Monomer-Monomer Interactions Drive the Prepore to Pore Conversion of a β-Barrel-forming Cholesterol-dependent Cytolysin*

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Perfringolysin O (PFO), a cholesterol-dependent cytolyis discusses large oligomeric pore complexes comprised of up to 50 PFO molecules. In the present studies a mutant of PFO (PFOY181A) has been identified that traps PFO in a multimeric prepore complex that cannot insert its transmembrane β-hairpins and therefore cannot form a pore. Remarkably, PFOY181A can be induced to insert its transmembrane β-hairpins if functional PFO is incorporated into the PFOY181A oligomeric prepore complex. Furthermore, the transition from prepore to pore appears to be an “all or none” process; partial insertion of the transmembrane β-barrel does not occur. Therefore, cooperative interactions between the monomers of the prepore drive the prepore to pore conversion that results in the formation of the transmembrane β-barrel.

Many pore-forming bacterial toxins, outer membrane porins, and autotransporter proteins of Gram-negative bacteria, as well as the outer membrane porins of mitochondria and chloroplasts, utilize amphipathic β-sheets to span the lipid bilayer. Although the structure of the membrane-spanning β-sheet has been elucidated for a wide variety of membrane proteins, the mechanism of its assembly into the membrane remains largely unexplored. Amphipathic β-sheets are used to penetrate and form pores in the membrane by several toxins that have been designated β-PFTs or “β-barrel pore-forming toxins” (1). Members of this family currently include Staphylococcus aureus α-hemolysin (2,3), Bacillus anthracis protective antigen (4,5), and Clostridium perfringens perfringolysin O (PFO) (6,7). In contrast to the porins that form a membrane-spanning β-barrel from a single protein, these toxins assemble a membrane-spanning β-sheet by the oligomerization of individual toxin molecules. Each monomer contributes one or two amphipathic β-hairpins (dependent on the specific toxin) to the formation of the transmembrane β-sheet. Hence, the β-barrel of these toxins is assembled at the membrane from many protein monomers rather than from a single protein, as are the porins. The mechanism of pore formation by the β-PFTs encompasses the following three basic steps: targeting to the membrane surface, oligomerization into a prepore complex, and the conversion of the prepore to the inserted pore complex by the insertion of the transmembrane β-sheet. However, it is likely that additional intermediate states exist for this transition, and these states will be important in defining the mechanism by which the β-PFTs form a pore in the membrane.

The size of the oligomeric complex, and therefore the size of the pore, varies dramatically for the various types of pore-forming toxins. Toxins such as aerolysin, α-hemolysin, and anthrax-protective antigen form heptameric oligomers and pores of 1–2 nm in diameter, whereas the cholesterol-dependent toxins (a family of β-PFTs whose cytolytic activity exhibits an absolute requirement for cholesterol), such as PFO, form oligomers of 40–50 monomers and pores of 20–30 nm (reviewed in Refs. 1 and 8). The formation of the prepore intermediate has been demonstrated for α-hemolysin (9,10), PFO (11–13), and anthrax-protective antigen (14), although the function of the prepore intermediate has not been determined.

In the present report we describe a novel mutant of PFO, PFOY181A, that is trapped in the prepore complex. Surprisingly, if PFOY181A monomers form a mixed oligomer with functional PFO monomers, the insertion of the transmembrane β-sheet of PFO, a cholesterol-dependent cytolyis (CDC), was significantly more rapid if the toxin monomers were first allowed to form the prepore complex. These observations suggested that formation of the prepore complex was a rate-limiting step and that the prepore complex is an obligatory intermediate in the insertion of the large transmembrane β-barrel formed by PFO.

In the present report we describe a novel mutant of PFO, PFOY181A, that is trapped in the prepore complex. Surprisingly, if PFOY181A monomers form a mixed oligomer with functional PFO monomers, the insertion of the PFOY181A β-hairpins occurs. Furthermore, these studies also reveal that insertion of the prepore appears to be an “all or none” process such that partial insertion of the prepore does not occur. These studies provide compelling evidence that monomers do not insert their transmembrane hairpins individually. Instead, cooperation between toxin monomers within the prepore complex is required for and drives the prepore to pore conversion, presumably in one concerted movement.

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† The abbreviations used are: PFO, perfringolysin O; TMH, transmembrane β-hairpin; FRET, fluorescence resonance energy transfer; AGE, agarose gel electrophoresis; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; NO-PC, 1-palmitoyl-2-stearoyl-17-doxyl-sn-glycero-3-phosphocholine; TMR, tetramethylrhodamine; CDC, cholesterol-dependent cytolyis; β-PFTs, β-barrel pore-forming toxins; NBD, 7-nitrobenz-2-oxa-1,3-diazole; AFM, atomic force microscopy.

