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EmrE dimerization depends on membrane environment

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ABSTRACT

The small multi-drug resistant (SMR) transporter EmrE functions as a homodimer. Although the small size of EmrE would seem to make it an ideal model system, it can also make it challenging to work with. As a result, a great deal of controversy has surrounded even such basic questions as the oligomeric state. Here we show that the purified protein is a homodimer in isotropic bicelles with a monomer–dimer equilibrium constant (K_{MD}^{2D}) of 0.002–0.009 mol% for both the substrate-free and substrate-bound states. Thus, the dimer is stabilized in bicelles relative to detergent micelles where the K_{MD}^{2D} is only 0.8–0.95 mol% (Butler et al. 2004). In dilauroylphosphatidylcholine (DLPC) liposomes K_{MD}^{2D} is 0.0005–0.0008 mol% based on Förster resonance energy transfer (FRET) measurements, slightly tighter than bicelles. These results emphasize the importance of the lipid membrane in influencing dimer affinity.

1. Introduction

Small multi-drug resistant transporters (SMR) are bacterial transporters with broad substrate specificity. They are located in the inner membrane and use the proton motive force to export lipophilic cations from the cytoplasm to the periplasm, thus conferring resistance to these compounds. As the smallest active transporters, SMR family members provide a unique system to study the mechanism of proton-coupled antiport [1–3]. Detailed biophysical and biochemical studies require using purified protein reconstituted into a variety of membrane or membrane-mimetic environments. Since structural and biochemical data has indicated that the minimal functional unit of EmrE is a homodimer [4–12], it is first necessary to understand the effect of environment in dimer stability.

EmrE dimerization has been previously studied in dodecylmaltoside (DDM) detergent [5]. Although EmrE binds substrate tightly in DDM, spherically shaped micelles may induce strain in protein quaternary structure, thus affecting the overall stability of an integral membrane protein oligomer. Bicelles provide an intermediate environment between micelles and liposomes where the protein is surrounded by lipids but in a solubilized form [13–18]. We have previously demonstrated, using ITC and NMR, that EmrE also binds substrate tightly when reconstituted into isotropic bicelles [17], with binding affinities that match those previously published for liposomes [19]. These studies confirmed that EmrE is properly folded into a functional form in bicelles and is able to interconvert between the inward- and outward-facing states necessary for substrate transport. However, the stability of the EmrE dimer itself has only been measured in detergent, and has not been quantitatively investigated in bicelles or lipid bilayers.

Like many other membrane proteins, EmrE function is affected by its environment. Even in the most native-like environment, lipid bilayers, the substrate binding affinity and transport rate of EmrE vary with lipid composition [20,21]. Recently, the oligomeric state of the close homolog TBsmr has been investigated using freeze fracture electron microscopy in different lipid environments and the oligomeric state correlated with differences in TBsmr activity [22]. Here we measure the dimerization affinity of EmrE in bicelles and liposomes to better understand the effect of environment on the functionally important dimerization of this unique and well-studied integral membrane protein.

It is common to assess the equilibrium binding of proteins by measuring the dissociation constant in aqueous solution as defined by three-dimensional units of concentration. However, integral membrane proteins are confined to the 2D lipid bilayer or membrane mimetic. Therefore, the dissociation constant depends on the protein:lipid or protein:detergent ratio and is best reported as mole percent (mol %) [23–28]. This K_{MD}^{2D} (mol %) is more useful for calculating monomer/dimer ratios of EmrE in solutions with different lipid concentrations, since only the lipid or detergent portion of the solution, which is accessible to the protein, is considered in the calculation. Using K_{MD}^{2D} for quantitative comparison, we demonstrate here that EmrE dimerization is affected by different membrane mimetic environments and show that

Abbreviations: SMR, small multidrug resistance; EmrE, *Escherichia coli* SMR protein; FRET, Förster resonance energy transfer; AUC, analytical ultra centrifugation; DDM, dodecylmaltoside; DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine; TPP^+, tetraphenylphosphonium+; TM, transmembrane

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the EmrE dimer is more stable in bicelles than micelles, and even more stable in lipid bilayers.

