BIOCHEMISTRY
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Biochemistry **2011,** *50,* 1599–1606 1599
DOI: 10.1021/bi1012386

Fluorinated Amphiphiles Control the Insertion of α-Hemolysin Pores into Lipid Bilayers†

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Received August 3, 2010; Revised Manuscript Received December 28, 2010

ABSTRACT: The insertion of fully folded and assembled ion channels and pores into planar lipid bilayers for electrical recording has been facilitated by the use of conventional detergents at a final concentration below the critical micelle concentration (CMC). After the desired number of channels or pores (often one) has been incorporated into a bilayer, it is important to prevent further insertion events, which is often done by awkward techniques such as perfusion. Here, we show that the addition of single-chain fluorinated amphiphiles (F-amphiphiles) with zwitterionic, simple neutral, and neutral oligomeric headgroups at a concentration above the CMC prevents the further insertion of staphylococcal α-hemolysin pores, MspA pores, and Kcv potassium channels into lipid bilayers. We found the commercially available F_6FC (fluorinated fos-choline with a $C_6F_{13}C_2H_4$ chain) to be the least perturbing and most effective agent for this purpose. Bilayers are known to be resistant to F-amphiphiles, which in this case we suppose sequester the pores and channels within amphiphile aggregates. We suggest that F-amphiphiles might be useful in the fabrication of bilayer arrays for nanopore sensor devices and the rapid screening of membrane proteins.

A fluorinated amphiphile (F-amphiphile)[1] comprises a polar headgroup and a hydrophobic tail that features a partially or largely fluorinated chain. Because fluorocarbons do not mix with the hydrocarbon chains of common bilayer lipids, F-amphiphiles do not in general solubilize membranes (*1*, *2*). This property opens up interesting possibilities for the use of F-amphiphiles in investigations of membrane proteins. For example, F-amphiphiles enhance the extent of insertion of the diphtheria toxin T-domain into preformed lipid bilayers by reducing the nonproductive aggregation of the protein (*2*, *3*). Other work has established that F-amphiphiles are compatible with the in vitro protein synthesis, folding, and oligomerization of the pentameric mechanosensitive channel MscL (*4*). The same report also suggested that F-amphiphiles facilitate direct and rapid incorporation of functional MscL into preformed lipid bilayers. Furthermore, nonionic or zwitterionic F-amphiphiles are considered less denaturing for proteins than their hydrocarbon-based counterparts (*1*, *5*, *6*). Previous reports have shown that some fluorinated amphiphiles, although unable to solubilize membranes, maintain the solubility of integral membrane proteins following their transfer from hydrogenated surfactants (*1*, *4*, *5*).

We have attempted to broaden the work of Popot and colleagues to determine whether the approach can be extended to enhance bilayer insertion of other membrane proteins. In this study, we used primarily α-hemolysin (αHL), which forms a 232 kDa heptameric protein pore (*7*). The crystal structure of the pore reveals a 14-strand transmembrane β-barrel capped by an extramembranous domain, which encloses a roughly spherical water-filled cavity (*7*). The αHL pore has been used for stochastic sensing (*8*), in studies of DNA sequencing (*9*), and for making functional droplet networks (*10*, *11*). We also examined MspA, a 157 kDa octameric porin from *Mycobacterium smegmatis* (*12*), and Kcv, a 42 kDa tetrameric potassium channel, which is largely α-helical. Kcv is encoded by the chlorella virus PBCV-1 (*13*, *14*).

By contrast with previous reports in which membrane protein insertion was enhanced, we find that at high concentrations of F-amphiphiles (roughly 5 times the CMC) the αHL pore becomes sequestered in the surfactant phase and is unavailable for insertion into lipid bilayers and liposomes. We also find that after the insertion of αHL pores into a lipid bilayer from a standard detergent, the addition of an F-amphiphile arrests further insertion without compromising bilayer stability or affecting the functional properties of pores that have already inserted. This phenomenon provides a means to control the number of functional αHL pores that insert into preformed planar bilayers and liposomes. Under the conditions of our experiments, MspA and Kcv behaved in a similar manner. While the behavior of the F-amphiphiles is not completely understood, our experiments show that the mechanism by which they prevent further insertion is unlikely to be denaturation of the remaining protein.

MATERIALS AND METHODS

Materials. Fluorinated fos-choline (F_6FC) and fluorinated octyl maltoside (F_6OM) (Figure 1) were obtained from Anatrace.

†This study was supported the Medical Research Council. P.R. is a recipient of a Felix Scholarship.

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[1]Abbreviations: αHL, α-hemolysin; βCD, β-cyclodextrin; CF, 5(6)-carboxyfluorescein; F_6TAC, $C_6F_{13}C_2H_4$-S-poly[tris(hydroxymethyl)-aminomethane]; CMC, critical micelle concentration; DPhPC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; F-amphiphile, fluorinated amphiphile; F_6OM, fluorinated octyl maltoside; F_6FC, fluorinated fos-choline; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HFTAC, $C_2H_5C_6F_{12}C_2H_4$-S-poly[tris(hydroxymethyl)aminomethane]; IVTT, in vitro transcription and translation; LUV, large unilamellar vesicles; MOPS, 3-(*N*-morpholino)propanesulfonic acid; rRBC, rabbit erythrocytes; WT, wild-type.

