**In vitro Unfolding and Refolding of the Small Multidrug Transporter EmrE**

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The composition of the lipid bilayer is increasingly being recognised as important for the regulation of integral membrane protein folding and function, both in vivo and in vitro. The folding of only a few membrane proteins, however, has been characterised in different lipid environments. We have refolded the small multidrug transporter EmrE in vitro from a denatured state to a functional protein and monitored the influence of lipids on the folding process. EmrE is part of a multidrug resistance protein family that is highly conserved amongst bacteria and is responsible for bacterial resistance to toxic substances. We find that the secondary structure of EmrE is very stable and only small amounts are denatured even in the presence of unusually high denaturant concentrations involving a combination of 10 M urea and 5% SDS. Substrate binding by EmrE is recovered after refolding this denatured protein into dodecylmaltoside detergent micelles or into lipid vesicles. The yield of refolded EmrE decreases with lipid bilayer compositional changes that increase the lateral chain pressure within the bilayer, whilst conversely, the apparent rate of folding seems to increase. These results add further weight to the hypothesis that an increased lateral chain pressure hinders protein insertion across the bilayer. Once the protein is inserted, however, the greater pressure on the transmembrane helices accelerates correct packing and final folding. This work augments the relatively small number of biophysical folding studies in vitro on helical membrane proteins.

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**Introduction**

Integral membrane proteins constitute approximately 30% of all proteins and yet, despite their abundance and importance, are underrepresented in biophysical and biochemical studies. Correct folding to a functional structure is vital not only in vivo but also in vitro for structural and functional studies. However, little is known about the factors driving folding of membrane proteins as only very few have proved amenable to folding investigations. Biophysical in vitro studies can lead to a wealth of information on folding, but rely on the ability to unfold and refold the protein. To date, this has been achieved for a handful of α-helical membrane proteins, including bacteriorhodopsin,¹,² the major light harvesting complex II of higher plants,³,⁴ and diacylglycerol kinase⁵,⁶ from Escherichia coli. Here, we optimise refolding of the small multidrug transporter from
The natural heterogeneous lipid bilayer environment of membrane proteins complicates folding studies. This environment consists of a central hydrophobic region of the lipid bilayer and a polar or charged headgroup region with aqueous solvent on either side of the bilayer. The protein thus interacts with all these differing solvents. Moreover, different lipid compositions consisting of various lipid headgroups, aliphatic chain lengths and saturation can have significant effects on proteins within the membrane. In vivo lipid compositions vary greatly between different organisms and subcellular organelles. In a native membrane, the lipid composition is reasonably well defined but is also regulated by the external environment. Such regulation is essential; for example, *E. coli*, which are incapable of synthesising certain lipids, have defects in membrane protein insertion and folding.

Structural and functional characterisation of membrane proteins requires isolation and purification of the protein of interest from their native membrane and will normally involve solubilisation of the membrane protein in a suitable detergent. Whilst purification in this way makes membrane proteins amenable to further biochemical characterisation, the change in environment can have drastic effects on protein folding, stability and activity. Membrane proteins can also be reconstituted from detergent into lipid vesicles of differing lipid compositions. Changes in the properties of several membrane proteins have been observed upon altering lipid bilayer composition. These can be either the result of general lipid bilayer properties or the specific effects of direct lipid–protein interactions. Mechano-sensitive channels such as the widely studied MscL have dissimilar opening properties in different bilayers. Lipid effects are not limited to this class of channel protein, whose natural function is to respond to changes in the bilayer. Lipid bilayer modulation of several enzymes involved in membrane lipid metabolism has also been observed (reviewed in Refs. 14,15). Furthermore, certain lipids, such as phosphatidylethanolamine (PE), are required for correct topogenesis of integral membrane proteins in *E. coli*; in the absence of these lipids, defects occur in the structures of lactose permease and γ-aminobutyric acid permease. Lipid bilayer effects are not necessarily limited to integral membrane proteins. For example, the activity of phospholipase C was shown to vary depending on lipid composition, and protein kinase C activity has been shown to be sensitive to small changes in lipid structure.

Physiological membranes consist of a number of lipids that impart different properties. This includes charge, in the case of the anionic lipids phosphatidylglycerol (PG) or cardiolipin in *E. coli* membranes, or zwitterionic phosphatidylcholine (PC) in eukaryotic bilayers. Changes in lipid composition also affect the lateral pressure profile of the membrane; there are outward pressures in the plane of the membrane in the chain and headgroup regions, with an equal and opposite inward pressure in the interfacial region. Although experimental studies quantifying lateral pressure within real membranes are limited, molecular dynamics simulations have shown a lipid composition dependence in the lateral pressure profile. Lipids such as PE form non-lamellar, hexagonal phases in water where the monolayers curve towards water. This contrasts with the flat monolayers of low curvature in a bilayer of lamellar lipids such as PC. Thus, incorporating PE lipids into a PC bilayer increases the monolayer curvature and the stored curvature elastic stress of the bilayer as the monolayers are forced flat in the bilayer structure. This also alters the lateral pressure profile and there is a relative increase in the outward chain pressure. These alterations in bilayer properties affect membrane protein folding. Increasing PE, and thus increasing stored curvature stress in the bilayer and lipid chain lateral pressure, results in decreased yields and rates of folding of bacteriorhodopsin. The stability of the potassium channel KcsA to trifluoroethanol denaturation is also affected by the lipid composition. Whilst global differences in the physicochemical properties of the bilayer have an effect on integral membrane protein properties, more specific interactions with lipids have also been observed. The *Rhodobacter sphaeroides* photosynthetic reaction centre contains a specifically associated molecule of cardiolipin, and the KcsA channel has a requirement for negatively charged PG headgroups for function, with PG also being specifically bound in the crystal structure. Different membrane mimetics and their properties can thus influence membrane proteins in various ways, and the properties of a membrane protein in one lipid composition or detergent may differ significantly from those in another.

Here, we investigate the folding of the small multidrug resistance protein EmrE. EmrE monomers have four transmembrane helices giving approximately 75% α-helical content overall. EmrE is capable of recognising and actively exporting a broad range of hydrophobic cationic substrates from the cell in a process that involves exchange with two protons. The protein has been well characterized, and when in the detergent n-dodecyl-β-d-maltoside (DDM), EmrE is stable for long periods of time and fully functional as a dimer.

In line with our previous work on bacteriorhodopsin and diacylglycerol kinase, we have systematically altered bilayer composition to address changes imbued upon the protein during folding. We use our previously developed lipid-based system to investigate the effects of two different lipid mixtures: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)/1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and DOPE/1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG). In the former, increasing DOPE increases the spontaneous monolayer curvature and lipid chain lateral pressure, which have been shown to hinder transmembrane insertion across bilayer vesicles during
Results

Unfolding in SDS and urea

CD and intrinsic protein fluorescence were used to monitor changes in EmrE structure in the presence of denaturants SDS and urea. The main criteria assessed for unfolding were a reduction in secondary structure, as shown by far-UV CD, and a loss of substrate binding activity.

