Conversion of a Mechanosensitive Channel Protein from a Membrane-embedded to a Water-soluble Form by Covalent Modification with Amphiphiles

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Covalent modification of integral membrane proteins with amphiphiles may provide a general approach to the conversion of membrane proteins into water-soluble forms for biophysical and high-resolution structural studies. To test this approach, we mutated four surface residues of the pentameric Mycobacterium tuberculosis mechanosensitive channel of large conductance (MscL) to cysteine residues as anchors for amphiphile attachment. A series of modified ion channels with four amphiphile groups attached per channel subunit was prepared. One construct showed the highest water solubility to a concentration of up to 4 mg/ml in the absence of detergent. This analog also formed native-like, α-helical homo-pentamers in the absence of detergent as judged by circular dichroism spectroscopy, size-exclusion chromatography and various light-scattering techniques. Proteins with longer, or shorter polymers attached, or proteins modified exclusively with polar cysteine-reactive small molecules, exhibited reduced to no solubility and higher-order aggregation. Electron microscopy revealed a homogeneous population of particles consistent with a pentameric channel. Solubilization of membrane proteins by covalent attachment of amphiphiles results in homogeneous particles that may prove useful for crystallization, solution NMR spectroscopy, and electron microscopy.

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Introduction

Structural characterization of membrane proteins has been extremely slow when compared to their water-soluble counterparts. This is in part due to difficulties in the recombinant production of membrane proteins, their poor stability in the absence of a lipid bilayer, the resulting presence of lipids and detergents in membrane protein preparations, and the difficulty in forming well-ordered crystals.1–3 Conversion of membrane proteins to a water-soluble form that eliminates the requirement for detergents or other amphiphiles could accelerate their structural and biophysical characterization. This approach is based on the proposal that water-soluble proteins may be considered as modified membrane proteins with covalently attached polar groups.4 To date, only the small, type I membrane protein phospholamban and the potassium channel KcsA have been successfully converted into a water-soluble form.5–8 A soluble form of the proper oligomeric state of these proteins was obtained by computational approaches and extensive mutagenesis of hydrophobic to hydrophilic surface residues. Efforts to convert the more complex seven helix-spanning proton pump bacteriorhodopsin using the same approach have been unsuccessful.9 Alternatively, addition of large polyethylene glycol polymers to bacteriorhodopsin produced water-soluble bacteriorhodopsin that was partially denatured in the absence of detergent or lipid.10 The conversion of aerolysine, a toxin that forms transmembrane channel in the plasma membranes of higher eukaryotic cells, to a water-soluble form was also recently reported.11

As an alternative approach, we reasoned that covalent attachment of amphiphiles to lipid-exposed sites on the protein surface might improve the homogeneity of membrane protein preparations, relative to detergent-solubilized forms, by precisely fixing the protein to amphiphile stoichiometry. This modification should then lead to well-defined constructs that are much more amenable to high-resolution structural studies by techniques such as X-ray crystallography and NMR spectroscopy. A particular strength of this approach is that the starting point for the modification is the native state of a membrane protein, which is advantageous, since no additional folding step is required after the modification.

To test this hypothesis, we covalently modified the mechanosensitive channel of large conductance of Mycobacterium tuberculosis (Tb_MscL) with an amphiphile. Mechanosensitive channels are ubiquitous in bacteria and play an important role in managing their transition from high to low osmotic environments. These channels open under osmotic stress, jettisoning water and solutes from the cytoplasm in an effort to prevent cell lysis during hypoosmotic shock.12 The crystal structure of Tb_MscL guided the design of the attachment sites for the amphiphiles.13 Tb_MscL forms a homo-pentameric structure organized into two domains, the transmembrane domain, which consists of two transmembrane helices (TM1 and TM2 from each monomer), and the cytoplasmic domain (Figure 1). The TM1 helix lines the lumen of the pore, packs against TM2, and makes little contact with the lipid bilayer. By contrast, TM2 (residues 69–89) traverses the lipid bilayer back to the cytoplasmic side on the perimeter of the TM1 helix bundle, and is responsible for most of the contacts with the lipid bilayer. Therefore, a subset of the lipid contacting residues on TM2 was chosen as the polymer attachment sites. Since the sequence of wild-type MscL does not contain any native cysteine residues, incorporation of non-native