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Published on Web 01/28/2011

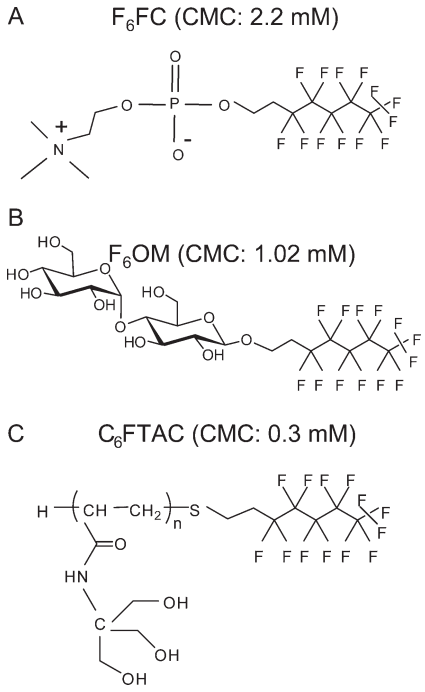

FIGURE 1: Chemical structures of the F-amphiphiles used in this study. (A) Fluorinated fos-choline (F_6FC), with a CMC of 2.2 mM (*28*). (B) Fluorinated octyl maltoside (F_6OM) with a CMC of 1.02 mM (*47*). (C) $C_6F_{13}C_2H_4$-S-poly[tris(hydroxymethyl)aminomethane] (F_6TAC) ($n \sim 7-8$), with a CMC of 0.3 mM (*4*). The CMC values quoted here most likely do not represent the concentrations at which spherical micelles are formed, which would be the case with hydrogenated amphiphiles. F-Amphiphiles probably form more elaborate aggregates above the CMC, as discussed in the text.

$C_6F_{13}C_2H_4$-S-poly[tris(hydroxymethyl)aminomethane] (F_6TAC) was a gift from the laboratory of J.-L. Popot. Stock solutions were made in water: F_6FC (100 mM), F_6OM (20 mM), and F_6TAC (30 mM).

Protein Purification. Heptameric WT αHL from *Staphylococcus aureus* (Wood 46 strain) in SDS buffer [20 mM sodium phosphate, 150 mM NaCl, and 0.3% (w/v) SDS (pH 8.0)], for liposome assays and planar bilayer recordings, was obtained as previously reported (*15*). Monomeric WT αHL, also for liposome assays and planar bilayer recordings, was also obtained from *S. aureus* by a modification of a previously reported protocol (*15*). After elution from the S-Sepharose FF XK-16 cation exchange column, the peak fractions containing the monomer were collected and concentrated to approximately 3 mg/mL by using ultracentrifugal filter devices with a 10 kDa cutoff (model 4321, Amicon) spun at 2900*g*. The protein concentration was determined from the absorbance at 280 nm (the OD_{280} of a 1.0 mg/mL solution is 1.95) (*16*). The monomer was purified further by chromatography on a Superdex 200 HiLoad gel filtration column (model 17107101, GE Healthcare), which was equilibrated and run with 10 mM Tris-HCl and 150 mM NaCl (pH 8.0), at a flow rate of 1 mL/min. The peak fractions were located by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), pooled, and concentrated to 1 mg/mL. The yield of the ~95% pure monomer, as judged by SDS−PAGE, was ~10 mg/L of culture.

The monomeric WT αHL used in the hemolytic assay was expressed in an *Escherichia coli* in vitro transcription and translation (IVTT) system (*E. coli* T7 S30 Extract System for Circular DNA, Promega) for 1 h at 37 °C with the complete amino acid mix. The solution was spun at 25000*g* for 15 min at 4 °C, and the supernatant containing the αHL monomers was retained.

The MspA (NNNRRK) mutant (GeneScript) was expressed in the IVTT system (50 μL) for 2 h at 37 °C in the presence of rRBCM (2 μL, 2 mg protein/mL) and [^{35}S]methionine. The membranes were recovered by centrifugation and solubilized in sample buffer. The proteins were then separated in an 8% SDS−polyacrylamide gel. The gel was dried without being heated onto paper (Whatman 3M) under a vacuum, and the MspA oligomer band was located by autoradiography. After rehydration in buffer [300 μL of 25 mM Tris-HCl (pH 8.0)], the paper was removed. The gel was crushed using a pestle and the slurry filtered through a QIAshredder column (Qiagen) by centrifugation at 25000*g* for 10 min.

The tetrameric potassium channel, Kcv, used for single-channel recording experiments was obtained after IVTT in the presence of [^{35}S]methionine as previously described (*17*, *18*).

Dye Leakage Assay. A 100 mM stock solution of 5(6)-carboxyfluorescein (CF, Sigma-Aldrich) was made in 150 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4 (pH 7.4) (*19*, *20*). The pH of the dye solution was readjusted to pH 7.4 with NaOH. Liposomes containing CF were made by extrusion. Soybean lecithin (20 mg, Calbiochem) was dissolved in chloroform (1 mL) in a round-bottom flask. The lipid was dried under N_2 to form a thin uniform layer and further dehydrated in a vacuum desiccator for 2−3 h. The lipid was then rehydrated by resuspension in a CF solution that had been diluted 50% with water (total volume of 300 μL) to give a final dye concentration of 50 mM. The flask was vortexed for a few minutes to ensure complete resuspension of the lipids, followed by five freeze−thaw cycles (liquid nitrogen/37 °C water bath). The suspension was then extruded 20 times through a 0.1 μm polycarbonate filter by using a mini-extruder (Avanti Polar Lipids) to yield large, unilamellar vesicles (LUV). Free CF was removed from the LUV suspension by size exclusion chromatography on a Sephadex G50 (Sigma Aldrich) column (1 cm × 20 cm), equilibrated, and eluted with 300 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4 (pH 7.4) (buffer A). Before dye release experiments, the freshly prepared liposome stock was diluted 50-fold in buffer A so that the maximal released CF fluorescence was within the range of the fluorimeter.

The release of CF, which is self-quenched within the liposomes, was assessed from the increase in fluorescence emission at 520 nm (excitation at 492 nm). At the end of each run, Triton X-100 was added to the cuvette (final concentration of 0.1%) to determine the maximal CF fluorescence. To determine the effects of the F-amphiphiles, αHL was added to buffer A containing the diluted LUV and an F-amphiphile. The final volume in the cuvette was 1 mL, and the amount of heptamer or monomer was 20 or 5 μg, respectively. The data were analyzed with Origin. The amount of released CF as a percentage of the total at time t is given by

$$R(t) = 100 \times [I(t) - I(0)]/[I(\infty) - I(0)]$$

where the fluorescence intensity at time t is $I(t)$, the initial fluorescence of the liposomes is $I(0)$, and the fluorescence after Triton X-100 addition is $I(\infty)$.