EmrE in DDM was diluted in buffer containing urea or SDS. No changes in secondary structure were observed in urea concentrations up to 10 M, nor were any changes seen in SDS concentrations up to 5%. A small reduction in secondary structure was only observed using high concentrations of both denaturants, in 10 M urea and 5% or 10% SDS. The decrease in secondary structure upon denaturation is difficult to quantify due to the absorbance of urea at low wavelengths. In 10 M urea/5% SDS, the CD signal intensity at 222 nm that is characteristic of α-helical structure decreased to 88% of the value of fully folded EmrE in DDM (i.e., about a 12% reduction in helicity). An ∼21% reduction in helix occurred in 10 M urea/10% SDS. Fig. 1 shows the far-UV CD spectrum of EmrE in DDM, together with those after denaturation in SDS and urea. Deconvolution of the CD spectra in DDM results in approximately 77% α-helical content, consistent with approximately 75% predicted α-helix calculated from NMR,36 ~72% from the current X-ray structure37 and 78–80% by FTIR.39

EmrE contains four tryptophans that dominate the protein fluorescence, with one (residue 31) localised to the periplasm, one (residue 45) on transmembrane helix 2 and two (residues 63 and 76) on transmembrane helix 3. The protein fluorescence band red-shifted (from 330 nm in DDM to 343 nm in urea/SDS; see Fig. 2a and b) and increased in intensity upon denaturation in 10 M urea/5% (w/v) SDS. Such changes are indicative of exposure of tryptophan residues to more hydrophilic environments and, thus, denaturation and unfolding. Much smaller changes in Trp fluorescence were observed in either urea or SDS alone with a maximum peak shift to 338 nm in the presence of only SDS or urea. The oligomeric state of 10 M urea/5% SDS denatured EmrE is unknown, but it is expected to be monomeric due to the observation of a monomer by Coomassie-stained SDS-PAGE, for which the SDS concentration is less denaturing than the conditions used here for unfolding and refolding (not shown).

Fig. 1. Far-UV CD spectra of unfolded and refolded EmrE. Reduction in 222-nm band of EmrE upon addition of 10 M urea with 5% (red line) and 10% (light blue line) (w/v) SDS. Broken line, recovery of native CD after refolding by dilution into DDM, from 10 M urea and 5% (w/v) SDS. Continuous black line, spectrum for functional EmrE in DDM that has not been denatured. Mean residue ellipticity values at 222 nm are −21,480, −18,908, −16,896 and −19,761 deg.cm².dmol⁻¹.residue⁻¹ for DDM, 10 M urea 5% SDS, 10 M urea 10% SDS and refolded, respectively. All spectra are averages of independent duplicate experiments. Data are not smoothed and were truncated at 205 nm due to high absorbance of urea. Protein and DDM concentrations were 37 μM and 0.08% (w/v), respectively.

Refolding from a urea/SDS-denatured state into detergent micelles

Experiments with buffer solutions containing 10 M urea/10% SDS proved impractical for refolding experiments due to urea and SDS solubility and high concentrations of residual SDS after dilution to remove denaturants. Therefore, EmrE in 10 M urea/5% SDS, which has lost a small amount (~12%) of α-helical structure but shows significant differences in native Trp fluorescence indicative of denaturation, was used for refolding experiments. The partially denatured EmrE in 10 M urea/5% SDS was refolded into DDM by two methods: (i) dilution into buffer containing DDM and (ii) removal of the denaturants on a Ni²⁺ affinity resin and elution in buffer containing DDM. Dilution of EmrE into DDM-containing buffer resulted in regain of the far-UV CD
The Trp fluorescence spectrum was also more native-like following dilution, with an emission maximum at 334 nm, compared to 330 nm for functional EmrE in DDM (and 343 nm for the urea/SDS-denatured state; see Fig. 2a and b).\(^4\) The differences in Trp fluorescence are attributed to solution effects of the residual denaturants in the refolded sample. Native Trp fluorescence was also recovered after the Ni\(^{2+}\) affinity/buffer exchange refolding. Exchange of denaturants for DDM using the latter method results in almost complete exchange of buffer components, whereas dilution of denatured EmrE in DDM results in residual urea and SDS of up to 0.5 M and 0.25% (w/v), respectively.

**Recovery of substrate binding activity upon refolding from urea/SDS**

Successful refolding was also assessed from the recovery of EmrE binding activity. The binding of the substrate tetraphenylphosphonium (TPP\(^+\)) was measured by fluorescence quenching\(^4\) and ITC. TPP\(^+\) binding results in a quenching of the native Trp fluorescence band of EmrE. Owing to the high binding affinity (low dissociation constant, \(K_d\)) of TPP\(^+\) binding to EmrE, the percentage of functional protein can be calculated.

EmrE refolding by Ni\(^{2+}\)-NTA/buffer exchange into DDM was determined to be very efficient, with approximately 100% recovery of protein determined by Trp fluorescence quenching by TPP\(^+\) (see Fig. 2c and Table 1). Table 1 shows the \(K_d\) values obtained for refolded EmrE. The \(K_d\) determined for TPP\(^+\) binding to EmrE, after refolding by Ni\(^{2+}\)-NTA/buffer exchange, using a tight-binding model, was determined to be approximately 4 nM compared to approximately 2.5±3 nM for functional EmrE in DDM at pH 8.5 (determined by Trp fluorescence quenching for EmrE that had not been denatured; see Table 1, rows 1 and 6). Although it is not possible to determine such tight binding with accuracy at the protein concentrations required for this assay, the values obtained are in agreement with other methods. ITC was used to determine a more accurate \(K_d\) for TPP\(^+\) binding for functional EmrE in DDM, giving a value of 19±2 nM at pH 7.5 (see Fig. 3a and Table 1, row 1) and 4.4±1.5 nM at pH 8.5 (see Table 1, row 4). This is similar to previously published values of EmrE: TPP\(^+\) binding affinity of approximately 5 and 30 nM at pH 8.5 and pH 7.5, respectively.\(^50\)–\(^51\) A binding stoichiometry of about 0.5 (i.e., one substrate binds per dimer) was found.
Table 1. Comparison of TPP⁺ binding affinities for EmrE under different refolding conditions determined by Trp fluorescence quenching or ITC