Figure 1. A, Ribbon diagram of Tb_MscL. Residues chosen for replacement with cysteine residues for subsequent modification are shown on one monomer. B, Model of cysteine-substituted MscL with residues shown only on monomer A. Residues above and below the region of interest are omitted for clarity. C, Top view of MscL with selected residues from all five monomers shown in ball-and-stick representation. The TM1 helices line the pore, and the TM2 helices are on the perimeter. Figures were generated using MOLSCRIPT32 and Raster3D.33
cysteine residues at specific sites allowed for specific modification of the channel protein by thiol-reactive reagents at these positions. The mild conditions during the chemoselective modification of these cysteine residues maintained the folded structure of the protein.14–18

The polyethyleneglycol-polyamide oligomers (PPO) consisted of multiple repeating units of succinic acid-(4,7,10)-trioxa-1,13 tridecanediamine. Discrete oligomers and polymers composed of this repeating unit have been used as inert peptide linkers19 and as enhancers of the pharmacokinetic properties of protein pharmaceuticals, respectively.20 Even short segments of the polymer greatly enhanced the solubility and handling of hydrophobic peptide segments, which is consistent with the presence of polyethylene oxide units in traditional detergents such as the Triton and Tween families. An additional advantage in the use of PPOs is the ability for assembly on a solid support (Scheme 1), which allows for incremental increases in polymer size. Solid-phase assembly also allows for the convenient incorporation of the maleimide and other chemoselective linkers on-resin.

Using this approach, we prepared five variants of MscL consisting of a mutant protein containing four cysteine residues in each subunit to which were attached one to five PPOs, respectively. Analysis of the PPO-modified constructs by several biophysical methods as well as electron microscopy (EM) demonstrated that Tb_MscL can be converted into a water-soluble form that retained native α-helical and pentameric structure.

Results

Protein design

The design of the oligomer-modified protein constructs was guided by the known three-dimensional structure of Tb_MscL. Coordinates for the Tb_MscL structure were obtained from the Protein Data Bank entry 1MSL.13 Four large hydrophobic surface residues (Leu73, Phe80, Phe84, and Phe88) in TM2 that faced the lipid bilayer were chosen for the attachment sites. Cysteine residues were introduced at these positions, resulting in fourfold

![Scheme 1](image)

**Scheme 1.** A, Synthesis of PPO: (i) succinic anhydride, DMAP; (ii) CDI-activation, (4,7,10)-trioxa-1,13-tridecanediamine, HOBT; (iii) repeat (i) and (ii); (iv) β-maleimidopropionic acid, NHS, DIC; (v) 3% TFA in DCM. B, Attachment chemistry: the maleimide group on the PPO reacts chemoselectively with a thiol group on the protein. C, Modification of folded MscL_4Cys after immobilization on Ni-beads. For clarity, only one subunit is shown with PPO1 modifications in the product. Cysteine residues are located only on TM2.
mutant MscL_4Cys (Figure 1). The expression level in *Escherichia coli* was similar to wild-type MscL. The mutant protein was found in the membrane fraction, and the same purification scheme could be used as for wild-type Tb_MscL. The MscL_4Cys had a nearly identical circular dichroism (CD) spectrum to Tb_MscL (Figure 3C), and a similar retention time by size-exclusion chromatography (SEC) in the presence of detergent (data not shown). As predicted, removal of detergent resulted in extensive protein aggregation, as seen for the wt Tb_MscL protein (data not shown). Based on these experiments, we assumed that the unmodified MscL_4Cys was expressed in a native-like, pentameric structure.