‡ All mutants of PFO in this study are derived from PFO C459A. PFOY181A is PFO C459A that contains an alanine substitution for tyrosine 181.
EXPERIMENTAL PROCEDURES

Generation and Purification of PFO and Its Derivatives—All amino acid substitutions were generated via PCR overlap mutagenesis using the gene for the cysteine-less derivative of PFO, PFO<sub>459A</sub>,<sup>3</sup> in which Cys-459 had been changed to an alanine (6). Expression and purification of PFO<sub>459A</sub> and its derivatives used in this study and the determination of their hemolytic activity were performed as described previously (6). For the mutants described above, expression and purification were performed in parallel in which unlabeled PFO(U)-replaced donor-labeled PFO to create the UA sample. The net donor emission was determined between 500 and 600 nm (excitation at 470 nm; bandpass at 4 nm) and subtracting the UA sample signal from the DA signal. Liposomes were then added, and the contents were mixed for 30 min at 37 °C prior to reading the emission. Quenching experiments were performed in the same manner using the acceptor-labeled PFO<sub>459A</sub> and for a mixture of 22 nm donor-labeled PFO<sub>459A</sub> and 22 nm acceptor-labeled PFO<sub>459A</sub>.

Fluorescence Lifetime Measurements—Time-resolved fluorescence measurements were made using an ISS (Urbania, IL) K2-002 multichannel four-channel cross-correlation phase and modulation spectrofluorimeter as described in Shepard et al. (6). Samples of 100–200 nM NBD-labeled PFO<sub>Y181A</sub> derivatives containing 0–400 nM functional PFO<sub>459A</sub> were incubated with an excess of liposomes (the molar ratio of protein to total lipids were 1:1000–1:370 at 37 °C before experiments began). Phase and modulation data were analyzed using GLOBALS UNLIMITED, obtained from the University of Illinois (Urbania, IL), as before (6). In samples containing liposomes, the lowest <i>x</i><sup>2</sup> value was obtained with a three-component fit consisting of a single exponential with a τ of 0.001 ns to correct for scatter and two discrete exponentials for the NBD emission lifetimes. The fractional contribution of the scattering determined by this analysis corresponded closely to the fraction of total signal intensity that resulted from Raman and Rayleigh scattering. In the absence of liposomes, the scatter component was omitted during data analysis.

Pore Formation Assay—β-Amylase from sweet potato and glutathione (Sigma) were labeled with fluorescein isothiocyanate (Molecular Probes, Eugene, OR) using the same procedures described in Heuck et al. (12) for carbonic anhydrase. Liposomes containing fluorescein-labeled β-amylose or glutathione were prepared as before for carbonic anhydrase (12). These liposomes (100 μM total lipids) were suspended in buffer A containing an excess of anti-fluorescein antiseraum that quenches fluorescein emission intensity by 80% upon binding to the dye. After thermal stabilization at 25 °C for 5 min, the initial fluorescence was determined (F<sub>0</sub>). The fluorescence was added at the indicated concentration to a final volume of 1.6 ml, and data collection was begun 15 s later, as described in Heuck et al. (12). Blank measurements were made using an otherwise identical sample that lacked the toxin, and the blank signal was subtracted from the corresponding sample signal.

Gel Electrophoresis—Denaturing agarose gel electrophoresis (SDS-Ag) was performed as described previously (11). Briefly, in all samples in which PFO was incubated with liposomes, the liposomes (cholesterol/POPC at 55:45 mol %) were incubated with PFO (8 μg of toxin for Coomassie-stained and 1.5 μg of TMR-labeled toxin for fluorescent gels) for 30 min at 37 °C. Oligomeric complexes were solubilized with sample buffer containing 2.2% (w/v) SDS and then separated on 1.5% (w/v) SeaPlaque-agarose (FMC, Rockland, ME) in SDS gel reservoir buffer (15). The gel was typically run at 100 V for 70–100 min. Gels were fixed (10% (v/v) acetic acid, 30% (v/v) methanol) overnight and then dried in a Hoefer gel dryer (San Francisco, CA). The dried gel was stained with Coomassie Brilliant Blue R and then destained to visualize the protein bands. TMR-labeled PFO or PFO<sub>459A</sub> were visualized on an UV transilluminator immediately after electrophoresis to detect the dye-labeled protein.