<table>
<thead>
<tr>
<th>Sample conditions</th>
<th>$K_d$ (nM)</th>
<th>$%$ Yield (of functional EmrE)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functional EmrE that has not been denatured</td>
<td>2.5±3</td>
<td>N/D</td>
<td>Trp fluorescence</td>
</tr>
<tr>
<td>0.08% (w/v) DDM</td>
<td>2.5±3</td>
<td>N/D</td>
<td>Trp fluorescence</td>
</tr>
<tr>
<td>0.08% (w/v) DDM</td>
<td>2.5±3</td>
<td>N/D</td>
<td>Trp fluorescence</td>
</tr>
<tr>
<td>2% DDM</td>
<td>19±2</td>
<td>0.48</td>
<td>ITC</td>
</tr>
<tr>
<td>No 6-His tag</td>
<td>33±2</td>
<td>0.53</td>
<td>ITC</td>
</tr>
<tr>
<td>100% DOPC</td>
<td>4.4±1.5</td>
<td>0.59</td>
<td>ITC</td>
</tr>
<tr>
<td>Ni²⁺-NTA immobilised</td>
<td>4.5±5</td>
<td>0.48</td>
<td>Trp fluorescence</td>
</tr>
<tr>
<td>EmrE refolded from 10 M urea 5% (w/v) SDS</td>
<td>6±1</td>
<td>0.52</td>
<td>Trp fluorescence</td>
</tr>
<tr>
<td>EmrE refolded from 10 M urea</td>
<td>3±3</td>
<td>0.67</td>
<td>Trp fluorescence</td>
</tr>
<tr>
<td>EmrE refolded from 5% (w/v) SDS</td>
<td>1.00×10⁻³</td>
<td>N/D</td>
<td>ITC</td>
</tr>
<tr>
<td>EmrE dilution refolded into DDM</td>
<td>1.00×10⁻³</td>
<td>N/D</td>
<td>ITC</td>
</tr>
<tr>
<td>T19-bimane dilution refolded into DDM</td>
<td>120±300</td>
<td>N/D</td>
<td>Trp fluorescence</td>
</tr>
<tr>
<td>C95-bimane dilution refolded into DDM</td>
<td>380±540</td>
<td>N/D</td>
<td>Trp fluorescence</td>
</tr>
<tr>
<td>EmrE in DDM with residual urea and SDS</td>
<td>0.98±10⁻³</td>
<td>N/D</td>
<td>ITC</td>
</tr>
</tbody>
</table>

N/D, values could not be determined due to the binding model used. Errors are 1 SD calculated from at least three independent experiments unless indicated otherwise.

- $^a$ TPP binding performed at pH 8.5.
- $^b$ Errors calculated from standard error of fitting.
- $^c$ Amount of functional protein calculated from control EmrE in the absence of initial denaturation (i.e., row 1).

Refolding EmrE into liposomes of different lipid compositions

In order to determine the effects of lipid composition, EmrE was refolded from 10 M urea/5% SDS, by dilution, directly into lipid vesicles. Residual detergent and urea were removed by dilution in a non-denaturing buffer and proteolipid vesicles were recovered by centrifugation. Negligible concentrations of denaturants are present after refolding into lipid vesicles by this method.
TPP\textsuperscript{+} binding to refolded EmrE in lipid vesicles was measured by ITC (see Fig. 4). The percentage recovery of functional protein can be determined by ITC independently of binding affinity. The stoichiometry of the EmrE–substrate interaction is known to be 0.5 (i.e., one substrate molecule binds to an EmrE dimer), and thus recovery of functional protein can be calculated as follows:

\[
\text{Fraction recovery} = \frac{n_{\text{ITC}}}{n}
\]

where \(n_{\text{ITC}}\) is the experimentally determined stoichiometry from ITC, and \(n\) is the known stoichiometry (0.5) of the interaction. The total protein associated with the lipid vesicles was quantified by solubilising proteolipid vesicles in chloroform/methanol.

Initially, TPP\textsuperscript{+} binding was tested on functional EmrE in DOPC lipid vesicles (i.e., reconstituted from DDM with no denaturation involved). Fig. 4 shows the resulting ITC data. Reconstituted, functional EmrE in DOPC was found to have a TPP\textsuperscript{+} binding stoichiometry of 0.47 (see Table 1, row 5), consistent with a dimer (expected \(n = 0.5\)). Ethidium binding was found to be much weaker, with a \(K_d\) of 1.11 \(\pm\) 0.25 \(\mu\)M in DOPC, but also exhibited a binding stoichiometry of 0.48. Recovery of EmrE function after refolding was determined by ITC studies of TPP\textsuperscript{+} substrate binding. Fig. 4c and d shows the results for TPP\textsuperscript{+} binding to EmrE refolded from urea/SDS into DOPC lipid vesicles. The \(K_d\) was found to be 43 nM (similar to that in DDM) and the yield of refolded, functional protein was 66\% (see Table 2a, row 1, left-hand columns). TPP\textsuperscript{+} binding to EmrE was not observed by ITC binding experiments in lipid vesicles prior to removal of residual urea and SDS.

Control heats of dilution for TPP\textsuperscript{+} binding experiments were determined from the heats of dilution at saturation of EmrE (e.g., 50–75 min in Fig. 4a) and subtracted from the heats of reaction for titration of TPP\textsuperscript{+} into vesicles containing protein. This avoids any variations in buffer composition between different samples. Control experiments were also performed with TPP\textsuperscript{+} and lipid vesicles containing no protein. An example of the latter at high TPP\textsuperscript{+} concentrations is shown in the inset to Fig. 4a, showing there is little variation in the heat of dilution over time. The actual magnitude of the heats of dilution depended not only on the TPP\textsuperscript{+} concentration (which varied with lipid composition and concentration of reconstituted or refolded EmrE, see below and Table 2) but also on buffer protonation effects. The binding of TPP\textsuperscript{+} to EmrE involves the compe-
Refolding EmrE

Fig. 4. TPP$^+$ binding to EmrE in DOPC measured by ITC. (a) Typical control isotherm for TPP$^+$ binding to EmrE reconstituted in DOPC that has not been denatured; (c) binding isotherm for EmrE refolded from 10 M urea/5% SDS into DOPC. The molar ratio of TPP/EmrE is shown on the x-axis corrected to 0.48 binding stoichiometry. (b and d) The corresponding integrated heats of binding for nondenatured and refolded EmrE, respectively (with the x-axis in d showing the raw data values, which in this case were used to determine the yield of refolded EmrE). Inset in (a) shows an example control binding isotherm for lipid vesicles in the absence of EmrE. The TPP$^+$ concentration in the control is higher than that used in the EmrE binding isotherms shown in (a), as higher TPP$^+$ concentrations were required under other lipid compositions due to variations in the amount of reconstituted or refolded EmrE (see Table 2). The heats of dilution are thus larger with higher TPP$^+$ concentrations.

EmrE was also refolded from a 10 M urea/5% (w/v) SDS state into lipid vesicles of either DOPC/DOPE or DOPG/DOPE, with varying mole fractions of DOPE, as well as DOPC/DOPG, with varying amounts of DOPG. Due to the non-bilayer forming properties of DOPE that result in phase separation at high mole fractions, a maximum of 0.7 mole fraction DOPE was used. As refolding efficiency was determined to be very low in certain lipid compositions, analysis of refolded EmrE was limited to analysis of functional protein by quantifying recovery of substrate binding capability.

The amount of functional EmrE recovered after refolding from a denatured state was observed to be dependent on the lipid composition: the 66% recovery of functional EmrE in DOPC lipid vesicles decreased significantly in the presence of PE to less than 1% in 0.5 mole fraction PE (Table 2a, left-hand columns). No TPP$^+$ binding was observed for EmrE in DOPC/DOPE lipid vesicles with 0.7 mole fraction PE. Similarly, upon refolding into DOPG lipid vesicles, 55% of functional EmrE was recovered. However, this decreased upon increasing the mole fraction of DOPE in DOPG/DOPE lipid vesicles,

tution of TPP$^+$ with protons for Glu14 in the binding pocket. Although the buffer conditions were identical for the ITC experiments (Tris–HCl, pH 7.5), there were small variations in buffer composition between refolded and reconstituted samples. The former refolded samples also contained residual denaturant buffer (acetate, pH 4.0) that is not completely removed in the refolding procedure. Heats of reaction using Tris–HCl buffer at pH 8.5 were also found to be smaller than those at pH 7.5, as the buffer becomes protonated by release of binding site protons at the latter pH but not at pH 8.5. Buffer effects probably contribute to the differences in the magnitudes of the heats of reaction between Fig. 4a and b compared to Fig. 4c and d. Additionally, the observed heats of each injection are larger in (c) than in (a), due the higher concentration of TPP$^+$ required in the former refolded samples, as the amount of EmrE is higher (66%; see Table 2) than in the latter reconstituted samples (39% EmrE). The determination of $K_d$ is not influenced by such changes in enthalpies, and thus we have used $K_d$ values to report on recovery of the binding activity of refolded EmrE.
with only 4% recovery in 0.7 mole fraction PE (see Table 2b, right-hand columns). Whilst the recovery of functional EmrE in DOPC or DOPG alone (with no DOPE) was very similar, lower yields were found for DOPC/DOPG mixtures (Table 2a, middle columns). The mole ratios of EmrE to lipid as well as lipid vesicles at different lipid compositions are given in Supplementary Table S2.

The affinity of the refolded EmrE for TPP⁺ varied with lipid composition. The $K_d$ for TPP⁺ in DOPC was 43 nM compared to 360 nM in DOPG, showing tighter binding of TPP⁺ in DOPC than in DOPG lipid vesicles. DOPE decreased the affinity for TPP⁺ in DOPC/DOPG lipid vesicles, as observed by an increase in $K_d$ (Table 2a). In contrast, DOPE increased the affinity for TPP⁺ in DOPG/DOPC lipid vesicles, with the $K_d$ decreasing from 360 nM in DOPG to 133 nM in DOPE/DOPG with 0.7 mole fraction DOPE. The actual values of binding enthalpies for TPP⁺ binding to refolded EmrE in different lipid compositions are given in Supplementary Table S1.

### Influence of lipid composition on reconstituted EmrE; substrate binding and secondary structure

In order to determine if the lipid effect on TPP⁺ binding affinity was a result of refolding EmrE from a denatured state, TPP⁺ binding was also measured for EmrE that had not been denatured. In this case, EmrE was reconstituted from a functional, DDM-solubilised state directly into lipid vesicles (see Table 2b). Similar trends in TPP⁺ binding were observed with lipid composition as for refolded EmrE. In a DOPC background, DOPE decreased the affinity for TPP⁺, with a $K_d$ of 19.0 nM observed in DOPC lipid vesicles, decreasing to 121 nM in DOPE/DOPC lipid vesicles with 0.7 mole fraction DOPE. Conversely, the $K_d$ decreased significantly upon addition of DOPE in a DOPG background; with the $K_d$ decreasing from 370 nM in DOPG to 67 nM with 0.3 mole fraction DOPE present (Table 2b). The functional yield of EmrE when reconstituted from either a functional DDM-solubilised state or a nonfunctional urea/SDS-denatured state shows a similar trend to previous observations of EmrE reconstitution. The enthalpies of TPP⁺ binding are given in Supplementary Table S1.

The secondary structure of EmrE in different lipid environments was monitored by ATR-FTIR, which can also determine the average alignment of transmembrane helices with respect to the plane of the bilayer. In order to maximise the amount of protein present in the lipid vesicles, functional EmrE in DDM was reconstituted into lipid vesicles, as yields of functional protein following refolding were low in certain lipid compositions. ATR-FTIR showed that the average helical tilt of EmrE with respect to the bilayer normal depended on lipid headgroup (see Fig. 5). In order to obtain aligned bilayers for these FTIR measurements, lipids with 1-palmitoyl-2-oleoyl (C18:1 and C16:0 chains) were used, rather than dioleoyl (two C18:1 chains). The average helical tilt angle was greater ($\sim 50^\circ$) in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) compared to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) ($\sim 38^\circ$). The tilt angle increased upon introducing PE into the PG background, but there was little change in PE/PC mixtures. We have also shown by CD spectroscopy that there is no discernable difference in the helical content of either refolded or reconstituted EmrE in any of the liposome compositions studied.
The kinetics of refolding EmrE from a denatured state in 10 M urea/5% SDS into lipid vesicles were measured by time-resolved fluorescence of probes attached at specific sites in the protein and native Trp fluorescence. Monochlorobimane (bimane) is a fluorescent probe that is sensitive to the local bilayer environment and is therefore ideal for monitoring insertion of transmembrane helices. Bimane is excited at 384 nm (i.e., independent of native Trp fluorescence that is excited at ∼290 nm) and has an emission band centred at ∼464 nm in hydrophilic environments, but this depends on the precise solvent conditions. Movement of the bimane label into a more hydrophobic environment is accompanied by a small (few nanometers) blue shift in bimane fluorescence emission to shorter wavelengths, as well as an increase in fluorescence intensity. We used the change in intensity to follow folding into lipids (as in our previous study demonstrating the use of bimane labels to follow insertion and folding into lipids49). Bimane was attached to individual Cys residues introduced near the midpoints of the four helices of EmrE (shown in Fig. 6a). Only single Cys mutants were used, with a single

![Fig. 6. Site-directed fluorescence labelling studies of EmrE folding. (a) Schematic diagram showing the location of monochlorobimane probes used in refolding experiments. (b) Time-resolved change in monochlorobimane fluorescence of labelled EmrE refolded from 10 M urea/5% SDS into DOPC liposomes. Data collected using stopped-flow mixing to dilute the denaturants. Upper black data curve is for singly labelled EmrE at position 41 on helix 2, with the residuals to a 2-exponential fit to the data shown below the fluorescence traces. The lower grey fluorescence data curve is a control trace, showing mixing of urea/SDS with DOPC liposomes, under the same conditions as for refolding by dilution, but in the absence of EmrE.](image)
The latter rate was the result of changes in the environment of the bimane label and which were due to mixing of 10 M urea/5% SDS buffer in the absence of EmrE or denatured EmrE without the presence of unlabelled EmrE, and thus represents an insertion/folding event. The insertion/folding rate measured by the bimane fluorescence was dependent on lipid composition and increased with increasing amounts of DOPE in DOPE/DOPC lipid vesicles. The increase in fluorescence was also observed (rate of the order of 0.050±0.006 to 0.113±0.028 s⁻¹, see Table 3) with an apparent rate constant of approximately 24 s⁻¹ (increasing DOPE, Fig. 6b). This rate is assigned to a functional insertion/folding event.