Single-Channel Recordings. The apparatus for planar lipid bilayer recording consisted of two compartments separated by a Teflon septum containing an aperture with a diameter of 100 μm. The aperture was pretreated with a solution of hexadecane in pentane (10%, v/v). For recordings with αHL, buffer A was used. For MspA, the buffer consisted of 1.0 M KCl and 10 mM Tris-HCl (pH 7.0), and for Kcv, the buffer consisted of 200 mM KCl and 10 mM HEPES (pH 7.0). To form a bilayer, buffer was

added to both compartments at a level below the aperture, and 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, Avanti Polar Lipids) in pentane (10 mg/mL) was added to each surface. The pentane evaporates to leave a lipid monolayer at the air−water interface. Sequential lowering and raising of the levels of the buffer solutions fold the two monolayers together to form a vertical lipid bilayer (*21*). The formation and quality of the bilayers were monitored by capacitance measurements. Currents were recorded under voltage-clamp conditions with Ag/AgCl electrodes.

Single-Channel Recordings with F-Amphiphiles. Control experiments without F-amphiphiles were initiated by the addition of the αHL heptamer or monomer (final concentration of 30 ng/mL), MspA (final concentration of 40 ng/mL), or Kcv (final concentration of 4 ng/mL) to the cis compartment (ground), the contents of which were stirred until insertion occurred. Experiments for examination of the inhibitory effects of F-amphiphiles on the insertion of WT αHL, MspA, or Kcv were initiated by the addition of protein to the cis compartment, which contained 1000 − V μL of electrolyte, where V is the volume of the F-amphiphile later added to the cis side after channel insertion had occurred. The contents of the cis compartment were stirred briefly after the addition of the F-amphiphile solution. To compensate for the addition of the F-amphiphile solution, an equal volume of water was added to the trans compartment. The low-pass Bessel filter of the amplifier was set at 1 kHz. Data were acquired at a sampling rate of 10 kHz. The data points for the $I-V$ curves, for the WT αHL heptamer, MspA, and Kcv, are the mean values from three separate single-channel experiments. The current values obtained in the presence of 10 mM F_6FC (cis) were normalized to the current values obtained in the absence of F_6FC to take into account the reduction in the ionic strength of the recording buffer caused by the addition of the F-amphiphile.

Single-Channel Recordings in β-Cyclodextrin Binding Studies. The recording buffer contained 1 M NaCl and 10 mM Na_2HPO_4 (adjusted to pH 7.5 with aqueous HCl). The experiments were initiated by the addition of ∼30 ng of the WT αHL heptamer to the cis compartment with stirring until a channel was inserted. βCD (40 μM) was added to the trans compartment. The internal low-pass Bessel filter of the amplifier was set at 5 kHz, and the acquisition rate was 20 kHz. τ_{on} and τ_{off} values for binding of βCD to the WT αHL pore, at ±40 mV, were obtained from dwell-time histograms fitted to single exponentials with Clampfit. To determine kinetic constants for the association and dissociation of βCD in the presence of the F-amphiphiles, we averaged data from three single-channel experiments.

Hemolytic Assay in the Presence of F-Amphiphiles. WT αHL (5 μL of IVTT protein), incubated for 10 min with either 10 mM F_6FC, 5 mM F_6OM, or 2 mM F_6TAC in a final volume of 10 μL, was diluted into MBSA [10 mM MOPS and 150 mM NaCl (pH 7.4) containing 1 mg/mL bovine serum albumin] in the first well of a microtiter plate (final volume of 100 μL). The protein was then subjected to serial 2-fold dilution in MBSA over the remaining 11 wells of the plate row (final volumes of 50 μL). An equal volume of 1% washed rabbit erythrocytes (rRBC) in MBSA was then added to each well, beginning with the most diluted lane. Hemolysis was followed for 1.5 h at 24 °C by monitoring the decrease in light scattering at 595 nm with a Bio-Rad microplate reader (model 3550-UV) running Microplate Manager version 4.0.

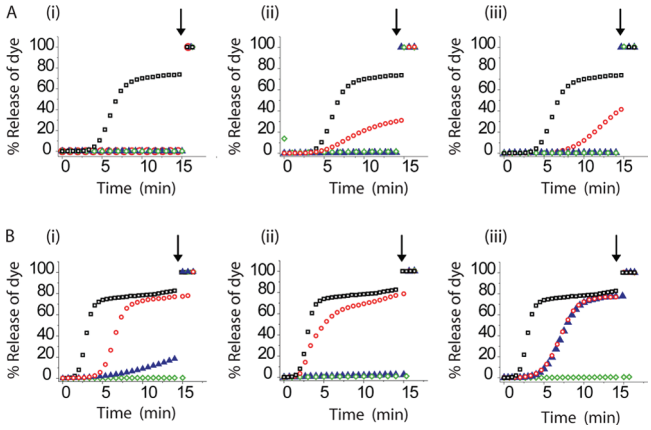

FIGURE 2: Dye leakage assays for determining the effects of F-amphiphiles on LUV permeabilization by αHL. The release of self-quenched CF causes an increase in fluorescence emission at 520 nm, which is monitored in the assays. (A) (i) Insertion of the αHL monomer in the presence of F_6FC. (ii) Insertion of the αHL monomer in the presence of F_6OM. (iii) Insertion of the αHL monomer in the presence of F_6TAC. (B) (i) Insertion of the αHL heptamer in the presence of F_6FC. (ii) Insertion of the αHL heptamer in the presence of F_6OM. (iii) Insertion of the αHL heptamer in the presence of F_6TAC. The traces are color-coded: insertion of αHL in the absence of an F-amphiphile (black squares), insertion of αHL below the CMC (red circles), insertion of αHL around the CMC (blue triangles), and insertion of αHL above the CMC (green diamonds). The arrow in each trace indicates the addition of Triton X-100 to the cuvette to fully lyse the liposomes. For further details, see Table 1.

RESULTS AND DISCUSSION

Effects of F-Amphiphiles on Membranes. Three different F-amphiphiles were tested in this study (Figure 1): F-fos-choline (F_6FC) with a zwitterionic headgroup, F-octyl maltoside (F_6OM) with a nonionic disaccharide headgroup, and $C_6F_{13}C_2H_4$-S-poly-[tris(hydroxymethyl)aminomethane] (F_6TAC) with a nonionic oligomeric headgroup (*4*). Earlier work suggested that F_6TAC neither solubilizes lipid bilayers nor interferes with protein synthesis at up to 50 times its CMC (*1*, *2*, *4*). In this work, F_6FC, F_6OM, and F_6TAC were tested for their effects on membranes in a hemolytic assay (*4*) and on planar lipid bilayers. All three F-amphiphiles lacked lytic activity toward 1% rRBCs at concentrations of up to 50 times the CMC (data not shown). In accordance with previous reports (*2*, *4*), F_6FC and F_6TAC did not affect the stability of preformed planar lipid bilayers, as determined by capacitance measurements, even at concentrations 5 times the CMC. However, the addition of F_6OM at concentrations above the CMC did decrease the stability of preformed bilayers, reducing the lifetime to less than 2 min (data not shown). Therefore, electrical recordings with planar bilayers were confined to F_6FC and F_6TAC. It should also be noted that we were unable to form or re-form DPhPC bilayers in the presence of any one of the three F-amphiphiles.

Effects of F-Amphiphiles on the Insertion of αHL into Liposomes. αHL is a pore-forming protein capable of transporting molecules as large as 2−4 kDa through lipid bilayers (*22*). Therefore, a dye leakage assay was chosen to monitor the incorporation of αHL into liposome (LUV) membranes. 5(6)-Carboxyfluorescein (CF) was incorporated into LUV at self-quenching concentrations (*23*, *24*). When pores are formed in the LUV, dye leakage occurs, generating a fluorescence signal. At the end of the assay, the maximal fluorescence signal is measured after the liposomes are lysed with detergent. We measured the lag phase before dye release, the initial rate of dye release, and the final

Table 1: Release of Carboxyfluorescein from Liposomes Induced by αHL Monomers and Heptamers in the Absence and Presence of F-Amphiphiles

	percent release of dye at end point[a] (%)		initial rate of release of dye[b] (% min^{-1})		lag time before initiation of release[c] (min)	
	monomer	heptamer	monomer	heptamer	monomer	heptamer
no addition of F-amphiphile	74	84	18	37	3.7	1.6
F_6FC						
above CMC[d]	0.2	0.6	nd[e]	nd[e]	nd[e]	nd[e]
at CMC[d]	0.2	20	nd[e]	2	nd[e]	2.8
below CMC[d]	0.3	78	nd[e]	23	nd[e]	4.3
F_6OM						
above CMC[d]	1	1	nd[e]	nd[e]	nd[e]	nd[e]
at CMC[d]	1	2.5	nd[e]	nd[e]	nd[e]	nd[e]
below CMC[d]	31	79	4	16	4.4	1.8
F_6TAC						
above CMC[d]	0.5	0.7	nd[e]	nd[e]	nd[e]	nd[e]
at CMC[d]	0.7	77	nd[e]	16	nd[e]	4.8
below CMC[d]	44	77	6.5	16	7.5	4.8

[a]The end point is given as the percentage of dye released. When release was still occurring after 15 min, the percentage release at that time is given. [b]The initial rate of dye release was calculated from the slope of the initial linear phase. [c]The lag time before the initiation of dye release was determined from the intercept of the initial linear phase with the *x*-axis. [d]The three concentrations of F-amphiphile were as follows: 0.5, 2.0, and 10 mM F_6FC; 0.2, 1.0, and 5.0 mM F_6OM; and 0.05, 0.3, and 2.0 mM F_6TAC. The concentration of liposomes was 25 μM (in lipid monomers). The protein concentrations were as follows: 5 μg/mL αHL monomer and 20 μg/mL αHL heptamer. [e]Not determined (in cases where there was no liposome permeabilization). The data shown are from a typical experiment (Figure 2).

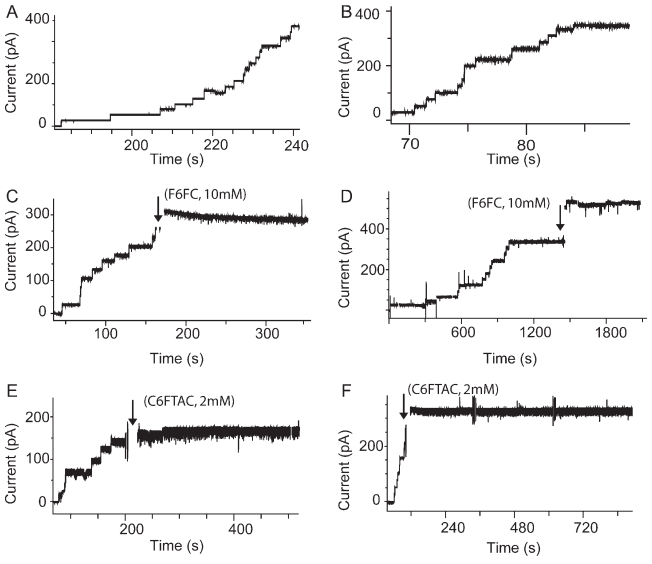

FIGURE 3: Effects of F-amphiphiles on αHL pore formation in planar lipid bilayers. (A) Multiple insertions of WT αHL heptamers (30 ng/mL, cis) in the absence of an F-amphiphile at 100 mV. (B) Multiple insertions of WT αHL heptamers (30 ng/mL, cis) in the presence of F_6FC (10 mM, trans) at 100 mV. (C and D) Arrest of the insertion of αHL heptamers (30 ng/mL, cis) and monomers (30 ng/mL, cis), respectively, at 100 mV, by the addition of F_6FC (arrow, 10 mM, cis). (E and F) Arrest of the insertion of αHL heptamers (30 ng/mL, cis) and monomers (30 ng/mL, cis), respectively, at 100 mV, in the presence of F_6TAC (arrow, 2 mM, cis). All recordings were made in buffer A.

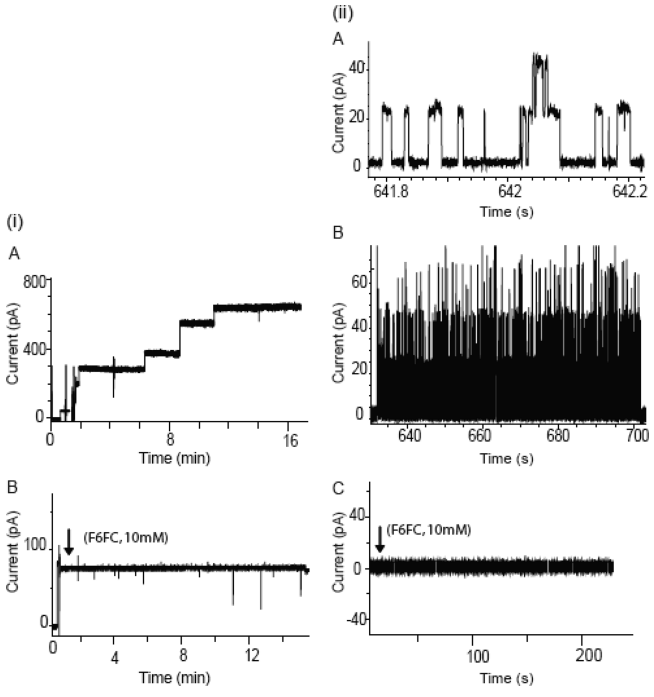

FIGURE 4: Effect of F_6FC on the insertion of the porin MspA and the potassium channel Kcv into planar lipid bilayers. (i) (A) MspA (NNNRRK mutant) pore activity in the absence of an F-amphiphile at 50 mV. (B) Effect of the addition of F_6FC (arrow, 10 mM, cis) after the insertion of an MspA pore. No further insertion events occur. The recording buffer for MspA consisted of 1.0 M KCl and 10 mM Tris-HCl (pH 7.0). (ii) (A) WT Kcv channel activity in the absence of an F-amphiphile at 100 mV. (B) Condensed view of the trace from which the expanded view in panel A was taken. Numerous individual openings are seen, with occasional coincident openings of two and three channels. (C) WT Kcv was added to the cis chamber just after the addition of F_6FC (arrow, 10 mM, cis) at 100 mV. No openings are seen. The recording buffer for Kcv consisted of 200 mM KCl and 10 mM HEPES (pH 7.0).

extent of release as a percentage of the maximal signal (Figure 2 and Table 1). Both the αHL monomer and the preformed αHL heptamer were examined in the presence of the F-amphiphiles, each at three concentrations: (i) below the CMC, (ii) at around the CMC, and (iii) above the CMC (Figure 2). To effect dye leakage, the αHL monomer must both assemble and insert (*25*) into the LUV bilayer, while the preformed αHL heptamer must undergo direct insertion from dilute detergent (*26*). At concentrations above the CMC, the F-amphiphiles completely prevented the action of both the αHL monomer and the αHL heptamer (Figure 2). Lower concentrations of the F-amphiphiles slowed αHL insertion to varying extents (Figure 2). F-Amphiphiles, including single-chain molecules, self-assemble above the CMC

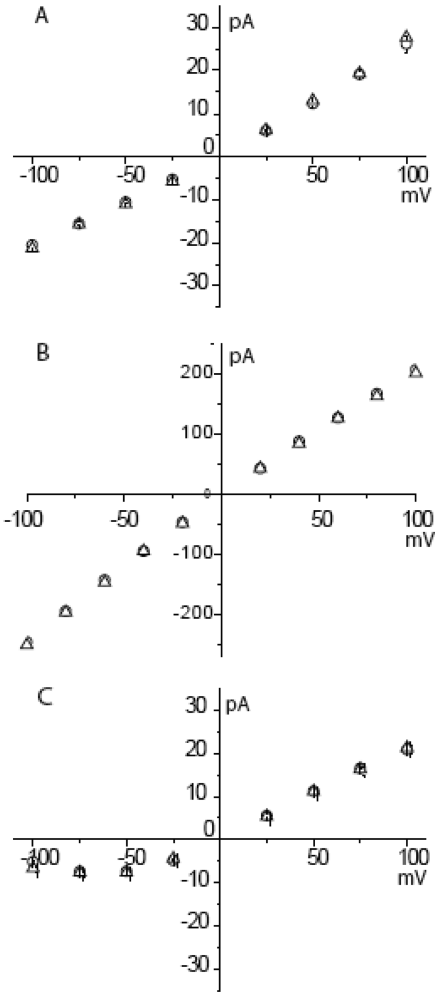

FIGURE 5: Effects of F_6FC on the $I-V$ curves of αHL, MspA, and Kcv in planar bilayers. (A) $I-V$ curve of a single WT αHL heptamer in the absence (○) and presence (△) of F_6FC (10 mM, cis). The recording buffer was buffer A. (B) $I-V$ curve of a single MspA pore in the absence (○) and presence (△) of F_6FC (10 mM, cis). The recording buffer for MspA consisted of 1.0 M KCl and 10 mM Tris-HCl (pH 7.0). (C) $I-V$ curve of a single WT Kcv channel in the absence (○) and presence (△) of F_6FC (10 mM, cis). The recording buffer consisted of 200 mM KCl and 10 mM HEPES (pH 7.0). For all $I-V$ curves, each data point is the mean ± standard deviation from three separate single-channel recordings.

to form extended structures, including tubules and structures containing bilayers such as unilamellar or multilamellar vesicles (*27*−*35*). The nature of these aggregates depends upon the concentration and structure of the amphiphile; only F-amphiphiles with bulky, branched oligosaccharide headgroups have been reported to form true micelles (*35*). Our experiments suggest that aggregates formed by F-amphiphiles prevent the insertion of both monomeric and heptameric αHL into lipid bilayers by sequestering the proteins. However, unlike the micelles formed by standard hydrocarbon-based detergents, the F-amphiphile aggregates are unable to solubilize lipid bilayers.

Effect of F-Amphiphiles on the Insertion of Membrane Proteins into Planar Lipid Bilayers. Preformed αHL heptamers and other membrane proteins with bound detergent [SDS in our case (see Materials and Methods)] have been reported to insert directly into lipid bilayers (*26*, *36*−*38*). By contrast, αHL monomers first oligomerize to form a heptameric prepore on the bilayer surface. The prepore then undergoes a conformational reorganization, forming a β-barrel during insertion into the bilayer (*25*, *39*).

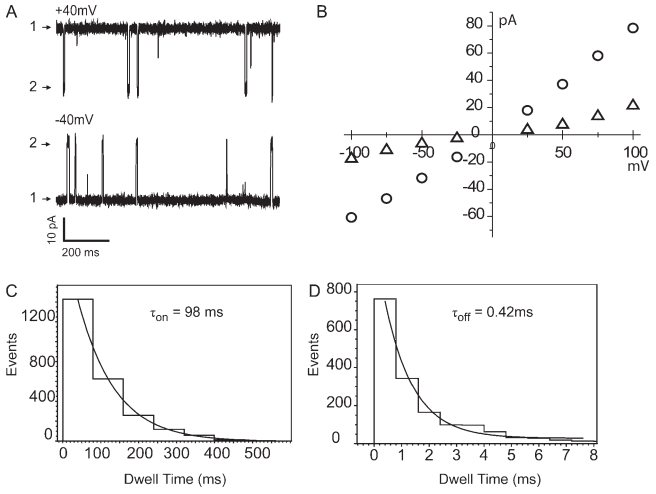

FIGURE 6: Effects of F_6FC on the binding of βCD to the WT αHL pore. (A) Representative single-channel current traces from a WT αHL heptamer at ±40 mV showing blockades by βCD (40 μM, trans) in the presence of F_6FC (10 mM, cis). Levels 1 and 2 indicate the unoccupied and occupied αHL pore, respectively. (B) $I-V$ curve of αHL in the absence (○) and presence (△) of bound βCD. (C and D) Representative dwell time histograms at 40 mV for the interaction of βCD (40 μM, trans) with WT αHL in the presence of 10 mM F_6FC (cis): τ_{on}, interevent interval; τ_{off}, βCD dwell time. The results from the kinetic analysis are summarized in Table 2. The recording buffer consisted of 1 M NaCl and 10 mM Na_2HPO_4 (pH 7.5).

In the absence of F-amphiphiles, multiple insertions of αHL heptamers (Figure 3A) and monomers (data not shown) were observed when these proteins were added to the cis compartment of the bilayer apparatus. When F-amphiphiles were present in the trans compartment, at final concentrations of 5 times the CMC, the rate of αHL insertion was comparable to the rate in the absence of the F-amphiphile (Figure 3B). By contrast, both F_6FC and F_6TAC, above the CMC in the cis compartment, caused complete arrest but not reversal of the insertion of both the αHL heptamer and monomer (Figure 3C−F). When either F_6FC or F_6TAC was present at a concentration below the CMC in the cis compartment, pore insertion events continued (data not shown).

The octameric porin MspA, which is also largely β-structure, behaved like αHL (Figure 4, panel i, A,B). Further, the α-helical membrane protein Kcv was also examined as a gel-purified tetramer. In three separate attempts, we found that no Kcv channels inserted into a bilayer when F-amphiphiles were present at concentrations above the CMC (Figure 4, panel ii, C), while channel insertion occurred in all three attempts in the absence of an F-amphiphile (Figure 4, panel ii, A,B). In neither case was insertion reversed. Because both major classes of membrane protein (β-barrels and α-helix bundles) are similarly affected under the conditions of our experiments, a common mechanism for the arrest of insertion must be invoked, and we favor sequestration within F-amphiphile aggregates, which may comprise bilayer structures (*27*−*35*). These experiments show that F-amphiphiles provide a useful means of controlling the number of proteins entering a lipid bilayer.

Effect of F_6FC on αHL and MspA Pores and Kcv Channels in Bilayers. To be of genuine utility in controlling insertion, an F-amphiphile should not affect the functional properties of pores and channels that have already inserted into bilayers. Gratifyingly, the addition of F_6FC, which is commercially available, above the CMC to the cis compartment did not cause blockades or changes in the gating of αHL pores, MspA pores, or Kcv channels that had already inserted into a bilayer.

Table 2: Conductance Values and Kinetic Constants[a] for the Binding of βCD to the αHL Pore in the Presence of F_6FC

	voltage (mV)	F_6FC[b]	$g_{\alpha HL}$ (pS)	$g_{\alpha HL,\beta CD}$ (pS)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (M)
1[c]	40	−	721 ± 6	253 ± 4	$(2.8 \pm 0.2) \times 10^5$	$(2.1 \pm 0.2) \times 10^3$	$(7.8 \pm 0.3) \times 10^{-3}$
2	40	+	721 ± 4	232 ± 4	$(2.5 \pm 0.4) \times 10^5$	$(2.4 \pm 0.3) \times 10^3$	$(9.6 \pm 0.4) \times 10^{-3}$
3[c]	−40	−	651 ± 4	240 ± 3	$(4.0 \pm 0.3) \times 10^5$	$(1.3 \pm 0.1) \times 10^3$	$(3.4 \pm 0.3) \times 10^{-3}$
4	−40	+	650 ± 3	257 ± 3	$(3.1 \pm 0.1) \times 10^5$	$(1.7 \pm 0.1) \times 10^3$	$(5.4 \pm 0.5) \times 10^{-3}$

[a] $g_{\alpha HL}$ and $g_{\alpha HL,\beta CD}$ are the unitary conductance in the absence of βCD and the unitary conductance with βCD bound in 1 M NaCl and 10 mM Na_2HPO_4 (pH 7.5), respectively. k_{on}, k_{off}, and K_d are quoted as the means ± standard deviation from three experiments. [b] The concentration of F_6FC was 10 mM in the cis compartment. [c] Values taken from ref *46*.

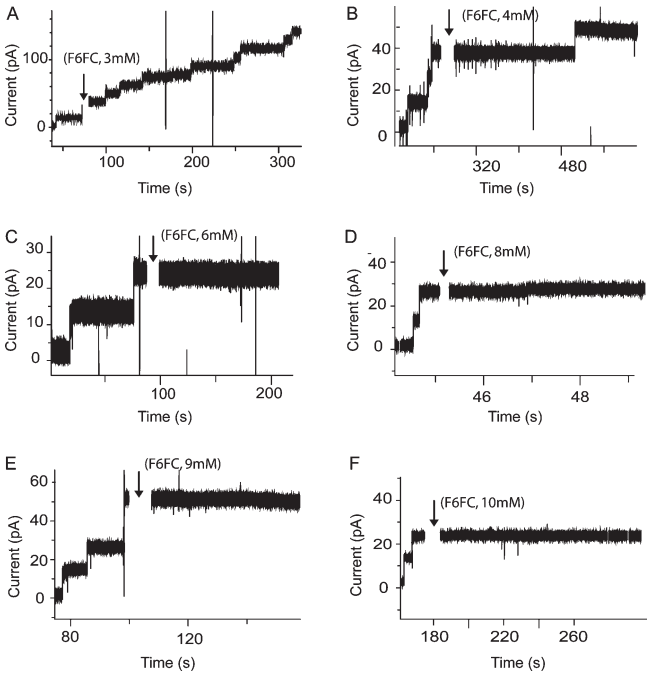

FIGURE 7: Titration with F_6FC (cis) to determine the concentration at which αHL heptamer insertion is blocked. The recording buffer was buffer A. The concentration of αHL heptamer in all cases was 30 ng/mL. The final concentrations of F_6FC were 3, 4, 6, 8, 9, and 10 mM (A−F, respectively). The arrows in the traces indicate the points at which F_6FC was added.

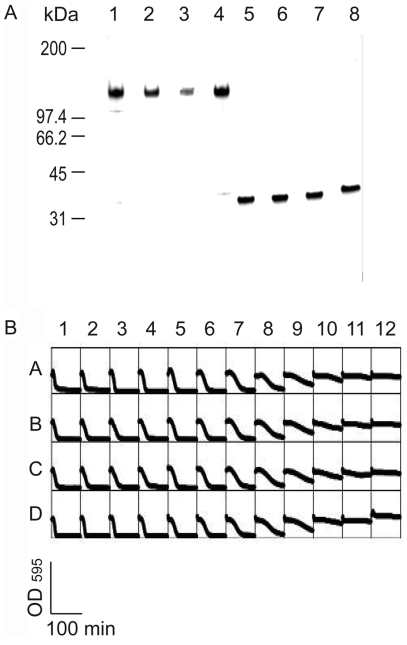

FIGURE 8: Stability of the αHL pore in the presence of F-amphiphiles. (A) The WT αHL monomer and heptamer were incubated at room temperature for 5 min with F-amphiphiles at concentrations 5 times the reported CMCs. Monomer and heptamer samples (~0.25 μg) were then run on 12% Bis-Tris SDS−polyacrylamide gels in XT-MES buffer at 200 V: lanes 1 and 5, WT αHL heptamer and monomer, respectively, in the absence of F-amphiphiles; lanes 2 and 6, WT αHL heptamer and monomer, respectively, incubated with F_6FC; lanes 3 and 7, WT αHL heptamer and monomer, respectively, incubated with F_6OM; lanes 4 and 8, WT αHL heptamer and monomer, respectively, incubated with F_6TAC. (B) Hemolytic activity of the αHL monomer in the presence of F-amphiphiles. The concentrations of F_6FC, F_6OM, and F_6TAC in lanes B1−D1 (after the addition of red cells) were 10, 5, and 2 mM, respectively. αHL prepared by IVTT (5 μL) was added to lanes A1−D1. The protein and amphiphile were then subjected to 2-fold serial dilution in 10 mM MOPS and 150 mM NaCl (pH 7.4) over the 11 remaining wells (final volume of 50 μL per well), followed by the addition of washed 1% rRBCs (50 μL) to each well. The concentration of the F-amphiphile in well 1 is above the CMC in each case. In all wells, the protein had been subjected to an F-amphiphile above the CMC before dilution.

Further, the single-channel $I-V$ curves of αHL, MspA, and Kcv were unchanged (Figure 5). We also examined the interaction of βCD (trans) with αHL pores in the presence of F_6FC (cis) (Figure 6). The values of k_{on} and k_{off}, and hence K_d, for βCD were comparable to previously reported values (Table 2). Therefore, although F_6FC prevents the insertion of proteins into lipid bilayers, it does not affect the functional properties of proteins that are already in them.