2. Materials and methods

2.1. EmrE expression, purification and reconstitution

Wild-type EmrE was expressed using a pET15b plasmid with an N-terminal 6×-His tag (a generous gift from Geoffrey Chang, Scripps Research Institute) and purified as previously described [17]. Briefly, BL21 (DE3) cells transformed with the EmrE-containing plasmid were grown in M9 minimal media. After harvesting the cells, they were lysed by sonication and the membrane fraction was solubilized with 40 mM DDM (Affymetrix Anatrace, Maumee, OH). EmrE was purified using Ni-NTA resin (Novagen/EMD Millipore, Darmstadt, Germany) and gel filtration chromatography in 10 mM DDM. The His-tag was cleaved using thrombin between the columns. Purified EmrE was then reconstituted into DLPC liposomes using AMBERLITE (Supelco/Sigma-Aldrich, St. Louis, MO) and the liposomes were broken into isotropic bicelles with the short chain lipid DHPC as previously described [17,18]. The T56C mutant of EmrE for fluorescent labeling was constructed using QUICKCHANGE (Stratagene/Agilent Technologies, Santa Clara, CA) and purified in an identical manner. This mutant had the three native cysteines mutated to serine in addition to the single added cysteine residue (C39S, C41S, C95S, T56C).

2.2. Sedimentation equilibrium analytical ultra-centrifugation (AUC)

AUC experiments were done using wild-type EmrE reconstituted in 56 mM DLPC/DHPC, q = 0.33, isotropic bicelles. In order to density match the 56 mM isotropic bicelles with the aqueous buffer [27,29,30], six otherwise identical bicelle samples (no protein) were prepared with increasing concentrations of D_2O (75, 80, 85, 90, 95, and 100%) in 20 mM potassium phosphate, 20 mM NaCl at pH 7.0, along with a reference sample in 100% H_2O. The samples were spun at 45,000 rpm in a Beckman Coulter XL-I analytical ultracentrifuge at 25 °C and the radial distributions of the samples were monitored using laser interference optics to identify the isopycnic point where the slope of the radial distribution is zero and buoyant molecular density of the bicelles is identical to the buffer. The same experiment was performed in the presence of 25 μM tetraphenylphosphonium (TPP^+) with identical results. 2, 5, 10, and 20 μM wild-type EmrE (concentration determined using the absorbance at 280 nm as in [17]) was reconstituted into bicelles in density matched buffer (98% D_2O). The samples were spun at 8000, 16,000, and 24,000 rpm for 15 h at 25 °C. 56 mM empty bicelles in the same buffer was used as a reference solution. The radial distribution of the protein at different concentration was monitored at 280 nm. The equilibrium radial distribution data were processed in SEDFIT and fit with the monomer–dimer self association model in SEDPHAT to extract the dissociation constant. The dissociation constant in concentration units (μM) was converted to mol % of the protein in bicelle constituting lipids. The partial specific volume of wild-type EmrE and the density of phosphate buffer containing 98% D_2O were estimated as 0.7673 ml/g and 1.10644 g/ml respectively using SEDNTERP program. Error analysis was done using the Monte-Carlo program in SEDPHAT.

2.3. FRET experiments

Determination of the two-dimensional monomer–dimer dissociation constant (K_{MD}^{2D}) in liposomes was performed by two different methods to ensure that samples were fully equilibrated and the results were independent of labeling efficiency. For the first method, 200 μM T56C-EmrE was labeled in 10 mM DDM micelles using an equimolar mixture of donor (Alexa488-maleimide) (Invitrogen/Life Technologies, Grand Island, NY) and acceptor (Alexa568-maleimide) (Invitrogen/Life Technologies, Grand Island, NY) in 20 mM potassium phosphate, 20 mM NaCl, 2 mM TCEP at pH 7.2. The labeled protein was purified from free dyes using Zeba desalting columns (Pierce) and reconstituted in DLPC liposomes at high protein/lipid ratio as described previously. The reconstituted labeled T56C-EmrE was then serially diluted with empty liposomes and multiple freeze–thaw cycles were performed to equilibrate the samples until no further FRET change was observed. Four diluted samples were made ranging from 1.5×10^{-03} to 3.0×10^{-06} protein:lipid ratio.