**PPO synthesis**

The synthesis of the PPOs used for protein modification is outlined in Scheme 1A. The PPOs were synthesized on a solid support by successively adding of succinic anhydride and (4,7,10)-trioxaoctadeca-1,13-tridecanediamine (TTD) to produce oligomers of various, discrete lengths. In a final step, N-β-maleimidopropionic acid was coupled to the PPO, providing suitable attachment chemistry to thiol-groups on the protein (Scheme 1B). High-pressure liquid chromatography (HPLC) and electrospray mass spectrometry (ES-MS) showed that the PPOs consisting of one to five subunits were >95% pure (data not shown) with yields between 30% and 40% based on the substitution of the starting resin (see Methods).

**Protein modification and analytical characterization**

Shown in Scheme 1C is an outline for the modification of MscL_4Cys with maleimide-modified PPOs, which was carried out after immobilization of the protein on Ni-NTA beads utilizing the N-terminal His$_{10}$-tag of the protein. Ni-NTA-beads were loaded with purified MscL_4Cys and washed with reaction buffer containing detergent to stabilize the protein in its native state. The maleimido-PPOs were added in detergent-containing buffer at high millimolar concentration and in large excess over cysteine residues in the MscL protein. The reaction proceeded fast, and no unmodified or partially modified protein could be detected by HPLC and ES-MS after 30 minutes at room temperature. The protein was then washed and eluted from the beads by imidazole in a detergent-free buffer. For the longer PPOs (three to five subunits), the modified protein was partially released from the Ni-beads during reaction with the maleimido-PPOs, leading to significantly lower reaction yields.

Five constructs of modified MscL_4Cys with one to five PPO subunits attached to each cysteine residue, named MscL_PPO1 through MscL_PPO5, could be synthesized using this approach, and in every case, the reaction was complete and a precisely controlled molecular structure was obtained. The identity and purity of MscL_PPO1 through MscL_PPO5 was assessed by ES-MS, SDS-PAGE and HPLC as demonstrated in Figure 2. The molecular mass (MW) of the modified MscL species was in good agreement with the theoretical MW (Table 1). In addition, the mass spectra of both MscL_4Cys and the modified MscL_4Cys species showed two mass adducts of +178 Da and +258 Da (Figure 2A–E). Digestion of the protein and LC-MS analysis identified these modifications as additions of a gluconyl- (+178 Da) and a 6-phosphogluconoyl group (+258 Da) at the N terminus. Spontaneous phosphogluconoylation of the His-tag has been reported for several proteins overexpressed in *Escherichia coli*.

HPLC analysis of MscL_PPO2 demonstrated the high purity of the reaction products. Due to the hydrophilic nature of the PPO-modified constructs, there was a decrease in retention time on a reversed-phase C4-column relative to the reactant (Figure 2G). All other constructs had comparable purity; however, the number of PPO subunits did not influence the retention time dramatically, indicating that modification with one PPO leads to the most significant increase in hydrophilicity. MscL_4Cys was also modified with iodoacetamide, iodoacetic acid, bromosuccinimide and iodosuccinimide. Ellman’s reagent (DTNB) was used to test for the presence of free unmodified cysteine residues, and showed that only ~25% of the cysteine residues were modified when the reaction was performed in solution. The reaction efficiency was increased when MscL_4Cys was modified on a Ni-column, resulting in ~50% of modified sites with iodoacetic acid. In the presence of detergent the reaction products showed the same retention time on a sizing column as wt Tb_MscL. However, removal of detergent from the modified proteins caused the

### Table 1. Properties of MscL with PPO modifications

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular mass of modification (g/mol)</th>
<th>Modification efficiency (%)</th>
<th>State without detergent</th>
<th>Oligomer:protein ratio</th>
<th>Molecular mass of modified protein (Da)</th>
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<tr>
<td>PPO1</td>
<td>472</td>
<td>100</td>
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<td>20,400</td>
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<tr>
<td>PPO2</td>
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<td>PPO3</td>
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<td>100</td>
<td>Partly soluble</td>
<td>0.23</td>
<td>22,819</td>
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<td>100</td>
<td>Aggregated</td>
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<td>PPO5</td>
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<td>100</td>
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protein to aggregate and elute in the void volume of the size-exclusion column, similar to the unmodified protein. The difference in efficiency of the PPO-modification reaction compared to the reactions using the halogen-substituted molecules might be explained by the amphiphilic nature of the PPO that allows it to penetrate more easily into detergent micelles around the immobilized MscL_4Cys.