Atomic Force Microscopy—Mutant PFO was added to supported lipid bilayers composed of egg phosphatidylcholine/cholesterol (Avanti Polar Lipids, Alabaster, AL) at a 1:1 molar ratio, following a procedure described previously (16). The protein was injected to a final concentration of ~15 μg/ml into a buffer of 5 mM dithiothreitol, 10 mM sodium phosphate (pH 7) covering the supported bilayer. After incubating for 45 min, the sample was extensively washed and then incubated with AFM under the same buffer. Imaging was performed in the contact mode with a NanoScope II AFM (Digital Instruments, Santa Barbara, CA) using oxide-sharpened “twin tip” Si<sub>n</sub>N<sub>n</sub> cantilevers with a spring constant of 0.06 nm. The typical scan rate was 7 Hz, and the applied force was minimized to 0.1 nN. The outer diameter of the rings was determined from the center-to-center distance between nearest neighbor complexes.

3This mutant PFO exhibits the same characteristics and hemolytic activity as native PFO except that the only cysteine of PFO, Cys-459, has been converted to alanine.
Monomer Cooperation Drives Membrane Insertion

RESULTS

Oligomer Formation by PFO<sup>Y181A</sup>—Tyrosine 181 of PFO<sup>C459A</sup> was substituted with alanine to yield the mutant PFO<sup>Y181A</sup>. This derivative was found to exhibit less than 1% of the hemolytic activity of PFO<sup>C459A</sup> on human erythrocytes (data not shown). PFO<sup>C459A</sup> lacks the only cysteine of PFO but exhibits the same activity as native PFO and is hereafter considered functional PFO (6). Tyrosine 181 is located immediately upstream of the first transmembrane β-hairpin of PFO (Fig. 1 and see Ref. 6). The basis for the loss of activity was initially unknown, although a previous study with the highly homologous streptolysin O suggested that mutations in the analogous region of streptolysin O abrogated the ability of this toxin to form membrane-bound oligomers (17). Therefore, we initially examined the ability of PFO<sup>Y181A</sup> to form oligomers on liposomal membranes.

PFO forms large SDS-resistant oligomeric complexes on cholesterol-containing membranes that can be visualized by SDS-AGE and electron microscopy (11, 13). The formation of oligomers by PFO<sup>Y181A</sup> on cholesterol-containing liposomes was investigated by SDS-AGE. As shown in Fig. 2A, PFO<sup>Y181A</sup> efficiently generated oligomers (Fig. 2A, lane 8). However, in contrast to PFO<sup>C459A</sup>, these oligomeric complexes were not stable to SDS unless they were chemically cross-linked with glutaraldehyde prior to the addition of SDS (compare lanes 6 and 8). Because the PFO<sup>Y181A</sup> cross-linked oligomers migrated more slowly than both uncross-linked (Fig. 2A, lane 2) and cross-linked PFO<sup>C459A</sup> oligomers (Fig. 2A, lane 4), the PFO<sup>Y181A</sup> prepropore oligomer is apparently either more structurally relaxed (i.e. less compact) than the inserted oligomer of PFO<sup>C459A</sup> and/or contains a few more monomers than the oligomer of the functional toxin. No oligomer was detected when monomers of PFO or PFO<sup>Y181A</sup> were incubated with an excess of cross-linker for 30 min in the absence of membranes (Fig. 2A, lanes 1 and 7, respectively). Therefore, as with functional PFO (i.e. PFO<sup>C459A</sup>), the formation of PFO<sup>Y181A</sup> oligomers was dependent on the presence of membranes.

Visualization of the PFO<sup>Y181A</sup> oligomers on cholesterol-containing membranes by atomic force microscopy (AFM) (Fig. 2B) demonstrated that the oligomers formed by this mutant have an internal diameter of ∼25 nm, similar to that of the oligomers formed by functional toxin (11, 13). Therefore, PFO<sup>Y181A</sup> can bind and oligomerize on the membrane surface, but its inability to lyse erythrocytes suggests that the mutant gets trapped in a prepore state.

PFO<sup>Y181A</sup> Cannot Insert Its TMHs into the Bilayer—Conver-

sion of the prepore complex to an inserted pore complex involves the insertion of two transmembrane β-hairpins (TMHs) per PFO molecule into the membrane (6, 7, 13). The interaction of these hairpins within the oligomeric complex of PFO generates a transmembrane β-barrel that is composed of up to 100 amphipathic β-hairpins (18).