For the second method, T56C-EmrE was reconstituted into liposomes at 2.5×10^{-02} P/L ratio and fluorescently labeled in the liposomes using the method previously developed to determine EmrE topology [17]. In this protocol, external cysteines are labeled first, followed by liposome permeabilization with detergent and then labeling of cysteines facing the interior of the liposome. The fluorescently labeled EmrE proteoliposomes were then divided into aliquots and 1.2% DDM was added to fully solubilize the proteoliposomes. Additional lipids were added to each sample to achieve P/L ratios ranging from 3.0×10^{-03} to 6.6×10^{-06} and proteoliposomes were reconstituted by removing the detergent with Amberlite as described previously [17]. For FRET measurements, each sample was excited at a wavelength of 490 nm and the emission spectrum was collected from 500 to 750 nm. Reference spectra were taken for solutions containing equal concentrations of DLPC liposomes and subtracted from the sample spectra.

FRET efficiency was calculated using the following equation [31]

$$E = 2I_A Q_D/(I_D Q_A + I_A Q_D) \quad (2)$$

where I_A and I_D are the intensity of acceptor and donor emission at 600 nm and 516 nm respectively, Q_D and Q_A are the quantum yields of donor (Alexa488; 0.92) and acceptor (Alexa568; 0.69) respectively, and both dyes label EmrE with equal efficiency.

The FRET efficiency (E) can also be expressed in terms of the ratio of dimer to the total protein concentration including both dimer (M_2) and monomer (M). Assuming that the monomer–dimer equilibrium is not affected by the dye labeling of the protein, the following relationship is obtained [31]

$$E = [M_2]/([M_2] + [M]/2) \quad (3)$$

where the denominator is the total protein concentration, C_T, expressed as moles of dimer per area for the 2D case. This can be expressed equivalently in terms of R, the EmrE:lipid ratio since only the lipid area is accessible to the highly hydrophobic EmrE. For the monomer/dimer equilibrium $K_{MD} = [M]^2/M_2$. This leads to

$$E = 1 - 0.5 * (E/R)^{1/2} K_{MD}^{1/2} \quad (4)$$

Eq. (4) shows that E vs $(E/R)^{1/2}$ has a linear relationship and the slope of the line $(-0.5 \times K_{MD}^{1/2})$ yields the dissociation constant.

3. Results

3.1. Oligomeric state of EmrE in the absence of substrate

Equilibrium analytical ultra-centrifugation (AUC) was used to assess the monomer–dimer equilibrium constant of wild-type EmrE in isotropic bicelles. This experiment measures the buoyant molecular weight of a molecule of interest. This includes both protein and membrane-mimetic in the case of an integral membrane protein like EmrE. To isolate the protein contribution, we density matched [27,29,30] the buffer to the bicelles at 98% D_2O for 56 mM DLPC/DHPC isotropic bicelles (q = 0.33) in phosphate buffer at pH 7.0 (Fig. 1A). Samples of wild-type EmrE were reconstituted into bicelles with a constant lipid concentration and different protein concentrations. The AUC data for all samples at 3 different rotor speeds was