Analytical characterization of PPO-modified proteins

The ability of the modified MscL proteins to retain α-helical secondary structure and pentameric quaternary structure was demonstrated by circular dichroism (CD) spectroscopy, size-exclusion chromatography (SEC) coupled with refractive index and multi-angle light-scattering (MALS) detection, dynamic light-scattering (DLS) and electron microscopy (EM). The Cys4 mutant of MscL, used as a reference, has a comparable structure relative to the wild-type protein as judged by its CD spectrum and melting behavior (Figure 3B and C).

Representative far-UV CD spectra for MscL_4Cys, MscL_PPO2 and MscL_PPO5 (in the presence and absence of detergent, respectively) are shown in Figure 3A. The typical CD-spectra of α-helical proteins with two absorption minima around 222 nm and 208 nm were observed in the presence of detergent for MscL_4Cys, as well as in the absence of detergent for MscL_PPO2 and MscL_PPO5. These spectra are consistent with the CD spectrum of wt Tb_MscL, indicating that the PPO-modified proteins retained the correct secondary structure (Figure 3C). MscL_PPO1, MscL_PPO3 and MscL_PPO4 exhibited comparable spectra. In fact, the spectra of all modified variants were indistinguishable from the CD spectra of MscL_4Cys or wt Tb_MscL in the presence of detergent. This finding indicates that

Figure 2. A–E, Electrospray-mass spectra of MscL_PPO1 through PPO5; F, SDS-PAGE of MscL_4Cys and MscL_PPO1 through MscL_PPO5; G, reverse-phase HPLC chromatograms of MscL_4Cys and MscL_PPO2 showing the decrease in retention time of MscL_PPO2 due to its more hydrophilic nature.
the introduction of the PPO, presumably pointing away from TM2, did not grossly disrupt the secondary structure of the protein. CD spectroscopy was further used to monitor thermal unfolding of wt Tb_MscL and MscL_4Cys in a detergent-containing buffer and of MscL_PPO2 in a detergent-free buffer, to assess the unfolding behavior and the relative stability of the two variants (see Figure 3B). A melting curve of very similar shape was obtained for both variants. The transition temperature observed for the first melting transition was lower for MscL_PPO2 (50°C) compared to MscL_4Cys (56°C). However, CD spectroscopy is not a good measure for the solubilization and aggregation state of the preparations (see below).

Size-exclusion chromatography in the presence and absence of detergent was performed to elucidate the oligomeric structure and aggregation state of the modified proteins. Figure 4A shows an overlay of the SEC-traces for MscL_4Cys and MscL_PPO2 in a detergent-containing buffer. The comparable elution times and peak-widths demonstrated that both proteins behaved similarly on this size-exclusion column. The apparent MW found for MscL_4Cys and MscL_PPO2 is around 200 kDa assuming a globular protein shape (as determined by calibration using molecular mass standards), which was about twice the expected MW of the channel pentamer. The difference between the apparent and calculated MW is most likely caused by the non-globular shape of the protein, the presence of detergent molecules, and the covalent modification with highly solvated polyethylene glycol-polyamide oligomers (in the case of MscL_PPO2).

Further purification of MscL_PPO2 resulted in a quite homogeneous population exhibiting a dominant single peak as shown in the complete size-exclusion chromatogram in Figure 4B and an enlarged section with the main peak in Figure 4C. Coupling of a multi-angle light-scattering (MALS) detector in combination with a refractive index (RI) detector to the SEC chromatography system allowed the measurement of absolute molecular mass for the protein portion, and for the protein–polymer complex. To determine the molecular mass of the protein part of MscL_PPO2, without including the PPO2 modification, the concentration was measured by using the absorbance at 280 nm (employing an extinction coefficient of 0.208 ml/(mg cm) for MscL_PPO2 determined by quantitative amino acid analysis and neglecting the very low absorbance of the attached PPOs at that wavelength). The measured molecular mass of