Table 3. Kinetic parameters for refolding bimane-labelled, urea/SDS-denatured EmrE into different lipid compositions

<table>
<thead>
<tr>
<th>Mole fraction DOPC</th>
<th>Mole fraction DOPE</th>
<th>Mole fraction DOPG</th>
<th>T19C Rate (s⁻¹)</th>
<th>Amplitude (V)</th>
<th>C41C Rate (s⁻¹)</th>
<th>Amplitude (V)</th>
<th>C95C Rate (s⁻¹)</th>
<th>Amplitude (V)</th>
<th>Trp fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC/DOPG</td>
<td>1.0</td>
<td>0.0</td>
<td>—</td>
<td>0.050±0.006</td>
<td>-0.028±0.001</td>
<td>0.049±0.008</td>
<td>-0.018±0.001</td>
<td>0.092±0.004</td>
<td>-0.013±0.001</td>
</tr>
<tr>
<td>(increasing DOPE)</td>
<td>0.7</td>
<td>0.3</td>
<td>—</td>
<td>0.073±0.005</td>
<td>-0.024±0.001</td>
<td>0.086±0.004</td>
<td>-0.013±0.001</td>
<td>0.076±0.001</td>
<td>-0.010±0.001</td>
</tr>
<tr>
<td>DOPC/DOPG</td>
<td>0.5</td>
<td>0.5</td>
<td>—</td>
<td>0.113±0.026</td>
<td>-0.010±0.002</td>
<td>0.131±0.02</td>
<td>-0.003±0.001</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>(increasing DOPG)</td>
<td>0.5</td>
<td>0.5</td>
<td>—</td>
<td>0.113±0.026</td>
<td>-0.010±0.002</td>
<td>0.131±0.02</td>
<td>-0.003±0.001</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>DOPG/DOPE</td>
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<td>—</td>
<td>0.7</td>
<td>0.073±0.005</td>
<td>-0.024±0.001</td>
<td>0.086±0.004</td>
<td>-0.013±0.001</td>
<td>0.076±0.001</td>
<td>-0.010±0.001</td>
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<tr>
<td>(increasing DOPE)</td>
<td>0.3</td>
<td>—</td>
<td>0.7</td>
<td>0.073±0.005</td>
<td>-0.024±0.001</td>
<td>0.086±0.004</td>
<td>-0.013±0.001</td>
<td>0.076±0.001</td>
<td>-0.010±0.001</td>
</tr>
</tbody>
</table>

Rates and amplitudes are shown for all data that resulted in recovery of functional protein as determined by ITC binding experiments. Data are not shown where this could not be distinguished from control in the absence of protein. The labelling efficiencies of different bimane label positions were determined to be 79%, 38%, 15% and 38% for T19C, C41C, L73C and C95C, respectively. Errors are 1 SD from three experiments.
(0.024 s\(^{-1}\) in DOPC) was of similar order of magnitude to that obtained by bimane fluorescence and increased with increasing mole fraction PE in PC, whilst the amplitude of this kinetic phase decreased (see Table 3), again consistent with changes observed by bimane fluorescence. Trp fluorescence changes were also observed in PG lipids; however, these were indistinguishable from controls where the mole fraction of PE exceeded 0.3. Relatively small fluorescence amplitudes were also observed for DOPC and DOPG mixtures, with the rate of insertion/folding increasing slightly with increasing DOPG.

**Discussion**

The small multidrug transporter EmrE can be denatured and refolded *in vitro*. This extends our earlier work on EmrE\(^{48,58}\) and firmly embeds EmrE as one of the few helical membrane proteins that can be recovered from a denatured state. It has previously been shown that functional EmrE can be recovered from protein solubilised in a chloroform/methanol mixture\(^{59}\) and lower concentrations of the denaturants SDS and urea.\(^{60}\) Here we extend these findings to demonstrate the recovery of functional protein from a denatured state in high concentrations of urea and SDS. In contrast to the other helical membrane proteins that have been successfully refolded, EmrE is more resistant to denaturation, particularly with regard to losses of helical structure. In the case of bacteriorhodopsin, there is a large reduction of helical content, of about 30–40%, in SDS concentrations greater than the critical micelle concentration of 0.2%. In contrast, SDS alone does not induce changes in EmrE secondary structure, and a reduction in helical content is only observed when both SDS and urea are present in very high concentrations of 10 M urea and 5% SDS. Even then, the reduction in helicity is small. Nevertheless, this denatured state has altered intrinsic fluorescence properties consistent with changes in structure upon unfolding and cannot bind substrate. EmrE in high SDS and urea is also most likely to be a monomeric state. Thus, the “refolding” we report here refers to the refolding of the small amount of denatured helical structure together with recovery of tertiary structure and formation of the substrate binding pocket and EmrE dimer.

The resistance of EmrE to the denaturants SDS and urea means that the “unfolded” state used in this study is highly structured, possessing considerable helical structure but altered tertiary and quaternary structure, which abolishes ligand binding. Even in low concentrations of SDS used for SDS-PAGE (≈8 mM), EmrE is observed as a monomer (not shown); as the substrate binding site is localised at the interface of the two monomers, this site is lost upon denaturation. In this respect, the state can be likened to the “molten globule” state of water-soluble protein folding.\(^{61}\) A molten globule state is an intermediate in a folding reaction and exhibits several experimental characteristics, including native-like secondary structure and altered tertiary structure. Molten globules are also characterised by increased side-chain flexibility and exposed hydrophobic residues. The observation of native secondary structure but altered tertiary structure is useful for membrane proteins.\(^{62}\) However, other aspects of molten globules such as exposed hydrophobic groups are not pertinent to membrane proteins as hydrophobic groups have to be exposed by a native, folded membrane protein to the hydrophobic bilayer interior. Another difference to note is that the urea/SDS-denatured EmrE state forms the starting point for the refolding experiments here and is not an intermediate. Rather, it is the experimentally accessible denatured state used as a starting state in the folding reaction that is accessible for study. As we have previously noted for folding studies of bR from a partly denatured state in SDS,\(^{63}\) this provides a different reference point from that normally used in water-soluble folding studies, where the starting states are usually considerably more denatured. The precise nature of these membrane protein denatured states is unknown and is nontrivial to determine.

Efficient (100%) refolding of EmrE occurs from this urea/SDS-denatured state in DDM micelles. The same 100% yield is recovered regardless of whether EmrE is refolded by dilution of the denaturants into DDM or by exchange into DDM on Ni\(^{2+}\) resin. Secondary structure is recovered and the refolded protein binds its substrate TPP\(^+\) with native-like affinity when denaturants are completely removed; the \(K_d\) for TPP\(^+\) of refolded EmrE in DDM is \(4 \pm 5\) nM with a calculated stoichiometry of 0.48 (see Table 1), the same order of magnitude as that of 19 nM for EmrE purified into DDM (and never denatured).

EmrE can also be refolded from its urea/SDS state into lipid vesicles. A refolding yield of 66% is obtained in DOPC lipid vesicles, which is similar to that (70%) for refolding bacteriorhodopsin from SDS into DOPC\(^{1}\) and a little higher than that (53%) for DGK refolded from acidic urea into DOPC.\(^{64}\) The substrate binding affinity for the folded EmrE in DOPC, with a TPP\(^+\) \(K_d\) of 43 nM, is similar to that for EmrE reconstituted into DOPC from pure protein in DDM, which exhibits a \(K_d\) for TPP\(^+\) of 19 nM. However, this \(K_d\) is sensitive to the lipid environment, and the residual SDS will partition into the DOPC lipid vesicles of the refolded EmrE, thus affecting the liposome properties.

