Lipid bilayer induces contraction of the denatured state ensemble of a helical-bundle membrane protein

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Defining the denatured state ensemble (DSE) and disordered proteins is essential to understanding folding, chaperone action, degradation, and translocation. As compared with water-soluble proteins, the DSE of membrane proteins is much less characterized. Here, we measure the DSE of the helical membrane protein GlpG of *Escherichia coli* (E. coli) in native-like lipid bilayers. The DSE was obtained using our steric trapping method, which couples denaturation of doubly biotinylated GlpG to binding of two streptavidin molecules. The helices and loops are probed using limited proteolysis and mass spectrometry, while the dimensions are determined using our paramagnetic biotin derivative and double electron–electron resonance spectroscopy. These data, along with our Upside simulations, identify the DSE as being highly dynamic, involving the topology changes and unfolding of some of the transmembrane (TM) helices. The DSE is expanded relative to the native state but only to 15 to 75% of the fully expanded condition. The degree of expansion depends on the local protein packing and the lipid composition. *E. coli*’s lipid bilayer promotes the association of TM helices in the DSE and, probably in general, facilitates interhelicical interactions. This tendency may be the outcome of a general lipophobic effect of proteins within the cell membranes.

For these three scenarios, solvent quality can be quantified by the Flory exponent, ν, in the relationship between the radius of gyration (Rg) and the number of monomeric units (N): Rg ∝ N^ν. The exponent is ν = 3/5, 1/2, and 1/3 for good, 0, and poor solvents, respectively. The ν value for self-avoiding random walk has been measured for unfolded proteins in elevated denaturant concentrations with small-angle X-ray scattering and Förster resonance energy transfer (9–13). For a variety of IDPs with protein-like sequences and the DSE of soluble proteins, ν values are typically above 1/2 under native conditions, much higher than anticipated given the general perception that soluble proteins undergo hydrophobic collapse prior to folding (12, 14, 15).

In contrast, the properties of the DSE are not as well characterized for membrane proteins. The folding of helical membrane proteins is thought to occur through at least two stages denatured state | membrane protein folding | GlpG | steric trapping | Upside simulation

Denatured states and intrinsically disordered proteins (IDPs) are involved in a variety of cellular events, serving as targets for degradation, chaperone action, translocation, and cell signaling (1–4). They also influence thermodynamic stability and direct early folding events (5). Thus, delineating the denatured state ensemble (DSE) has been a subject of extensive study (1). For water-soluble proteins, DSE can be described as a collection of disordered conformations that interconverts on a timescale faster than folding (6).

For the quantitative description of the DSE, polymer theory has proven useful (7, 8). For a given polymer/solvent combination, the solvent quality can be classified into three limiting regimes depending on the relative strengths between intrachain and chain–solvent interactions (7). In a “good” solvent, chain–solvent interactions are more favorable than the intrachain interactions and the DSE is well described as a self-avoiding random walk. In a “poor” solvent, the intrachain and chain–solvent interactions are balanced such that the intrachain attraction cancels out the expansion caused by excluded chain volume and the protein behaves as a random walk. In a “poor” solvent, the intrachain interaction exceeds the chain–solvent interactions, inducing contraction or even collapse of the chain into a globule.

**Significance**

The conformational properties of denatured states of proteins affect their folding, turnover, translocation, and chaperone action. We find that the denatured state ensemble of a helical membrane protein is richer than depicted in the classic two-stage model of membrane protein folding. Some helices undergo topological changes and partial unfolding in the denatured state. In the bilayer, the ensemble is compact relative to the fully expanded state, indicating that the bilayer may not act as a good solvent. This behavior, however, varies depending on the properties of the system, including the local protein sequence, helical and linker lengths, and lipid composition. This study provides insights into the lipid bilayer as a solvent that mediates the assembly and function of membrane proteins.

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(16). In Stage I, hydrophobic segments in a polypeptide chain insert into the lipid bilayer as transmembrane (TM) helices. In cells, insertion is cotranslationally mediated by a translocon complex (17, 18). In Stage II, TM helices associate into a compact native structure. The DSE for helical membrane proteins has been regarded as the conformational ensemble prior to formation of the native structure (19), in which individual TM helices can diffuse around within the bilayer, although the extent may be limited by the length of the interconnecting loops and residual interhelical contacts. The concept of solvent quality and Flory exponent is harder to quantitatively apply to membrane proteins due to the physical constraints of the quasi-two-dimensional (2D) bilayer and the difficulty in treating the denatured chains as simple polymers.

The underlying principle of the two-stage model is that each stage is driven by distinct driving forces. In Stage I, the formation and insertion of TM helices are favored by the hydrophobic nature of TM segments and the high desolvation cost of having unpaired moieties for backbone hydrogen bonding (H bonding) within the bilayer (17, 20). The role of water in TM helix association likely is less important in Stage II once the individual TM helices have been dehydrated and are favorably solvated with lipids (21). Nonetheless, water may play a role when TM helices have partially hydrated polar residues (22). The association of TM helices requires good van der Waals packing or polar interactions (21, 23–25) and is also known to be modulated by the physical properties of the lipid bilayer (e.g., the lipid packing density and the lipid deformation induced by the hydrophobic mismatch between the protein and the bilayer) (26–29). Thus, a combination of various molecular forces determines how favorable TM helix association is and whether the lipid bilayer functions as a good solvent for the DSEs of membrane proteins.

So far, the DSEs of helical membrane proteins have been characterized under denaturing conditions induced by urea or guanidine hydrochloride (30, 31), anionic detergents including sodium dodecyl sulfate (SDS) (24, 32–36), or mechanical force (37, 38) in various hydrophobic environments (e.g., micelles, lipid–detergent mixtures, or lipid bilayers). These studies suggest that, relative to native states, DSEs are expanded but their TM helices are constrained by interaction sites. The degree of expansion also depends on the choice of denaturant and may be limited by the size of micelles (30, 33–36). Lipid bilayers provide a distinct but biologically relevant 2D environment. As a result, the conformational features of the DSE obtained under denaturing conditions may be different from those in cellular membranes.

Here, we reconstitute the DSE of a helical-bundle membrane protein, Escherichia coli (E. coli) GlpG in a native-like lipid bilayer, and study its conformation without denaturant. GlpG is a member of the rhomboid intramembrane protease family with high kinetic stability (SI Appendix, Table S1) (36–40). As with any stable protein, the characterization of the DSE under native conditions is challenging due to its low population and short lifetime (12). We generate GlpG’s DSE using our steric trapping method, which couples the spontaneous denaturation of a doubly biotinylated protein to the simultaneous binding of two bulky monovalent streptavidin (mSA, 52 kDa) molecules at ambient temperature (Fig. 1A). By combining multiple experimental measurements and molecular dynamics (MD) simulations to probe the flexibility and physical dimensions of the DSE at the local and global levels, we demonstrate that the DSE is highly dynamic, involving extensive helical fraying and changes in membrane topology. The DSE expands relative to the native state but retains a degree of compactness in the lipid bilayers. The degree of expansion further depends on the local stability in the native state and the lipid composition.

Results

Reconstitution of the On-Pathway DSE in Lipid Bilayers. To measure the properties of the DSE, we applied our steric trapping method (Fig. 1A) (36). GlpG is labeled with biotin tags at two specific residues that are spatially close in the folded state but on different structural segments. The first mSA can bind unhindered to either biotin tag. The binding of a second mSA, however, is sterically disallowed since the two biotins are close in the native state, but the binding is allowed when the protein is denatured. Taking advantage of the stability and long lifetime of the mSA–biotin complex (K_d(biotin) = 10^{-14} M; k_{off(biotin)} = days) (41), this method enables the trapping and characterization of the DSE even under native conditions.

Previously, we have shown that the TM domain of GlpG (resides 87 through 276) can be separated into the N-terminal TM helices (TM1–L1–TM2–L2–TM3–L3) and C-subdomains (TM4–L4–TM5–L5–TM6), each having distinct folding behaviors (Fig. 1B) (36). The three sites selected for biotinylation were located at the N and carboxyl termini and on the middle loop (M) connecting the helices TM2 and TM3 (Pro95N, Val267C, and Gly172N, respectively) (36). These three sites located on the same side of the protein were used pairwise to disrupt either the entire protein (with the residue pair 95N267C) or just the N- or C-subdomain (with 95N172M or 172M267C). Each of the three pairs was substituted with a pair of cysteine residues that were then labeled with the thiol-reactive biotin derivative possessing a spectroscopic probe (fluorescent pyrene, BnTyr, or a nitroxide spin label, BnRG) (Fig. 1C and SI Appendix, Fig. S1) (36). Neither conjugation of the biotin derivative to each single cysteine variant nor the binding of an mSA molecule to each biotin label inhibited the proteolytic activity of GlpG relative to wild type (WT) for the model substrate LytM2 (the second TM segment of an E. coli lactose permease, LacY) (SI Appendix, Fig. S2A) (36). We reconstituted sterically denatured GlpG into lipid bilayer environments: 1) Large negatively charged bicelles (i.e., discoidal bilayer fragments edge-stabilized by detergent) composed of dic14:0-phosphatidycholine (DMPC), dic14:0-phosphatidylglycerol (DMPG), and 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS) (molar ratio = 3:1:1.4); 2) Liposomes composed of E. coli phospholipids (SI Appendix, Fig. S3) to provide a native-like lipid environment for E. coli GlpG. Based on the cryoelectron microscopic (cryo-EM) images of the bicelles and liposomes, the average diameters were 190 ± 40 Å and 510 ± 220 Å, respectively (Fig. 1A).

We initially attempted to obtain the DSE using steric trapping with native GlpG reconstituted in bicelles and liposomes. However, incubation with excess mSA did not yield noticeable denaturation for 1 wk when GlpG activity was used as a folding indicator, consistent with GlpG having high kinetic stability in bilayers (SI Appendix, Fig. S4). However, denaturation was achieved in dodecylmaltoside (DDM) detergent micelles for all three constructs within 48 h of incubation with mSA (SI Appendix, Figs. S2B and S4). With doubly conjugated BnRG, the activities of the 95N172M, 172M267C, and 95N267C constructs in micelles were reduced by 57 ± 15%, 74 ± 9%, and 88 ± 7% of their native enzymatic activity, respectively (SI Appendix, Fig. S2C). This activity loss is correlated with the level of double biotinylation (42 ± 2%, 55 ± 2%, and 60 ± 1% for 95N172M, 172M267C, and 95N267C, respectively) (SI Appendix, Figs. S1 and S2C), implying that double binding of mSA results in a loss of activity and the residual activity level stems from incomplete biotin labeling.

For reconstitution in bilayers, sterically denatured GlpG prepared in micelles (Fig. 1A) was transferred to bicelles or to liposomes by direct injection (for the liposomes, detergents were removed using polystyrene beads, SI Appendix, Fig. S3). To verify incorporation of GlpG into bicelles, we employed a
fluorescence-quenching assay (Fig. 2A). Here, the double cysteine variants were labeled with fluorescent BtnPyr (Fig. 1C). Native and sterically denatured GlpG were injected into the bicelles containing the quencher-labeled lipid, \( \text{dC}_{18:1\text{c9}} \)-phosphatidylethanolamine (DOPE)-dabcyl. After injection, pyrene fluorescence from both native and denatured GlpG was quenched to a level close to that observed for full incorporation.

To verify the incorporation of GlpG into liposomes, we employed a liposome-flotation assay (Fig. 2B and SI Appendix, Fig. S5A). Native and denatured GlpG were doubly labeled with BtnPyr and reconstituted in the liposomes containing the fluorophore-labeled lipid, \( \text{dC}_{18:1\text{c9}} \)-PE (DPPE)-rhodamine. Both native and denatured GlpG coalesced with the liposomes after centrifugation in a sucrose gradient, indicating membrane association. They were also resistant to sodium carbonate extraction, indicating membrane integration (SI Appendix, Fig. S5B).

Next, we tested whether the denaturation status of GlpG initially prepared in micelles was retained after reconstitution in bilayers (Fig. 2C and SI Appendix, Fig. S6). To trap the denatured state, we used BtnRG to doubly biotinylate GlpG. This biotin label forms a disulfide linkage to cysteine, which can be broken with the addition of a reducing agent, dithiothreitol (DTT). After reconstitution of the GlpG samples containing mSA, the activities relative to the doubly biotinylated native forms (95N/172M and 172M/267C, and 95N/267C) in bicelles were 12%, 40 ± 17%, and 12 ± 7%, respectively, and 56 ± 20%, 49 ± 14%, and 0 ± 4% in liposomes, similar to the values in micelles. Thus, sterically denatured GlpG in micelles largely remained denatured after transfer to bilayer environments.

We tested whether denatured GlpG in the lipid bilayers can refold after the release of the steric repulsion between bound mSA molecules (Fig. 2C). Upon addition of DTT to dissociate the BtnRG labels bound with mSA from GlpG, the activity relative to the native form increased to >70%. Thus, sterically denatured GlpG in the bilayers can refold to its native structure and is not a dead-end product incapable of refolding.

**Limited Proteolysis Reveals DSE Flexibility.** To investigate the conformational features of GlpG's DSE in a native-like lipid bilayer and other hydrophobic environments, we employed limited proteolysis using Proteinase K (ProK). ProK is a robust endopeptidase that nonspecifically proteolyzes water-exposed unstructured regions rather than structured or membrane-buried regions (42, 43). We biotinylated GlpG with BtnRG so that the addition of DTT would remove the mSA molecules from GlpG and its fragments, simplifying the analysis of proteolyzed GlpG with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) or mass spectrometry.

Upon denaturation, the conformational flexibility and solvent accessibility increased throughout the protein. Time-dependent proteolysis was measured using SDS-PAGE for native and sterically denatured GlpG trapped for the three

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**Fig. 1.** Steric trapping strategy to reconstitute denatured GlpG in the lipid bilayers. (A, Left) Doubly biotinylated GlpG was first denatured using steric trapping by the addition of excess mSA in micelles. Sterically denatured GlpG was transferred to bicelles or liposomes. (Center) