prepore complex. In this state, neither of the TMHs in the disulfide-trapped prepore complex were embedded in the membrane, even though both TMHs were capable of normal insertion into the membrane upon reduction of the Cys\(^{190}\)-Cys\(^{57}\) disulfide bond. Therefore, the disulfide linkage effectively allowed us to synchronize the major insertion of the molecules into a single state, the prepore. By allowing the oxidized PFO\(^{C190-C57}\) mutant to form a prepore complex on liposomal membranes prior to reduction, we could dramatically increase the rate of membrane insertion of both TMH1 and TMH2 (Fig. 7). This dramatic increase in the rate of insertion of the TMHs also corresponded nicely with the increased rates of hemolysis of erythrocytes (Fig. 2) and of pore formation (14) that were also observed when oxidized prepore complexes were allowed to form prior to DTT addition. Thus, TMH insertion, pore formation, and hemolysis proceeded rapidly upon reduction of the disulfide bond because the majority of the PFO molecules were already assembled into the prepore state and therefore primed for synchronous insertion into the membrane.

These results are consistent with the prepore model (Fig. 8) because the insertion of the transmembrane β-sheet should occur more rapidly if the prepore complex is first allowed to form on the membrane before insertion is initiated. It had been suggested previously that a significant energy barrier might prevent the simultaneous insertion of the large β-sheet of the CDCs, and that the synchronized insertion of so many β-hairpins would make insertion of the CDCs via a prepore-based mechanism unlikely (24). However, by arresting the pore formation process after the assembly of the oxidized PFO\(^{C190-C57}\) prepore complex and then synchronizing the insertion of the transmembrane β-hairpins, upon addition of reducing agent, we have shown that quite the opposite effect is observed; formation of the prepore significantly enhances the rate of insertion of the transmembrane β-sheet. Since the membrane insertion of the large β-sheet of the prepore complex is far more rapid if the prepore is first allowed to assemble, the rate-limiting step in cytolysis is a step involved in the formation of the prepore complex, not the insertion of the transmembrane β-hairpins.

PFO pore formation therefore involves the assembly of a prepore complex that, once an insertion-competent size is reached, can initiate membrane insertion of the transmembrane spanning β-sheet. The exact size of an insertion competent prepore complex is currently unclear, although based on SDS-Age, EM, and planar bilayer analyses shown here and elsewhere (13), it must be significantly larger than the heptameric structures observed for pore-forming toxins such as α-hemoly-
sin (11), aerolysin (26, 27) and anthrax protective antigen (25). Shepard et al. (13) observed that the channels formed by PFO in planar bilayers exhibited conductances of 4–6 nanosiemens, which are 20–40 times larger than that observed for the smaller toxin pores (28, 29). However, whether or not a complete ring must form prior to membrane insertion of the transmembrane β-sheet remains to be determined unambiguously.

By synchronizing the insertion of the prepore β-hairpins, we have been able to provide direct evidence that prepore complex formation facilitates the insertion of the transmembrane β-sheet for a pore-forming toxin. Therefore, the prepore state is an important intermediate in the assembly and insertion of the transmembrane β-sheet of the CDCs. In addition, the data support the remarkable concept that the PFO oligomer complex can possibly coordinate the simultaneous insertion of up to 100 β-hairpins to form the transmembrane β-sheet. Therefore, the prepore mechanism is sufficiently flexible to accommodate the insertion of both large (PFO and the CDCs) and small (α-hemolysin, anthrax protective antigen) membrane-spanning β-sheets.

REFERENCES

1. Alouf, J. E. (1999) in Bacterial Toxins: A Comprehensive Sourcebook (Alouf, J., and Freer, J., eds) 2nd Ed., pp. 443–456, Academic Press, London
2. Bhakdi, S., Tranum-Jensen, J., and Szegoleit, A. (1984) in Bacterial Protein Toxins (Alouf, J. E., Fehrenbach, F. J., Freer, J. H., and Jeljaszewicz, J., eds) Vol. 24, pp. 173–180, Academic Press, London
3. Olofsson, A., Hebert, H., and Thelestam, M. (1993) FEBS Lett. 319, 125–127
4. Prigent, D., and Alouf, J. E. (1976) Biochim. Biophys. Acta 433, 422–428
5. Watson, K. C., and Kerr, E. J. (1974) Biochem. J. 140, 95–98
6. Rossjohn, J., Feil, S. C., McKinstry, W. J., Tweten, R. K., and Parker, M. W. (1997) Cell 89, 685–692
7. Shatursky, O., Heuck, A. P., Shepard, L. A., Rossjohn, J., Parker, M. W., Johnson, A. E., and Tweten, R. K. (1999) Cell 99, 203–209
8. Shepard, L. A., Heuck, A. P., Hamman, B. D., Rossjohn, J., Parker, M. W., Ryan, K. R., Johnson, A. E., and Tweten, R. K. (1998) Biochemistry 37, 14563–14574
9. Walker, B., Krishnasastri, M., Zorn, L., and Bayley, H. (1992) J. Biol. Chem. 267, 21762–21766
10. Van der Goot, F. G., Pattus, F., Wong, K. R., and Buckley, J. T. (1993) Biochemistry 32, 2636–2642
11. Song, L. Z., Hobaugh, M. R., Shustak, C., Cheley, S., Bayley, H., and Gouaux, J. E. (1996) Science 274, 1859–1866
12. Sellman, B. R., Kagan, B. L., and Tweten, R. K. (1997) Mol. Microbiol. 23, 551–558
13. Shepard, L. A., Shatursky, O., Johnson, A. E., and Tweten, R. K. (2000) Biochemistry 39, 10284–10293
14. Heuck, A. P., Hotze, E., Tweten, R. K., and Johnson, A. E. (2000) Mol. Cell 6, 1233–1242
15. Hazes, B., and Dijkstra, B. W. (1988) Protein Eng. 2, 119–125
16. Darst, S. A., Ribi, R. O., Pierce, D. W., and Kornberg, R. D. (1988) J. Mol. Biol. 203, 269–173
17. Harris, R. W., Sims, P. J., and Tweten, R. K. (1991) J. Biol. Chem. 266, 6936–6941
18. Luesmih, U. K. (1970) Nature 227, 680–685
19. Walker, B., Braha, O., Cheley, S., and Bayley, H. (1995) Chem. Biol. 2, 99–105
20. Rossjohn, J., Tweten, R. K., Rood, J. I., and Parker, M. W. (1999) in Bacterial Toxins: A Comprehensive Sourcebook (Alouf, J., and Freer, J., eds) 2nd Ed., pp. 406–420, Academic Press, London
21. Bhakdi, S., Weller, U., Walev, I., Martin, E., Jonas, D., and Palmer, M. (1993) Mol. Microbiol. Immunol. 182, 167–175
22. Hamman, B. D., Chen, J. C., Johnson, E. E., and Johnson, A. E. (1997) Cell 89, 535–544
23. Gottschalk, S., Tweten, R. K., Smith, L. C., and Woo, S. L. C. (1995) Gene Ther. 2, 498–503
24. Palmer, M., Harris, R., Freytag, C., Kehoe, M., Tranum-Jensen, J., and Bhakdi, S. (1998) EMBO J. 17, 1598–1605
25. Petosa, C., Collier, R. J., Klimpel, K. R., Leppa, S. H., and Liddington, R. C. (1997) Nature 385, 833–838
26. Wilmsen, H. U., Leonard, K. R., Tichelaar, W., Buckley, J. T., and Pattus, F. (1992) EMBO J. 11, 2457–2463
27. Moniatte, M., van der Goot, F. G., Buckley, J. T., Pattus, F., and van Dorsselaer, A. (1996) FEBS Lett. 384, 269–272
28. Menestrina, G. (1986) J. Membr. Biol. 90, 177–190
29. Chakraborty, T., Schmidt, A., Nortmers, S., and Benz, R. (1996) Infect. Immun. 64, 2127–2132