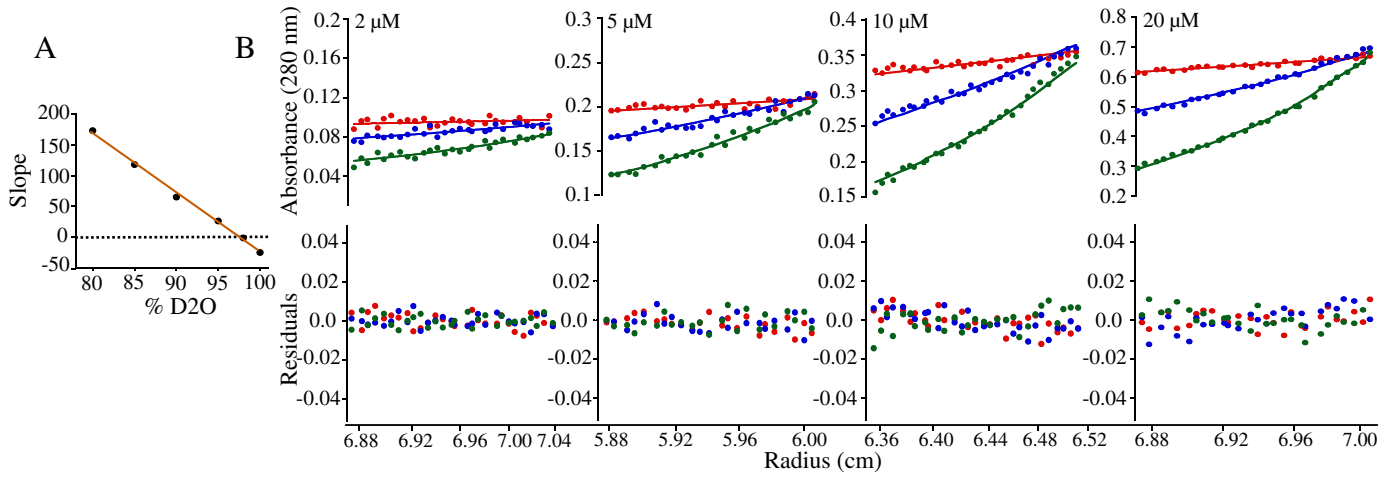

Fig. 1. Monomer–dimer equilibrium of wild type EmrE in isotropic bicelles assessed by analytical ultracentrifugation. Sedimentation equilibrium experiments were performed using wild-type EmrE in isotropic bicelles at 25 °C. (A) Isotropic bicelles (56 mM total lipid concentration, q = 0.33) were density matched with 20 mM potassium phosphate, 20 mM NaCl, pH 7.0 at 98% D_2O. This eliminates the contribution of the bicellar buoyant molecular weight from the protein-bicelle system so that only the sedimentation properties of protein are measured. (B) Radial distribution of wild-type EmrE at 8000 (red circles), 16,000 (blue circles), and 24,000 (green circles) rpm at 25 °C in isotropic bicelles in density matched buffer. The EmrE concentration is indicated in the figure and the bicelle lipid concentration is constant. The data is fit with a simple monomer–dimer self-association model using SEDPHAT (solid lines) with a monomer–dimer equilibrium constant of 4 ± 2 μM or ~0.006 mol%. The lower panel shows the residuals.

well fit with a simple monomer–dimer equilibrium model using SEDPHAT (Fig. 1B). The monomer–dimer equilibrium was converted to K_{MD}^{2D} with the assumption that EmrE is restricted to the long-chain lipid region of the bicelle, resulting in a K_{MD}^{2D} of 0.006 ± 0.003 mol%. The assumption that EmrE only samples the long-chain portion of a perfectly disk-like bicelle with no mixing of long- and short-chain lipids is clearly an imperfect approximation [32–34], so this represents an upper limit for K_{MD}^{2D}. At the other extreme, consideration of the total lipid concentration places a lower limit on K_{MD}^{2D} of 0.002 mol%. Converting the published dimerization affinity for EmrE in micelles to K_{MD}^{2D} using the reported 3D K_D and detergent concentration results in 0.8–0.95 mol% for wild-type EmrE in DDM micelles without substrate [5]. The exact value within this range depends on the extent of delipidation of the sample. This indicates that the EmrE dimer is at least 100× more stable in discoidal lipid bicelles than spherical micelles and demonstrates the importance of lipid environment for stabilizing the EmrE dimer.

3.2. TPP^+-bound EmrE in isotropic bicelles

Equilibrium AUC was performed in the presence of saturating tetraphenylphosphonium (TPP^+) to determine the monomer–dimer equilibrium of substrate-bound EmrE. Under conditions otherwise identical to the substrate-free EmrE experiments, a simple monomer–dimer equilibrium model was again sufficient to fit the data for TPP^+-bound EmrE and a nearly identical K_{MD}^{2D} of 0.003–0.009 mol% of DLPC was obtained. This is not unexpected since dimerization has been proposed to be dominated by transmembrane helix 4 (TM4)-TM4 interactions that involve 'knob–hole' packing between the hydrophobic residues of the helices [35–38] and substrate binding does not directly involve TM4.

3.3. EmrE oligomerization within the membrane bilayer

Determination of two dimensional equilibrium constants in lipid membranes is not trivial and has only been performed in a few cases [23,28,39–42]. In order to assess the oligomeric state of EmrE in bilayers, we explored techniques that might be suitable for measuring the monomer–dimer equilibrium under these conditions. EmrE has four tryptophan residues, only one of which (W63) is essential for activity, and the change in intrinsic tryptophan fluorescence upon substrate binding has been used previously to determine substrate affinity [43]. However, the intrinsic tryptophan fluorescence is linear over a wide range of concentrations, and therefore does not change significantly upon dimerization (data not shown). We therefore used a Förster resonance energy transfer (FRET) based assay [31] to obtain the 2D dimerization constant (K_{MD}^{2D}) for EmrE in liposomes. In this assay, T56C-EmrE labeled with donor and acceptor (Fig. 2A) and was reconstituted into liposomes at different protein:lipid ratios (R). These experiments were performed twice using different labeling conditions and mixing protocols to ensure full equilibration of the protein and lipid in the bilayers, as described in detail in the methods. In the first protocol, we labeled EmrE in detergent micelles, reconstituted into liposomes at high P/L ratio and then used multiple freeze thaw cycles to equilibrate the proteoliposomes with additional lipid in a dilution series. In the second protocol, we fluorescently labeled EmrE in permeabilized liposomes, and then fully solubilized the proteoliposomes with excess detergent to ensure complete mixing with additional lipids to create the dilution series. All the detergent was then removed using hydrophobic beads to reform proteoliposomes at each protein:lipid ratio. The first protocol minimizes handling of the samples, while the second protocol achieves higher labeling efficiency of EmrE and ensures more complete equilibration of the samples. Triplicate samples were made using each protocol and the K_{MD}^{2D} was determined from the measured FRET efficiency (E) using Eq. (4) [31]. Both methods result in the same K_{MD}^{2D} (Fig. 2B and *inset*): 0.0005 ± 0.0002 mol% by method 1 and 0.0008 ± 0.0004 mol% by method 2 for TPP^+ bound EmrE.

4. Discussion and conclusion

The results presented in this article explore the oligomerization of the small multidrug resistance transporter, EmrE. It is important to consider that integral membrane proteins reside in a two dimensional space in the lipid bilayer [39,40,44]. Therefore, a concentration reported in units of volume is not really relevant since the protein is restricted to the membrane portion of the sample. The change in effective concentration upon membrane association and the thermodynamic effects of reduced dimensionality are widely recognized as important factors