The refolding of EmrE into lipid vesicles is affected by lipid composition, in line with our previous studies on the influence of lipids on membrane protein refolding. Increasing the proportion of DOPE in DOPE/DOPC lipid vesicles and in DOPE/DOPG lipid vesicles decreases the yield of refolded EmrE. We have previously shown that the refolding yield of bR also decreases as the amount of PE increases in PE/PC lipid vesicles.\(^{65}\) We assigned this to an increase in the lateral chain pressure in the PE/PC bilayer as the proportion of PE lipid increases, which hinders insertion of a transmembrane protein into and across the bilayer-lipid vesicles. In addition, we
have previously noted that the activation energy for insertion of a transmembrane helix increases with the proportion of PE.\textsuperscript{14} There can also be specific effects of lipids on folding; for example, we have previously shown that PG lipids give a 3-fold enhancement of the folding yield of another \textit{E. coli} protein, DGK.\textsuperscript{5} However, we find no such improvement of EmrE folding, with yields in DOPG being similar to DOPC (55% compared to 66%). EmrE resides in the cytoplasmic membrane of \textit{E. coli}, which contains predominantly ~70–75 mol% PE lipids, with the rest being dominated by PG. Thus, although PG is a native \textit{E. coli} lipid, it does not play a specific role in the correct folding of this \textit{E. coli} protein, EmrE. The DOPE and DOPG lipids used here have two identical chains (C18 with one unsaturated bond), whilst the PE and PG lipids of the native membrane have differing chain lengths and unsaturation. Moreover, there are other lipids present \textit{in vivo}, such as cardiolipin, and the lipid composition may be asymmetric across the two membrane leaflets. Our experimental system allows us to use a maximum of 70% mole fraction DOPE before the non-bilayer properties of DOPE cause the synthetic DOPE/DOPG bilayers to phase separate and start to lose bilayer structure. This 70% DOPE, 30% DOPG is similar with regard to headgroup composition to the native \textit{E. coli} membrane, but differs in chain type and in the lack of other lipids such as cardiolipin. Our mixtures do, however, allow us to monitor lateral pressure effects due to PE on insertion \textit{in vitro} and folding of EmrE. Whilst increased PE content hinders insertion, this is a reflection of the \textit{in vitro} experiment involving folding from urea and SDS. In contrast, \textit{in vivo} EmrE will most likely insert co-translationally via the translocon apparatus.\textsuperscript{63} The increased lateral chain pressure caused by PE lipids has been found to decrease the rate of insertion of a transmembrane helix or protein across a PE/PC bilayer, as well as the yield of insertion. In contrast to EmrE, the rate measured by a monochlorobimane label attached at the centre of one of three transmembrane helices increases as the proportion of PE in the PE/PC bilayer increases (see Table 3). This suggests that this label is not reporting purely on transmembrane insertion. One folding event that we have previously suggested would be accelerated by the increased lateral chain pressure of PE is transmembrane helix packing.\textsuperscript{64} Thus, the fluorescence label is most likely monitoring an event dominated by this faster helix packing/reorientation. We suggest that the packing is coupled to insertion with the increase in mono-chlorobimane fluorescence, reflecting the more hydrophobic environment in the bilayer centre.

Lipids affect the functional activity of integral membrane proteins,\textsuperscript{11,15} and in an earlier study, we have shown that EmrE is susceptible to lateral pressure changes, with binding and transport of the substrate ethidium decreasing with increasing DOPE in DOPE/DOPC lipid vesicles.\textsuperscript{45} In order to assess whether PE has the same influence on refolded EmrE as on purified, native-like EmrE, we compared the refolded state to EmrE that had never been denatured. This latter protein had been reconstituted into lipid vesicles from a fully functional state in DDM. TPP\textsuperscript{+} binding affinity showed the same dependence on PE for both refolded and reconstituted EmrE; the affinity decreased as DOPE was incorporated into DOPE/DOPC lipid vesicles but increased as DOPE was incorporated into DOPG lipid vesicles (see Table 2). In both EmrE samples, the TPP\textsuperscript{+} affinity was greater in DOPC than in the anionic DOPG. In a DOPC background, the incorporation of DOPE causes an increase in chain lateral pressure, which therefore results in an apparent decrease in binding affinity. This could be due to a difference in conformational mobility of EmrE and the ability of the binding site to interact with substrate. Structural studies have shown a change in structure of EmrE upon substrate association.\textsuperscript{52}

Conversely, in a DOPG background, the opposite effect is observed with an increasing concentration of DOPE. TPP\textsuperscript{+} binding affinity increases as the mole fraction of DOPE is increased. This cannot be directly interpreted as a lateral pressure effect, since there is insufficient data on the lateral pressure behaviour of PE/PG mixtures in buffer. Moreover, although DOPG forms bilayers, buffers and ionic strength have a greater influence on its phase behaviour than for DOPC, reflecting the different charge and solvation properties of the PG headgroup. DOPG clearly influences TPP\textsuperscript{+} binding, significantly decreasing the binding affinity as compared to DOPC. This difference is likely to be due to the charge differences of the bilayer headgroups, with the anionic PG either interacting with charged residues on the surface of EmrE or influencing the negatively charged binding site of EmrE, where E14 within the membrane region has been found to be essential for function.\textsuperscript{57} However, no background, nonspecific binding was observed for TPP\textsuperscript{+} to lipid vesicles of any lipid composition. ATR-FTIR implies that PG headgroups also cause a change in the average orientation of EmrE within the bilayer (see Fig. 5).

Here we have focussed on refolding EmrE from a denatured state in high urea and SDS concentrations, and with a slight reduction in secondary structure. This extends earlier reports that hinted that refolding may be possible, but used less denatured states that would have had near-native secondary structure.\textsuperscript{48} The secondary structure of EmrE is particularly resistant to denaturation by chemical denaturants; however, this is not unique as stability to SDS and urea has been previously observed for the potassium channel KcsA.\textsuperscript{68} This high helical stability in EmrE implies that structural changes associated with drug efflux are likely to be whole-scale rigid-body motions and rearrangement of transmembrane helices, rather than subtle changes to individual secondary structure elements. ATR-FTIR data from this study identified changes in helical tilt in different lipids, which are consistent with some degree of flexibility in EmrE manifesting itself in the different lipid bilayers. Structural data on EmrE also imply a degree of conformational mobility. Cryo-electron microscopy and X-ray crystallographic data, collected for
EmrE in different conditions, are also consistent with different rearrangements of secondary structure elements in different environments.\textsuperscript{37,52,69}

Helical membrane protein folding has proved difficult to study with regard to establishing refolding conditions as well as with the lack of experimental methods to monitor the folding. The inherent stability of EmrE further complicates folding experiments due to the very high concentrations of denaturants required for unfolding. The particularly high concentrations of urea and SDS affect mixing rates in stopped-flow experiments, influence lipid vesicles and are harder to dilute and remove than lower denaturant concentrations, which in turn limits the sensitivity of folding experiments by reducing the final concentration of protein. We have shown that ITC is ideal for quantifying binding activity of a refolded protein within a lipid environment. ITC is not affected by liposome size and light scattering like optical techniques, and it can provide a large amount of information about a protein–substrate interaction. Substrate binding would also have no requirement for orientation, as even if binding sites are only accessible from one side of the bilayer, only accessible sites are measured. A particular advantage of ITC is that the concentration of functional protein can be determined if the stoichiometry of binding is known. In the case of EmrE, the binding stoichiometry has been previously determined\textsuperscript{40,52} as one molecule of substrate associating with a dimer of EmrE.