Figure 3. A, CD spectra of MscL variants at 20°C: MscL_4Cys in detergent-containing buffer; MscL_PPO2 and MscL_PPO5 in detergent-free buffer. B, Melting curves of wt Tb_MscL, MscL_4Cys (with detergent) and MscL_PPO2 (no detergent) monitored by circular dichroism at 223 nm and pH 7.5. C, CD-spectra of wtMscL and MscL_4Cys (in the presence of detergent) indicating the identical secondary structure of both proteins.
84(±10) kDa is in very good agreement with the 93 kDa calculated for non-aggregated, pentameric MscL (Figure 4C). The main source of error in the MW determination for the polymer–protein complex is the uncertainty of the dn/dc value of the protein–polymer complex, which is a critical parameter in this form of absolute molecular mass determination. Using a dn/dc value of 0.178 ml/g, an absolute molecular mass of 146 kDa (calculated MW for PPO-modified pentameric MscL is 108 kDa) was determined across the peak for MscL_PPO2 (Figure 4C). The dn/dc value of MscL_PPO2 was calculated, based on the known protein to polymer ratio, from the dn/dc value for proteins (0.185 ml/g) and the dn/dc value determined for PPO2 (0.132 ml/g) using the RI-detector. Although the resultant molecular mass is not fully consistent with a pentameric channel, it certainly suggests the absence of subunit monomers, dimerization, or higher-order aggregation.

A size-exclusion chromatogram for MscL_PPO5 is presented in Figure 4D. Similar to the wild-type protein in the absence of detergent, and all MscL variants modified with PPO1, PPO3, and PPO4 (data not shown), a low retention time (corresponding to a higher apparent MW) and a very broad peak were observed. This observation is most likely due to aggregation of the pentameric channel into higher oligomeric states in all channels other than MscL_PPO2. MALS analysis showed a mass distribution between 5.7 MDa and 1.5 MDa over the width of the first peak. Similar results were also found for all molecules modified with polar cysteine-reactive small molecules. These results demonstrated that MscL_PPO2 was a fully-solubilized integral membrane protein that formed a native-like pentameric assembly. By comparison, MscL_PPO1 and MscL_PPO3 through MscL_PPO5 or small-molecule modified MscL_4Cys displayed aggregation. We speculate that the shortest chains do not provide sufficient hydrophilicity to solubilize the protein, and that the longer chains induce micelle-type assemblies of the subunits as evidenced by the higher molecular mass of the resulting oligomers.

Dynamic light-scattering experiments were performed with MscL_PPO2 in order to obtain an orthogonal measure of the hydrodynamic size of

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**Figure 4.** Size-exclusion chromatography of different MscL variants on a Superdex 200 column. A, Overlay of elution profiles of MscL_4Cys and MscL_PPO2 in buffer containing detergent. B, MscL_PPO2 in detergent-free buffer. C, Magnification of MscL_PPO2 peak at 23.6 minutes from B. The circles indicate the molecular mass of the eluting species as measured by MALS. MW including PPO2 modification (red) and MW of protein only (black). D, MscL_PPO5 in detergent-free buffer. The circles indicate the molecular mass as determined by MALS across the first peak.
MscL_PPO2. The hydrodynamic radius of the majority of the sample population (86%) was 58 Å, which correlates to an apparent MW of 206 kDa assuming a spherical shape. This result was in close agreement with the apparent MW found by SEC (~200 kDa). However, 14% of the sample’s population showed a much higher MW (R_h: 280 Å; > 8000 kDa) that was possibly due to aggregation of MscL_PPO2 during sample storage between size-exclusion and light-scattering experiments (data not shown).

Electron microscopy of MscL_PPO2

Electron microscopy of negatively stained MscL_PPO2 showed a homogeneous population of particles (Figure 5a) with a size that was consistent with the expected dimensions of wt Tb-MscL. Individual channels (Figure 5b, left) could be clearly discerned from these images and subsequently averaged. Visual inspection of an average of 29 individual images showed typical EM features of a pentameric channel (Figure 5b, center), which suggested 5-fold symmetry (Figure 5b, right). Subsequently, a rotational correlation analysis of top views was performed for 4-fold, 5-fold, 6-fold and 7-fold symmetry (Figure 5c). The results were most consistent with a particle having 5-fold symmetry, as expected for a pentamer. In summary, the EM images clearly support the notion that MscL_PPO2 is a soluble membrane protein with defined, pentameric symmetry that is amenable to structural analysis.