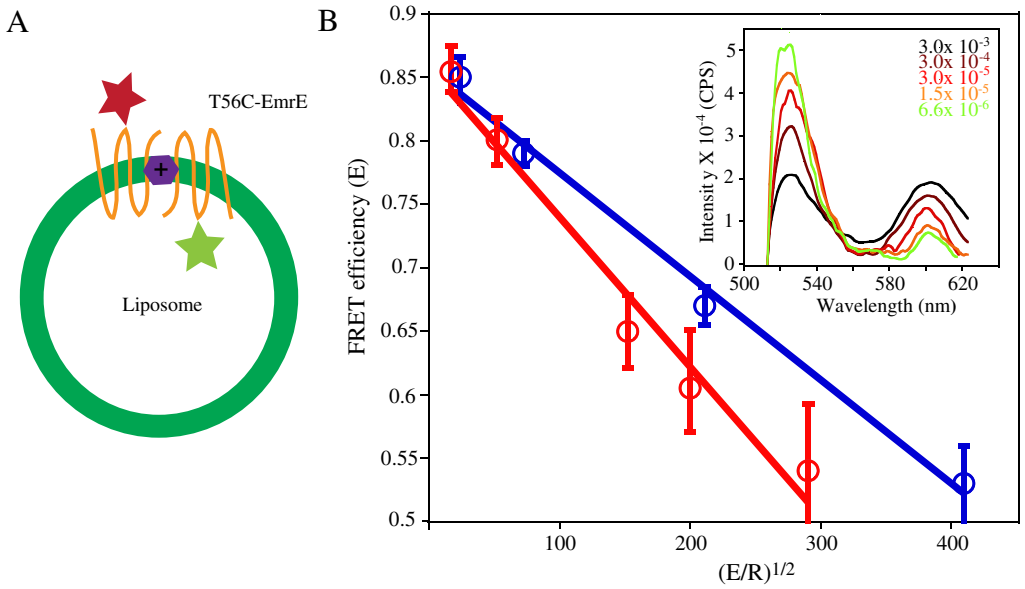

Fig. 2. 2D monomer–dimer equilibrium of EmrE in liposomes. The figure shows FRET studies to evaluate the 2-dimensional monomer–dimer equilibrium constant (K_{MD}^{2D}) of EmrE in DLPC liposomes. (A) The cartoon shows the donor (Alexa488, green star) and acceptor (Alexa568, red star) labeled T56C-EmrE FRET construct. This construct was prepared using two different methods as described in the text. Briefly, in method 1 EmrE was labeled in detergent, reconstituted into liposomes, and freeze–thaw cycles were used to equilibrate samples at different protein/lipid ratio. In method 2, EmrE was labeled in permeabilized liposomes and mixed with additional lipids under solubilizing detergent concentrations to produce samples with different protein/lipid ratio, which were then reformed into proteoliposomes via detergent removal. (B) FRET efficiency (E) versus $(E/R)^{1/2}$, where R is the protein to lipid ratio, is linear. The slopes ($-K_{MD}^{1/2}/2$) yield a K_{MD}^{2D} of 0.0005 ± 0.0002 mol% using method 1 (blue) and 0.0008 ± 0.0004 mol% using method 2 (red) with triplicate samples. The inset shows the raw FRET data at different protein to lipid ratios as labeled in the figure using method 2.

influencing biological processes such as signaling and adhesion [39–42]. Despite this, the monomer–dimer equilibrium constants for membrane proteins are frequently reported in three-dimensional units. Extreme care must be taken in extrapolating these values to different lipid or detergent conditions, since changing detergent or lipid concentration changes the effective membrane-mimetic space available to the protein. Because of this partitioning, mole fractions provide the most useful units for considering protein association within a membrane-mimetic environment [45]. We therefore report the EmrE dimerization affinity, K_{MD}^{2D}, in mol % relative to lipid or detergent. These units allow simple comparison of dimerization affinities between different lipid and detergent conditions to understand the influence of membrane-mimetic environment on EmrE self-association.