To determine whether the transmembrane hairpins of PFO<sup>Y181A</sup> were inserted into the membrane, we individually analyzed the membrane exposure of residues in each PFO<sup>Y181A</sup> TMH. The residues responsible for the formation of the transmembrane β-sheet have been rigorously mapped for PFO by the use of multiple independent fluorescence techniques (6, 7). Therefore, selected residues that face the membrane in TMH1 (Ser-194 and Ala-215) and TMH2 (Lys-288 and Ile-303) were substituted with cysteine and then modified via their sulfhydryl groups with the NBD fluorescent dye. Because the fluorescence emission of NBD is strongly quenched by water, its emission intensity and lifetime increase as it moves into a nonpolar environment. NBD is therefore an excellent indicator for membrane insertion of the transmembrane β-sheet of PFO (6, 7, 11–13).

The changes in the emission spectra of NBD dyes positioned at the above four locations are shown in Fig. 3 (left column) as functional PFO (PFO<sup>C459A</sup>) moves from its water-soluble monomer state to a membrane-inserted pore complex. By comparison, the emission spectra (Fig. 3, right column) and lifetimes (Table I) of NBD located at these same positions in PFO<sup>Y181A</sup> did not change significantly as soluble PFO<sup>Y181A</sup> monomers were allowed to oligomerize on liposomes. Thus, the Y181A mutation allows the prepore oligomer to form, but it prevents the prepore complex from inserting its hairpins into the membrane and thereby prevents the formation of the pore.

Y181A Can Form Mixed Oligomers with Functional PFO—Because the PFO<sup>Y181A</sup> oligomer did not make the transition from a prepore to pore complex, it presented an excellent opportunity to determine directly if neighboring monomers
within the prepore complex cooperate to help drive the conversion of the prepore complex to a fully inserted pore complex. If such monomer-monomer interactions are important in enabling this event, then mixing PFO Y181A with the PFO C459A mutant may induce PFO Y181A to insert its TMHs and lead to pore formation. Therefore, we initially determined whether PFO Y181A could form a mixed oligomer with PFO C459A using fluorescent resonance energy transfer (FRET). FRET is a non-destructive approach to demonstrate a close proximity between proteins and has been used previously by us to demonstrate the association of PFO monomers in the oligomeric complex (13, 19, 20).

As discussed in detail under “Experimental Procedures,” the association of two proteins can be detected by labeling one with a fluorescein dye (the donor dye) that can transfer its excited state energy to a rhodamine dye (the acceptor dye) attached to the second protein. This transfer of energy, and therefore the loss of donor emission intensity, only occurs when the donor and acceptor dyes are very close (<100 Å), so no FRET would be observed at the concentration of PFO used in our experiments unless the proteins had associated with each other. As shown in the top panel of Fig. 4, the addition of cholesterol-containing liposomes to an equimolar mixture of donor- and acceptor-labeled PFO molecules results in a large decrease in donor intensity. As we have shown previously (13, 19), this reduction in donor emission intensity is due to FRET that occurs as the PFO monomers associate to form the oligomeric pore complex. A similar reduction in donor emission intensity occurs upon adding liposomes to donor- and acceptor-labeled PFO Y181A (Fig. 4, middle panel). In addition to the cross-linked oligomer analysis and the AFM analysis (Fig. 2), the FRET provides spectral evidence that the PFO Y181A molecules are associating into oligomeric complexes on the membrane surface. Furthermore, PFO C459A and PFO Y181A form mixed oligomers when mixed in
the presence of membranes (Fig. 4, bottom panel). The FRET observed in the lower panel shows that an equimolar mixture of PFOC459A and PFOY181A forms complexes on the membrane that are spectroscopically indistinguishable from the complexes formed by homogeneous mixtures of PFOC459A or PFOY181A.