Mode of Action. The cessation of membrane protein insertion by F_6FC appears to be effective only at F_6FC concentrations significantly above the CMC. F_6FC concentrations of 3 and 4 mM, which are just above the reported CMC value of F_6FC (2.2 mM), are not as effective in stopping the membrane insertion of αHL heptamers (Figure 7A,B) when compared with higher concentrations of F_6FC, which completely prevent αHL heptamer insertion (Figure 7C−F).

F-Amphiphiles might work by denaturing the uninserted protein in the aqueous phase. However, SDS−PAGE suggests that the αHL heptamer does not undergo denaturation after coming into contact with F-amphiphiles, as there is no dissociation to monomers (Figure 8A). The integrity of the monomer in the presence of or after treatment with the F-amphiphiles above the CMC was confirmed by a hemolytic assay (Figure 8B). The ability of αHL monomers to act on rabbit erythrocytes under conditions under which they will not insert into pure lipid bilayers might be explained by the presence of strong receptors on the red cells that promote irreversible binding and assembly (*40*, *41*), in competition with sequestration by F-amphiphile aggregates.

We have proposed sequestration within F-amphiphile aggregates as a plausible mechanism for the arrest of insertion into lipid bilayers of the membrane proteins tested here (Figure 9).

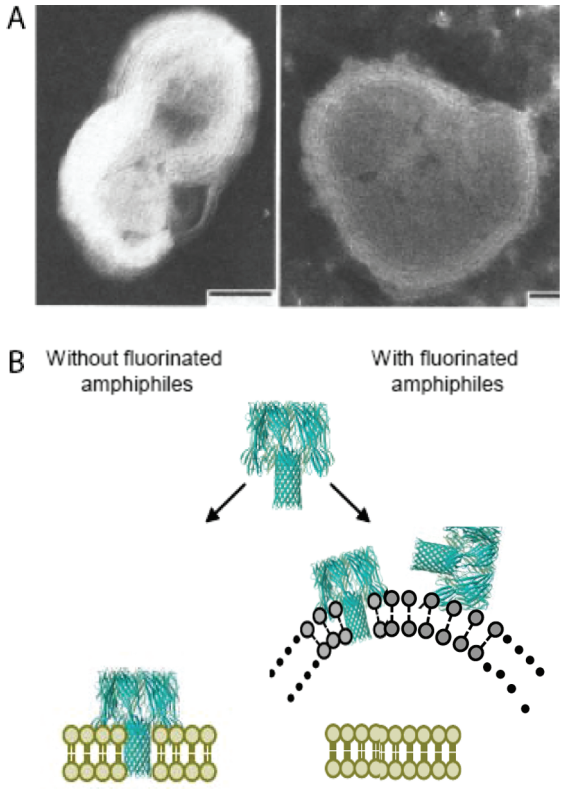

FIGURE 9: Aggregates formed by F-amphiphiles and the proposed mechanism of protein sequestration by them. (A) TEM of isolated multilayer vesicles in a dilute dispersion (0.5%, w/v) of F_8FC after gentle shaking by hand at room temperature (*28*). The bar is 50 nm. F_8FC is a single-chain F-alkyl phosphocholine amphiphile with a $C_8F_{17}C_2H_4$ chain [cf. the $C_6F_{13}C_2H_4$ chain in F_6FC (Figure 1)]. (B) Proposed mechanism of action in which membrane proteins partition partially or fully into the aggregates formed by F-amphiphiles (gray) and are therefore unavailable for insertion into lipid bilayers (yellow).

This seems reasonable as proteins have been reported to have a higher affinity for F-amphiphiles than for hydrocarbon-based amphiphiles (*42*, *43*). Under our experimental conditions with a low lipid concentration (micromolar range), the αHL, MspA, and Kcv proteins are suggested to partition quickly onto or into the F-amphiphile aggregates, which are present at higher concentrations (millimolar monomers), and remain unavailable for insertion into lipid bilayers. Ladokhin and colleagues have carefully examined the effects of F-amphiphiles on the insertion into bilayers of diphtheria toxin T domain and annexin B12 (*2*, *3*, *44*). In these cases, insertion was reversible and high concentrations of the F-amphiphile removed the proteins from bilayers. The proteins that we have tested bind tightly to bilayers and cannot be removed by F-amphiphiles, although insertion ceases in the presence of these agents, suggesting that the systems are under kinetic rather than thermodynamic control (*44*) in the cases that we have examined.

CONCLUSIONS

Previous reports have shown that F-amphiphiles (e.g., F_6TAC and HFTAC) can assist in the insertion of proteins such as diphtheria toxin and MscL into lipid bilayers most likely by preventing their aggregation in solution (*2*, *4*). We attempted to extend this work by examining the effects of two commercially available F-amphiphiles, F_6FC and F_6OM, as well as the previously reported F_6TAC, on the insertion of the αHL pore, the MspA pore, and the Kcv potassium channel into bilayers. At all concentrations of the F-amphiphiles tested, we found an inhibitory effect on insertion, suggesting that optimal solubilization had already been achieved for these proteins, which was also the case for annexin B12 (*44*). Further, at concentrations 5 times the CMC and at low lipid concentrations, the F-amphiphiles completely prevented the insertion of αHL, MspA, and Kcv into planar lipid bilayers. We also found that the F-amphiphiles had no effect on the properties of proteins that had already inserted into bilayers. Therefore, addition of an F-amphiphile might be used to control membrane protein insertion without resorting to methods such as perfusion. The approach might prove useful in single-channel studies of membrane proteins in planar bilayers, and in the manufacture of chips for the rapid screening of membrane proteins or for use as sensor arrays (*45*), where one difficulty has been to control the number of proteins in each element of the chip.

ACKNOWLEDGMENT

We thank Jean-Luc Popot for the F_6TAC sample.

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