In the available structures of EmrE, only one pair of helices (TM4) is tightly associated, with limited additional contact between monomers in the absence of substrate [4,6,7]. Low-resolution cryoelectron microscopy studies of substrate-bound and apo EmrE indicate that small but significant changes occur in the overall structure upon substrate binding. EPR measurement of spin-label mobility and accessibility indicate that the apo protein is more dynamic and is stabilized by substrate binding [46]. This is supported by more recent solid-state NMR experiments suggesting greater heterogeneity in the apo state [47], while solution NMR studies reveal a single structure when bound to TPP^+ [17]. Our results confirm that EmrE is a dimer in bicelles and that substrate-free and substrate-bound EmrE have nearly identical monomer–dimer equilibria. Thus, even though substrate binding stabilizes a single compact 3D structure of EmrE [46,47] it is not critical for dimer stability. This confirms the importance of TM4 [6,35–38] for dimer stability of small multidrug resistance transporters, as has been extensively studied in the EmrE homolog Hsmr [35]. Our results emphasize that substrate specific interactions in the binding pocket are not required for dimer affinity. This observation is interesting, since the dynamic nature of apo EmrE has been suggested to have functional importance in enabling multidrug recognition. With the TM4 dimerization motif sequestered from the substrate-binding pocket it is possible to maintain a stable dimer, as required for transport activity, while allowing significant flexibility in the substrate-binding pocket. Indeed, solid-state NMR experiments observed a diversity of lineshapes suggesting differences in apo EmrE dynamics across the protein [47].

It is clear from our data that bicelles provide a better membrane mimic for biophysical and biochemical studies of solubilized EmrE than detergent since the dimerization affinity in bicelles (0.002–0.009 mol%) is 100 times tighter than in micelles (0.8–0.95 mol%). The dramatic difference in dimer stability between detergent and bicelles confirms the importance of the membrane-mimetic environment for proper quaternary structure of integral membrane proteins, particularly small proteins such as EmrE with limited inter-monomer interaction. Dimer stability has functional implication as well. While EmrE binds substrate tightly in DDM detergent, this measure of EmrE function is highly sensitive to detergent type and concentration. Early studies of EmrE:substrate stoichiometry indicated a 3:1 or 4:1 ratio rather than the now-accepted 2:1 ratio, most likely due to incomplete dimerization of EmrE under the assay conditions [4,48]. Furthermore, although EmrE binds substrates with high affinity both in the detergent DDM and in bicelles, suggesting both provide a “good” environment and stabilize properly folded protein. Our results demonstrate that more complete consideration of EmrE oligomerization as well as substrate binding reveal bicelles to be a better membrane mimetic than detergent micelles due to the enhanced stabilization of the functional EmrE dimer.

The two dimensional monomer–dimer equilibrium constant obtained for EmrE in DLPC liposomes provides the first direct experimental measure of EmrE K_{MD}^{2D} in full lipid bilayers. Unsurprisingly, lipid bilayers provide the best environment for EmrE when evaluated in terms of dimer stability, with dimerization affinity in liposomes (0.0005–0.0008 mol%) another 10-fold tighter than in bicelles. It is likely that dimerization affinity is further affected by the exact composition of the lipid membrane. When the EmrE homolog, TBsmr, is reconstituted into liposomes, ethidium transport activity depends on the lipid composition and increased activity is correlated with increased oligomerization [22]. EmrE transport activity in liposomes is itself dependent on the lipid composition, with significant differences in transport rate when phosphatidylglycerol (PG) or

phosphatidylethanolamine (PE) lipids are included in the liposome [20,21]. Lipid curvature strain, lateral pressure, and lipid charge may affect protein insertion, folding, oligomerization, dynamics, and function, as has been extensively studied for individual TM helices, anti-microbial peptides, rhodopsin and others [49–57]. Our results provide a dramatic demonstration of the importance of the proper physico-chemical environment for EmrE dimer stability. The very tight dimerization affinity of EmrE in lipid bilayers suggests EmrE dimers do not dissociate once formed and makes it unlikely that SMR monomers would exchange between dimers. Future studies will be needed to determine whether lipid composition alters EmrE activity through effects on structure, oligomerization, or functional dynamics and measure EmrE dimerization directly in the native membrane.

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