**Mixed Oligomers of Functional PFO and PFOY181A Are Stable to Dissociation with SDS**—The FRET analysis showed clearly that PFOC459A and PFOY181A are capable of associating on the membrane to form mixed oligomers. However, the oligomers of PFOY181A are completely dissociated by SDS unless covalently linked by a cross-linker, whereas functional PFO (PFOC459A) forms SDS-resistant oligomers (Fig. 2). We therefore considered the consequence of forming the mixed oligomer on the stability of the oligomeric complex: could functional PFOC459A induce structural changes in PFOY181A that might result in a SDS-stable oligomer or would the oligomer remain sensitive to SDS? To visualize the incorporation of PFOY181A into mixed oligomers by SDS-Age, PFOY181A was labeled with TMR via the sulfhydryl group of a cysteine substituted for aspartate 30 in PFOY181A (Fig. 5). As expected, in the presence of cholesterol-containing liposomes (11, 13), functional PFOC459A (also labeled at D30C) with TMR (Fig. 5, lane 3) formed SDS-resistant oligomers. Also, as expected from the results in Fig. 2, homogeneous oligomers of labeled PFOY181A were not resistant to SDS (Fig. 5, lane 4); only monomer and incomplete oligomers were observed. It should be noted that the partially dissociated oligomers of PFOY181A are in comparably small quantities as they are not observed for PFOY181A in the Coomassie-stained gels in Fig. 2A. The intermediate-sized oligomers for un-cross-linked PFOY181A are only observed when a sensitive detection system, as in the case of the fluorescently tagged proteins, is employed to visualize the oligomers.

When unlabeled functional PFO (PFOC459A) was mixed with an equimolar amount of rhodamine-labeled PFOY181A (Fig. 5, lane 5), we observed that about 50% of the TMR-labeled PFOY181A was incorporated into SDS-resistant oligomers. Increasing the ratio of PFOC459A to a 4-fold molar excess over PFOY181A converted nearly all of the TMR-labeled PFOY181A into an SDS-stable oligomer (Fig. 5, lane 6). Therefore, the functional PFO was clearly affecting the nature of the mixed oligomer such that this pore complex exhibited the characteristic resistance to dissociation by SDS that is observed for a homogeneous oligomer of PFOC459A.

**The Insertion of the Transmembrane β-Hairpins of PFOY181A in Mixed Oligomers**—The results in Fig. 5 showed that as the proportion of functional PFO (PFOC459A) in a mixed oligomer of PFOC459A and PFOY181A increases, the fraction of the oligomer that exhibits resistance to dissociation by SDS also increases. Therefore, we suspected that the functional PFO in the mixed oligomers was forcing PFOY181A to undergo structural transitions that allowed it to form a more stable oligomer and possibly to insert its transmembrane hairpins. To test the latter possibility directly, we examined the membrane insertion of the TMHs of PFOY181A in the presence of various molar ratios of functional PFO.

Membrane-bound oligomers were formed by mixing various ratios of unlabeled PFOC459A with each of four derivatives of PFOY181A that were labeled with NBD at positions S194C, A215C, K285C, or I303C and then incubating the toxin mixtures with excess liposomes. PFOY181A TMH insertion was then determined by the magnitude of the increase in the emission intensity of the NBD probe at each of these locations. As shown in Fig. 6A, the emission intensity of the NBD increased significantly in all cases as the ratio of functional PFO (PFOC459A) to PFOY181A increased. These results demonstrated that the TMHs of PFOY181A were inserting into the membrane in the presence of functional PFO, whereas, as shown in Fig. 3, PFOY181A alone cannot insert its TMHs. Hence, as the concentration of functional PFO increased in the mixed oligomers, it forced the insertion of the TMHs of PFOY181A.

Although we have previously demonstrated that these four residues, among many other residues in these transmembrane β-hairpins, face the membrane in functional PFO (6, 7), we confirmed the membrane location of these residues for PFOY181A in the mixed oligomers by determining the accessibility of the NBD probe to collisional quenching by a nitroxide moiety attached to a phospholipid acyl chain (7-NO-PC) that is restricted to the hydrophobic core of the membrane (6, 7, 13). Mixed oligomers were again formed with each of the four NBD-labeled PFOY181A derivatives using a 4-fold molar excess of unlabeled functional PFO (PFOC459A) to stimulate maximal insertion of the PFOY181A hairpins. The emission spectra were then taken before and after incubation of these mixtures with cholesterol/POPC (55:45 mol %) liposomes or with cholesterol/POPC/7-NO-PC (55:25:20 mol %) liposomes (Fig. 6B). In all cases, the fluorescence intensity of the NBD probe was quenched more than 75% by the membrane-restricted nitroxide, a result that is only possible if the NBD is positioned within the nonpolar interior of the membrane.