Discussion

The goal of our study was to establish whether a membrane protein could be solubilized by covalent attachment of amphiphiles to its lipid-exposed surfaces. Using Tb_MscL as a test case, and PPO1 through PPO5 as amphiphiles, we demonstrated that this approach does indeed lead to well-defined, homogeneous and monodisperse preparations of a
soluble membrane protein with preservation of native secondary and quaternary structure, even though ultimately a high-resolution structure determination would be the best tool to confirm the native structure. Furthermore, it is intrinsically challenging to demonstrate correct function of the solubilized protein due to the inability to perform conductance measurements on a randomly oriented and diffusing protein in solution.

Cysteine scanning experiments on Tb_MscL (R.B.B., unpublished results) and other proteins (e.g. lactose permease\textsuperscript{23,24} and sensory rhodopsin II\textsuperscript{25}) provided evidence that cysteine substitutions are well tolerated at most positions in the sequence of integral membrane proteins without a significant reduction in expression levels. In most cases, such modifications do not prevent membrane insertion and folding. For many protein families of interest, the surface-exposed cysteine residues may be known by sequence alignment and homology modeling, mutagenesis work, and to some degree by hydropathy plots. Several important classes of membrane proteins, such as GPCRs, ion channels, porins, and transporters from some families, have prototypical structures solved that allow for “educated guesses” on the placement of cysteine residues. Free cysteine residues that are buried away from the protein surface will likely not be modified by the bulky solubilizing group, and therefore may not pose a problem. Existing surface cysteine residues are desirable for modification, since no mutation is needed. Therefore, we expect that cysteine incorporation for amphiphile attachment may be applicable to many membrane proteins of interest.

After expression with a His-tag or equivalent affinity tag, the desired protein can be modified on the solid phase with multiple short precision polyamides as described here. The complete labeling of the membrane protein that was achieved with all PPO variants is in contrast to the incomplete labeling observed for small, polar molecules. This avoids complications arising from the separation of labeled and unlabeled species, and is most likely due to the ability of the PPO to incorporate into the detergent micelle, and thus to induce a high local reactant concentration. The resulting constructs can then be assayed for aggregation behavior using techniques such as size-exclusion chromatography, light-scattering or analytical centrifugation. Experiments along these lines are currently underway to investigate the generality of the approach.

The method described here has the advantage over other approaches like mutagenesis of hydrophobic residues that the starting point for solubilization is a folded membrane protein, and no further folding or refolding of the protein is necessary after solubilization. Compared to single amino acid substitutions the PPOs used for the labeling reaction are relatively large (PPO2, ~774 Da, Table 1) and influence the overall MW of the protein considerably. However, this type of modification is small when compared to the size of detergent micelles typically used for membrane protein solubilization. A dodecyl-\(\beta\)-d-maltopyranoside (DDM) micelle, for example, is of the order of 50 kDa,\textsuperscript{26} while all 20 PPO2 units present on the surface of pentameric MscL_PPO2 comprise only ~15.5 kDa. This accounts for only 17% of the total mass of the protein–polymer construct (Table 1). The fixed stoichiometry of the PPO–protein complex is in stark contrast to the variable detergent micelle–protein complex. Due to the oligomeric structure of ion channels such as MscL, a total of 20 PPO attachment sites were created by mutating four residues per channel subunit. Whereas we have not attempted to solubilize MscL with fewer PPO2 attachment sites, it will be of interest to investigate what the minimum modification number will be to achieve solubility in aqueous buffer.

In summary, the approach we have developed allows for manipulation of the position of the cysteine mutations in the sequence, the ability to change the number of modification sites, and the ability to tune physical properties by the choice of the modifying reagent. We expect that the versatility of these features could be customized for solubilization of other membrane proteins, provided that they can be generated in sufficient amounts. It should be particularly useful for oligomeric membrane proteins, where several exposed cysteine residues are generated by each mutation. Although still to be realized experimentally, the solubilization of membrane proteins as homogeneous, monodisperse oligomers should help ameliorate the limitations of NMR spectroscopy and crystallization of membrane proteins caused by the presence of a large and variable number of detergent molecules in the solubilizing micelle that result in disorder and heterogeneity. This methodology might thus help to accelerate the pace of membrane protein structure determination, and allow the study of membrane protein function in solution in the absence of lipids and detergents.

**Methods**

**Mutagenesis and protein expression**

The largest four hydrophobic side-chains (residues L73, F80, F84, and F88) facing the lipid bilayer were chosen for the initial modification study, resulting in the fourfold cysteine mutant MscL_4Cys protein (Figure 1). MscL_4Cys protein was constructed by several rounds of inverse PCR,\textsuperscript{27} using a *M. tuberculosis* MscL gene in plasmid pet19b (Novagen). All mutants were transformed into BL21-DE3 (Invitrogen) host and verified by sequencing. Four cysteine residues per monomer gave rise to 20 cysteine residues available for modification on the protein surface of the pentamer (Figure 1C).

**MscL_4Cys purification**

Purifications were performed as described.\textsuperscript{13}
Tryptic digest

To facilitate further characterization of MscL_4Cys the protein was reduced (1 mM Tris(2-carboxyethyl)-phosphine (TCEP), 90°C, 15 minutes), alkylated (50 mM iodoacetamide at room temperature for one hour) and digested (TPCK-treated porcine trypsin, Promega). The tryp tic peptides were subjected to LC-MS/MS analysis on a QSTAR Pulsar mass spectrometer (MDS Sciex) operating in positive ion mode. The tryp tic peptides were separated using nano-liquid chromatography (Ultimate, LC Packings), at a flow rate of 300 nL/minute. Separation of peptides was achieved by using a gradient of 2% (v/v) buffer 1 (acetoni trile, +0.1% (v/v) formic acid) to 40% (v/v) buffer 1 in buffer 2 (water, +0.1% formic acid) over 30 minutes on a 75 μm ID, C18 Pepmap column. MS acquisitions were recorded for one second followed by four seconds collision-induced dissociation (CID) acquisi tions on ions selected by preset selection parameters of the information-dependent acquisition (IDA) method. Collision energies were adjusted according to the charge state and mass of the precursor ions. All data were processed using Protein Prospector version 4.3 (UCSF) and Mascot (Matrix Sciences) software.

PPO synthesis

Polyethyleneglycol-polyamide oligomers (PPOs) were synthesized on SASRIN™ resin (Bachem) starting with the coupling of succinic anhydride (10 eq, Fluka) in the presence of N,N-dimethyl-4-aminopyridine (10 eq) for 30 minutes. The resin was washed with dimethylfor mylam ide (DMF) and treated with a 0.5 M solution of 1,1-carbonyldiimidazole in DMF for 30 minutes and then washed with DMF. (4,7,10)-Trioxa-1,13-tridecanediamine (TDD) was activated with a 0.5 M 1-hydroxybenzotriazole (HOBT) solution in DMF and added to the resin for 30 minutes. After washing the resin with DMF, another succinic anhydride unit was added to the resin activated with 0.5 M TDBT solution and diisopropylcarbodiimide and 2-hydroxyethylamin e (DEIA) for 30 minutes. Further extension of the oligomer chain was achieved as described. For attachment of the chemoselective linker, 3 eq of N-β-maleimidopropionic acid (BMAP) were used to modify the free amino group of the oligomer. BMAP was preactivated for 30 minutes with equal amounts of N-hydroxysuccinimide (NHS) and disopropylcarbodiimide and coupled to the resin for 30 minutes to produce maleimido-PPO. The PPO-resin was washed with DMF, transferred into dichloromethane (DCM) and dried in vacuo for cleavage with 3% (v/v) trifluoroacetic acid (TFA) in DCM (2×20 minutes). After removal of the resin beads, the DCM/TFA-mixture was evaporated and the remaining oil was dissolved in 5% (v/v) acetonitrile in water containing 0.1% TFA for HPLC purification on an RP-C4-column with a gradient from 25% (v/v) buffer 6 (IPA:ACN:TFE:H2O, 12 : 6 : 5 : 2 by vol., +0.1% TFA) to 100% buffer 6 in buffer 4. The intact protein mass was measured by ES-MS on an API III triple-quadrupole mass spectrometer (PE Sciex) operating in positive ion mode. The molecular mass was deconvoluted from the envelope of multiply charged ions.

Circular dichroism spectroscopy

CD spectra were collected on a JASCO J-600 Spectro polemter equipped with a thermoelectric unit using a 1 mm path-length cell. Thermal melts were monitored at 223 nm. Data were collected every 1 deg. C with an equilibrium time of two minutes and an averaging time of 30 seconds. Wavelength scans were performed at 20°C collecting the data every 1 nm (200–260 nm) with an averaging time of one second, and three scans were averaged for each measurement.

Size-exclusion chromatography with on-line light scattering, absorbance and refractive index detectors

SEC was performed on an Äkta-FPLC system (Pharmacia) equipped with a zinc lamp for detection at 214 nm.
using a Superdex200 10/30 column (Pharmacia). Buffer 5 containing only 5% glycerol was used as a running buffer for experiments where detergent was required and detergent-free buffer 5 (5% glycerol) for all other experiments. Samples were run at a flow rate of 0.4 ml/minute or 0.5 ml/minute, respectively. SEC experiments with on-line light-scattering and refractive index detection were performed on an Agilent 1100 system coupled to a miniDawn multi-angle light-scattering and an Optilab refractive index detector (both Wyatt Technology) using a Superdex 200 10/30 column (Pharmacia). Molecular mass was calculated with a dn/dc value of 0.185 for proteins and 0.132 for PPO2 using the Astra software package (Wyatt Technology).

Dynamic light scattering

Dynamic light-scattering data were collected with a DynaPro MS/X instrument using a 12 µl-quartz scattering cell at Alliance Protein Laboratories. The instrument was calibrated with latex sphere size standards (81 (± 2.7) nm diameter, Duke Scientific Corp.). Thirty ten second data accumulations were recorded and averaged. Data analysis was performed with Dynamics version 5.26.56 and 6.3.01. The class diameter, Duke Scientific Corp.). Thirty ten second data accumulations were recorded and averaged. Data analysis was performed with Dynamics version 5.26.56 and 6.3.01. The collected data were corrected for the higher viscosity due to glycerol in the buffer used.29 Samples were purified by SEC as described above prior to analysis.

Electron microscopy and image analysis

Aliquots (~4 µl) at ~0.7 mg/ml were allowed to adhere for about one minute to carbon-coated, 400-mesh copper grids that had been rendered hydrophilic by glow discharge. The grids were washed for one minute with three successive drops of 25 mM Tris–HCl (pH 8.0), 200 mM NaCl, 10% glycerol and then exposed to three successive drops of 2% (w/v) uranyl acetate for one minute (Ted Pella, Tustin, CA). Images at 60,000× magnification were recorded on Kodak SO163 film under low electron dose conditions using a CM120 electron microscope (Philips Electron Optics/FEI, Eindhoven, The Netherlands) operating at 100 kV. Micrographs that showed uniform staining and minimal astigmatism and drift were digitized using a SCAI scanner (Carl Zeiss, Inc.) at 7 µm per pixel. The pixels were binned to 14 µm per pixel, which corresponded to 2.3 Å at the level of the specimen. Particles were masked, centered and classified using EMAN software.30 The class having the appearance of top views was analyzed for rotational symmetry using Spider software.31

Crystallization trials

We have conducted a series of crystallization trials on MscL_PPO2, but to date these have not identified any promising leads (J. Choe & D.C.R., unpublished results). Efforts in this direction are on-going; it is likely that the crystallizability of these solubilized preparations will be sensitive to the size and chemical nature of the modifying groups that will require further experimentation to address.

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