It has proved challenging to unfold and refold membrane proteins \textit{in vitro}, which means that there is a much poorer knowledge of the factors that drive their folding as compared to water-soluble proteins. The inability to refold or maintain the correct fold of a membrane protein also has serious implications for structural and functional work that is reliant on stable, functional protein \textit{in vitro}. Hydrophobic, helical membrane proteins have turned out to be especially problematic to refold as they tend to aggregate and denature irreversibly. Here we have shown that a protein in this category, EmrE, can be successfully refolded with 100% efficiency in detergent micelles. Moreover, the protein can be refolded into lipid vesicles and the folding is affected by lipid bilayer properties, in line with our earlier hypotheses.\textsuperscript{1,2,66} This study provides further evidence that PE lipids alter folding in accordance with their influence on the lateral pressure profile of the bilayer. Thus, an increase in PE and the associated chain lateral pressure lowers the refolding yield of EmrE as also found previously for bacteriorhodopsin.\textsuperscript{1} The refolding work on EmrE is also a starting point for understanding the folding and assembly of multidrug transport proteins in general. These are proteins that are designed to recognise an array of substrates, which implies a degree of flexibility in their binding pocket and overall structure. Our results are consistent with such flexibility as they reveal very stable helical structure, but tertiary structure and, thus, helix orientation and packing arrangements are susceptible to alterations in lipid properties.

\section*{Materials and Methods}

All chemicals, unless otherwise specified, were of the highest grade and purchased from Sigma-Aldrich. DDM and octyl-β-D-glucoside (OG) were purchased from Anatrace, and DOPC, DOPG and DOPE were from Avanti Polar Lipids.

\subsection*{EmrE expression and purification}

EmrE-His was cloned into pT7-7, with overexpression and purification performed as previously described.\textsuperscript{70,71} Briefly, cultures were harvested and disrupted using a cell disrupter. Membranes were isolated by centrifugation, solubilised at 4 °C for at least 2 h in 25 ml of 40 mM Tris–HCl (pH 8.2), 100 mM NaCl, 10 mM 2-mercaptoethanol and 4% (w/v) DDM. Solubilised material was diluted 1:1 with water and insoluble material was removed by centrifugation at 35,000 g for 30 min at 4 °C. NaCl and imidazole were added to final concentrations of 350 and 15 mM, respectively, and DDM-solubilised protein was consequently incubated with 1.5 ml of Ni-NTA agarose (QIAGen) for 1.5 h at 4 °C. Nonspecifically bound protein was removed by washing with 20 mM Tris–HCl (pH 8.3), 400 mM NaCl, 15 mM imidazole, 0.1% DDM and 5 mM 2-mercaptoethanol. This wash was repeated with a lower concentration of NaCl (20 mM). EmrE was eluted with 20 mM Tris–HCl (pH 8.3), 25 mM NaCl, 200 mM imidazole, 0.1% DDM and 5 mM 2-mercaptoethanol. Eluted protein was concentrated in an Amicon Ultra 50,000 MWCO centrifugal concentrator (Millipore) and applied to a HiLoad Superdex 200 gel filtration column (Amersham) equilibrated with NH\textsubscript{4}DDM buffer [15 mM Tris–HCl (pH 7.5), 190 mM NH\textsubscript{4}Cl, 0.08% (w/v) DDM and 5 mM 2-mercaptoethanol]. Eluted EmrE was concentrated to approximately 1–2 mM as determined by A\textsubscript{280} of 1 mg ml\textsuperscript{-1} = 2.56.\textsuperscript{40} Snap frozen in liquid N\textsubscript{2} and stored at −80 °C until required. EmrE without a His tag, used to determine if dimer formation was influenced by the 6-His tag, was produced as described above with the exception of additional purification steps after gel filtration. Thrombin (100 U) and CaCl\textsubscript{2} (10 mM) were added to approximately 5 mg of EmrE, with a thrombin cleavage site inserted in the His tag linker region, and incubated overnight at room temperature. Protein was exchanged into 20 mM Na-acetate pH 5.5 and 0.08% DDM (w/v) and passed through a 1-ml HiTrap HP SP (Amersham) column. Digested protein was collected in the flow-through; concentrated to 1 ml and exchanged into 15 ml of 20 mM Na-phosphate buffer, 150 mM NaCl, 15 mM, 0.08% DDM (w/v); and applied to ~1 ml of Ni-NTA resin (QIAGen). After 1-h incubation, unbound protein was concentrated and purified by gel filtration as described above. EmrE without a His tag was used only to determine the stoichiometry of the functional EmrE oligomer in DDM micelles (described in Table 1). All other experiments utilised EmrE-His.

\subsection*{Mutagenesis and fluorescence labelling}

Single cysteine mutants of EmrE for labelling were created using the Quikchange mutagenesis kit (Stratagene). All mutants were identical with native EmrE-His with the
exception of mutation of three native cysteine residues to serine. EmrE mutants C41C, C95C (containing a single native cysteine, with all other cysteines mutated to serine), L73C and T9C were used for monochlorobimane labeling experiments. Monochlorobimane in methanol was added at a 20-fold molar excess over EmrE in 20 mM NaPi (pH 7.2), 200 mMimidazole, 2.5% (w/v) SDS and 5 M urea. Protein was incubated at room temperature overnight, and excess label was removed by buffer exchange of labelled EmrE into 20 mM Tris–HCl (pH 8.5), 150 mM NaCl and 0.5% (w/v) SDS. Labelled EmrE was then bound to a 1-ml HisTrap column (Amersham); washed with 20 mM Tris–HCl (pH 8.3), 10 M urea and 5% (w/v) SDS; and eluted with elution buffer [20 mM Na-acetate (pH 4.0), 10 M urea and 5% (w/v) SDS]. The concentration of eluted EmrE was determined from absorbance at 280 nm. Bimane concentration and labelling stoichiometry were determined by absorbance at 394 nm. The labelling efficiencies of different bimane label positions were determined to be 79%, 38%, 15% and 38% for T9C, C41C, L73C and C95C, respectively. Protein was stored at −80 °C prior to use in refolding experiments.

Reconstitution of EmrE in lipid vesicles from DDM

Lipid vesicles were prepared according to methods similar to those previously published.12 Lipid mixtures of DOPC, DOPE and DOPG at 50 mg ml⁻¹ were prepared in cyclohexane, evaporated to a thin film at room temperature and freeze-dried overnight. Dried lipids were rehydrated at 2.5% (w/v) SDS and 5% (v/v) cyclohexane, evaporated to a thin film at room temperature for 30 min. Lipid vesicles were diluted 90-fold of protein associated with Ni²⁺ resin by buffer exchange and removal of denaturants. For the latter Ni²⁺ refolding, purified protein was diluted to approximately 30 μM in 1 ml of DDM buffer [20 mM Tris–HCl, 150 mM NaCl and 0.08% (w/v) DDM] and incubated with 50 μl of Ni²⁺-NTA agarose (Qiagen) for 1.5 h at 4 °C. Ni²⁺-NTA agarose beads were recovered by centrifugation and buffer was exchanged for denaturing with 10 M urea, 5% (w/v) SDS wash buffer for denaturation. Alternatively, for control binding experiments, DDM buffer was unchanged. Ni²⁺ beads were recovered and refolding of EmrE was initiated by buffer exchange into 3 × 1-ml changes of DDM buffer. Refolded and control EmrE were eluted in DDM buffer containing 200 mMimidazole.