The extent of PFOY181A TMH insertion into the membrane was quantified by fluorescence lifetime analysis of the NBD emission when the probes were located at either position 215 in

| Liposomes Position 215, NBD | Position 305, NBD |
|-----------------------------|-------------------|
| tL | tR | mol % C459A in sample | mol % NBD buried in membrane | tL | tR | mol % C459A in sample | mol % NBD buried in membrane |
|---|---|----------------|----------------|---|---|----------------|----------------|
| − | 3.9 | 0.9 | 1.3 | 3.3 | 0.6 | 4.1 |
| + | 2.2 | 0.9 | 4.9 | 0 | 0 | 0.9 | 2.5 |
| + | 20 | 6 |
| + | 33 | 9 |
| + | 9.0 | 1.6 | 8.1 | 50 | 41 |
| + | 67 | 43 |
| + | 80 | 62 | 9.6 | 1.9 | 3.0 | 50 | 41 |
| + | 67 | 49 |
| + | 80 | 62 |

The distribution of the NBD probe lifetimes in samples with an NBD located at either position 215 (TMH1) or 303 (TMH2) of PFOY181A was determined as described under "Experimental Procedures." In all cases, less than 4% of the signal was due to scattering. When liposomes were present in the sample, the lifetime data were best fit (i.e., the analyses have the lowest χ²) to two discrete lifetimes for NBD, tL and tR, that represent NBD probes in a non-aqueous milieu with a long lifetime or an aqueous milieu with a short lifetime, respectively, and one scatter component (τ fixed at 0.001 ns) (6, 7). The "mol % buried" column shows estimates of the fraction of dyes in each sample that have lifetimes ≥0.9 ns and hence are buried in the nonpolar interior of the membrane. A τ corresponding to a residue buried in the membrane interior (τ > 6 ns) was not observed in the absence of functional PFO (PFOC459A). In those samples, more than 82% of the NBD probes are in an aqueous environment. The minor component of slightly longer lifetimes (<4 ns) represents probes exposed to less aqueous environments within the protein (7).
TMH1 or position 303 in TMH2. In Table I we show that whenever the mol % of functional PFO reached or exceeded 50%, more than 40% of the NBD probes, and hence the TMHs of PFOY181A, in the sample were inserted into the membrane, as indicated by their long lifetimes (40% 9 ns). At a 4:1 ratio of PFOC459A to PFOY181A, more than 60% of the NBD probes in the samples make the transition to a long lifetime. These experiments demonstrate that significant fractions of both TMH1 and TMH2 of PFOY181A are inserting into the membrane in the mixed oligomers. Therefore, functional PFO can evidently drive structural transitions in PFOY181A that result in the insertion of its TMHs. Because the extent of conversion of these mixed oligomers to an inserted pore complex is dependent on the amount of functional PFO in the mixed oligomers, it suggests that only those oligomers with sufficient functional PFO can overcome the energetic barrier posed by the presence of the Y181A mutation and undergo the prepore to pore transition. Based on the lifetime data, 40% of the prepore complexes do not have enough functional PFO to insert into the membrane under the conditions of the experiments. Does the Pore Size Change in the Mixed Oligomers?—Because the conversion of the mixed prepore oligomers to an inserted pore complex is dependent on the concentration of functional PFO in the mixture, we examined the nature of the pore formed by these mixed oligomers. Previous studies (11, 13) have suggested that the prepore complex must attain an insertion-competent size to form a pore in the membrane. To determine whether the pores formed by mixed oligomers containing the PFOY181A mutant were significantly reduced in size, we used liposomes that had encapsulated either fluorescein-labeled glutathione or fluorescein-labeled β-amylose. Initially we examined the release of these markers by a homogeneous mixture of PFOY181A, in the sample were inserted into the membrane, as indicated by their long lifetimes (>9 ns). At a 4:1 ratio of PFOC459A to PFOY181A, more than 60% of the NBD probes in the samples make the transition to a long lifetime. These experiments demonstrate that significant fractions of both TMH1 and TMH2 of PFOY181A are inserting into the membrane in the mixed oligomers. Therefore, functional PFO can evidently drive structural transitions in PFOY181A that result in the insertion of its TMHs. Because the extent of conversion of these mixed oligomers to an inserted pore complex is dependent on the amount of functional PFO in the mixed oligomers, it suggests that only those oligomers with sufficient functional PFO can overcome the energetic barrier posed by the presence of the Y181A mutation and undergo the prepore to pore transition. Based on the lifetime data, 40% of the prepore complexes do not have enough functional PFO to insert into the membrane under the conditions of the experiments. Does the Pore Size Change in the Mixed Oligomers?—Because the conversion of the mixed prepore oligomers to an inserted pore complex is dependent on the concentration of functional PFO in the mixture, we examined the nature of the pore formed by these mixed oligomers. Previous studies (11, 13) have suggested that the prepore complex must attain an insertion-competent size to form a pore in the membrane. To determine whether the pores formed by mixed oligomers containing the PFOY181A mutant were significantly reduced in size, we used liposomes that had encapsulated either fluorescein-labeled glutathione or fluorescein-labeled β-amylose. Initially we examined the release of these markers by a homogeneous mixture of PFOC459A. As seen in Fig. 7A, little difference was observed in the release of the two markers demonstrating that the functional PFO formed a large pore that stimulated the release of both the large and small markers at similar rates. As expected, a homogeneous mixture of PFOY181A and functional PFO forms a large pore that stimulates the release of both the large and small markers at similar rates.
PFOY181A did not induce the release of GSH (Fig. 7A) or β-amylase (not shown). We then compared the release kinetics for fluorescein-glutathione (600 Da) and fluorescein-β-amylase (200,000 Da) from liposomes treated with mixtures of functional PFO and PFOY181A containing 50, 66, or 80 mol % of PFOY181A. Very little difference in the release kinetics was observed for glutathione and β-amylase, even though the hydrodynamic radii of these two molecules differ greatly. Thus, each pore created by the toxin mixtures was large enough to pass glutathione and β-amylase with approximately equal efficiency. These results strongly indicate that the reduction in the rate of fluorescence-detected pore formation observed with mixed oligomers (Fig. 7) occurs because of a reduced rate in the prepore to pore transition but not to a reduction in the average pore size. Therefore, as the ratio of PFOY181A to functional PFO increases fewer complexes can make the transition to a functional pore suggesting that only those complexes that reach an insertion-competent size, and that contain sufficient functional PFO to overcome the inhibition posed by the presence of the PFOY181A monomers, can make the transition.

DISCUSSION

The functional consequence of the Y181A mutation in PFO is that PFOY181A oligomers cannot make the transition from the prepore to the pore complex. The Y181A mutation therefore
raises the activation energy required for PFO insertion into the membrane. Yet the PFO\textsuperscript{Y181A} monomers within the oligomer are able to make this transition if the prepore complex contains a significant number of functional PFO molecules. Thus, functional PFO molecules are able to induce the proper conformational changes in the nonfunctional PFO\textsuperscript{Y181A} molecules such that they can insert their transmembrane \(\beta\)-hairpins. By monitoring emission intensities, fluorescence lifetimes, and accessibilities to membrane-restricted collisional quenchers of NBD probes positioned within the TMHs of PFO\textsuperscript{Y181A}, we were able to demonstrate unequivocally that as the fraction of functional PFO increased in the mixed oligomers, an increasing fraction of the PFO\textsuperscript{Y181A} TMHs moved into the membrane. These data show, for the first time, that interactions between the monomers of a CDC prepore complex are required to drive the conversion of the prepore complex to the inserted pore complex. 

FRET, AFM, and SDS-AGE oligomer analyses showed that PFO\textsuperscript{Y181A} is fully capable of forming homo-oligomers and that it forms oligomeric complexes similar in size to those of functional PFO. Furthermore, we have shown that PFO\textsuperscript{Y181A} is capable of oligomerizing with functional PFO to form mixed oligomers. Whereas the oligomer of PFO\textsuperscript{Y181A} was clearly dissociated by SDS, mixed oligomers with sufficient functional PFO are almost completely stable to SDS. However, it was necessary to use a 4-fold molar excess of functional PFO to induce the conversion of all of the mixed oligomers to an SDS-stable form, suggesting that the barrier to stable oligomer formation posed by the Y181A mutation is significant. Because the PFO\textsuperscript{Y181A} proteins form less stable prepore complexes, the monomer-monomer affinity in the mutant prepore complex is reduced relative to that in the wild-type PFO oligomers. The molecular basis of the reduced affinity is not yet known, but the fact that this tyrosine is conserved in all CDCs suggests that the aromatic ring may be important in stabilizing monomer-monomer interactions and effecting the conformational changes necessary to accomplish the prepore to pore conversion. Consistent with this conclusion, PFO mutants with Tyr-181 replaced with Phe are significantly more active in pore formation than mutants with Tyr-181 replaced with Ile, Ala, Cys, and Thr (data not shown).

Because the prepore complex formed by PFO\textsuperscript{Y181A} is unable to insert its TMHs into the membrane, PFO\textsuperscript{Y181A} cannot undergo the proper conformational changes that are required for the alignment and insertion of the TMHs. One possible explanation for the observed behavior of PFO\textsuperscript{Y181A} is that it is unable to align its TMHs with those of adjacent subunits in the prepore complex. The absence of hydrogen bonds between adjacent

\textsuperscript{4} E. M. Hotze and R. K. Tweten, unpublished data.