Steady-state and time-resolved fluorescence

Steady-state measurements of EmrE unfolding/refolding and substrate binding were performed in a Fluoromax-2 fluorimeter using for Trp measurements, a 3 mm path length cuvette and an excitation wavelength of 295 nm, with scanning emission between 315 and 400 nm typically using 1–5 nm excitation and emission slits and a step size of 1 nm.

Time-resolved refolding of EmrE was performed in an Applied Photophysics stopped-flow device using an excitation wavelength of 295 or 384 nm for tryptophan, and bimane, respectively. A bandwidth of between 2.3 and 4.7 nm was used for all experiments with long-pass emission filters, cutting off below 305 and 405 nm for Trp and bimane respectively. Asymmetric, stopped-flow mixing was used for all refolding experiments, with a 1:10 dilution of EmrE at 50 μM to lipid vesicles at 6.25 mg ml⁻¹, resulting in a final concentration of 5 μM EmrE and 5.6 mg ml⁻¹ lipid in buffer containing 15 mM Tris–HCl (pH 8.3), 190 mM NaCl, together with residual denaturant concentrations of 0.5% (w/v) SDS and 1 M urea. Either two or four experiments were examined for consistency, and the resulting data were averaged. Time-resolved fluorescence data were analyzed by fitting to a sum of exponential functions using Gnuplot software. Differences in the viscosity of the two solutions (i.e., denatured, high-urea solution and refolding DDM micelles) resulted in unreliable data approximately 50 to 100 s after initial mixing, resulting in significant limitations in the amount of data that could be interpreted as protein folding data. This was observed as a large change in the baseline, which we interpret as back-mixing, by diffusion into the stopped-flow cell after a certain time. These artefacts were also observed in protein-free controls but did not affect data prior to 50 s. The quality of the fits to the exponential functions was assessed by analysis of residuals.

CD spectroscopy

CD measurements were performed at the Daresbury Synchrotron Radiation Source. The synchrotron source has a higher intensity than a conventional CD spectrometer, thus increasing the signal to noise and spectral range attainable into the far UV for samples containing lipids and urea, both of which absorb in the far UV. For unfolding, EmrE at 370 μM in 20 mM NaPi buffer at pH 7.8 and 0.08% (w/v) (1.6 mM) DDM (critical micelle concent-
concentration ≈ 0.17 mM) was diluted to 37 μM in 20 mM NaPi buffer containing DDM to 0.08% (w/v), urea or urea/SDS. Samples were incubated at 30 °C for at least 30 min prior to measurement. Duplicate measurements using a 1-nm wavelength increment and slit width were collected for all samples. Equilibrium refolding was performed on the same samples by dilution of denatured EmrE to 0.3 μM in 20 mM NaPi and 0.08% (w/v) DDM followed by measurement in a 10-mm cuvette. All CD measurements were otherwise performed in a 0.1-mm cuvette (Helma) at 25 or 30 °C for lipid and refolding samples, respectively, with a resolution and increment of 1 nm. Duplicate samples were prepared and the resulting scans averaged.

Isothermal titration calorimetry

ITC experiments were performed on a VP-ITC calorimeter (MicroCal) using one initial injection of 7.5 μl of substrate followed by 17 injections of 15 μl. All samples were thoroughly degassed before use. Substrate concentrations of between 20 and 40 μM TPP⁺ and 100 to 200 μM ethidium were used depending on the lipid composition and the percentage of EmrE; it was necessary to use higher concentrations of substrate in experiments with a greater percentage of nonfunctional EmrE mutant, E14C. Control experiments using TPP⁺ and vesicles in the absence of EmrE (e.g., Fig. 4a, inset) utilised higher concentrations of TPP⁺ to ensure that the concentration range of control experiments encompassed the range of TPP⁺ used in EmrE binding experiments. Due to the inherent variability in sample preparation and the difference in empty vesicle properties compared to vesicles containing EmrE, subtraction of control experiments could result in a small offset from zero at substrate saturation, but this did not significantly affect fitting. The resulting K_d also agreed with that determined by subtracting the heats of TPP⁺ dilution at saturation of EmrE. After correction for TPP⁺ dilution, TPP⁺ binding data were fitted to single-site binding using Origin 5.0 (MicroCal).

ITC binding assays provide a quantitative measure of substrate binding and have the advantage of not only providing the affinity of an interaction but also the quantity of functional protein. As substrate binding is a prerequisite for transport, it can be used as a quantitative measure of function. Although binding may not necessarily correlate with transport activity, lipid-dependent differences in TPP⁺ binding agree well with the lipid dependence of methyl viologen transport despite differences in the reconstitution protocol required for ITC binding and pH-driven transport assays.

Table 4. Summary of concentrations of EmrE, DDM and lipid used in reconstitution and refolding experiments

<table>
<thead>
<tr>
<th>Refolding method</th>
<th>Measurement method</th>
<th>EmrE⁺ (μM)</th>
<th>DDM (% w/v)</th>
<th>Lipid⁺ (mg ml⁻¹)</th>
<th>Residual urea/SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refolding into DDM micelles</td>
<td>Dilution CD</td>
<td>0.3</td>
<td>0.08</td>
<td>—</td>
<td>80 mM urea/0.04% SDS</td>
</tr>
<tr>
<td></td>
<td>Dilution Trp fluorescence</td>
<td>5</td>
<td>0.08</td>
<td>—</td>
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</tr>
<tr>
<td></td>
<td>Dilution ITC TPP binding</td>
<td>8</td>
<td>0.2</td>
<td>—</td>
<td>0.5 M urea/0.25% SDS</td>
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<tr>
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<td>—</td>
<td>Trp fluorescence</td>
<td>30</td>
<td>0.08</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>ITC TPP binding</td>
<td>0.5, 5, 50</td>
<td>0.08</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>ITC TPP binding</td>
<td>10</td>
<td>0.08</td>
<td>—</td>
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<tr>
<td></td>
<td>—</td>
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<td>40</td>
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<td>—</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
<td>40</td>
<td>0.08</td>
<td>—</td>
</tr>
<tr>
<td>Refolding into lipids (stopped flow)</td>
<td>Dilution Fluorescence</td>
<td>5</td>
<td>—</td>
<td>5.63</td>
<td>1 M urea/0.5% SDS</td>
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<td>—</td>
<td>ITC TPP binding</td>
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<td>6.25</td>
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<td>ITC Ethidium binding</td>
<td>50</td>
<td>—</td>
<td>12.5</td>
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</tbody>
</table>

* Protein and lipid concentrations are those initially used in refolding or reconstitution experiments and may differ from those concentrations after reconstitution in the samples used for measurements, due to dilution or less than 100% recovery of proteolipid sample by centrifugation. Calculated final concentrations are described in Materials and Methods.

Concentrations are assumed to be zero due to buffer exchange; however, residual concentrations of denaturants may still be present.
Supplementary Data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.jmb.2009.08.039

References


Refolding EmrE