Fig. 7. 

Release kinetics of markers with significantly different hydrodynamic radii from liposomes treated with mixed oligomers. Liposomes loaded with either fluorescein-labeled glutathione or fluorescein-labeled \(\beta\)-amylase were treated with various ratios of PFO and PFO\textsuperscript{Y181A}. The release of the fluorescein-tagged markers from the liposomes was detected by the quenching of the fluorescein emission by anti-fluorescein antibody that was present in the extra-liposomal milieu of the liposome suspension. A, the release of fluorescein-labeled glutathione and \(\beta\)-amylase from liposomes by 100 nM PFO and the release of fluorescein-glutathione by 100 nM PFO\textsuperscript{Y181A} are shown. The plateau values differ for fluorescein-labeled glutathione and \(\beta\)-amylase because the antibodies quench the former more efficiently than the latter. B–D, liposomes loaded with either fluorescein-labeled glutathione (solid line) or \(\beta\)-amylase (dashed line) were then exposed to 100 nM functional PFO mixed with 100 nM (B), 200 nM (C), or 400 nM (D) of PFO\textsuperscript{Y181A}. 

A, the release of fluorescein-labeled glutathione and \(\beta\)-amylase from liposomes by 100 nM PFO and the release of fluorescein-glutathione by 100 nM PFO\textsuperscript{Y181A} are shown. The plateau values differ for fluorescein-labeled glutathione and \(\beta\)-amylase because the antibodies quench the former more efficiently than the latter. B–D, liposomes loaded with either fluorescein-labeled glutathione (solid line) or \(\beta\)-amylase (dashed line) were then exposed to 100 nM functional PFO mixed with 100 nM (B), 200 nM (C), or 400 nM (D) of PFO\textsuperscript{Y181A}.
Monomer Cooperation Drives Membrane Insertion

TMHs would prevent a concerted movement of the TMHs into the bilayer and the formation of the pore. However, functional toxin monomers in an oligomer with the PFOY181A induce the proper conformational changes in this mutant so that its transmembrane β-strands align and form intra- and inter-strand hydrogen bonds, and thereby allow the concerted insertion of the β-sheet to proceed.

In the homo-oligomer of PFOY181A, the TMHs did not detectably insert into the membrane (Fig. 3; Table I), but successful insertion of the PFOY181A hairpins occurred with increasing efficiency as the fraction of functional PFO in the mixed oligomers was increased. Thus, the insertion of the PFOY181A TMHs could only result from the effects of the neighboring functional PFO monomers in the mixed prepore oligomer. This result further showed that PFOY181A insertion could not be initiated with only a few functional monomers within the prepore oligomer (cf. a domino effect) but instead required a substantial fraction of functional PFO (≥50%) to induce the required conformational changes in PFOY181A necessary to overcome the misalignment and transition energy barrier imposed by the Y181A mutation (Fig. 6; Table I). It is also important to note that the liposome release assays showed that the average size of the pore did not decrease when fewer functional PFO molecules were present in the mixed oligomers (Fig. 7). As the PFO:PFOY181A ratio decreased fewer of the mixed oligomers contained a sufficient level of functional PFO to induce the prepore to pore transition. Thus, the mixed prepore complexes did not appear to partially insert into the membrane and form small pores that would have been reflected by a difference in the rate of release of the small (GSH) and large (β-amylase) markers. Instead, the insertion of the prepore appeared to be an all or none process, i.e. the prepore to pore transition occurred only if the prepore was of a sufficient size and a sufficient number of functional molecules were present to overcome the barrier posed by the presence of PFOY181A in the mixed oligomers.

The experiments reported here demonstrate directly that TMH insertion into the bilayer does not occur independently for PFO. Instead, TMH insertion is coupled with the insertion of its neighbors to create the β-barrel in the bilayer. Monomer-monomer interactions therefore not only promote insertion but cooperative interactions between PFO monomers appear to be required to drive TMH insertion and β-barrel formation. The model of β-barrel biogenesis derived from this study of PFO may be a paradigm for the creation of other β-barrel pores in membranes. For example, the formation of the TOM complex in the outer mitochondrial membrane may involve a cooperative and concerted insertion of more than one Tom40p molecule (21, 22). Experiments can now be done to ascertain the generality of cooperative TMH insertion in the biogenesis of β-barrel pore-forming proteins.

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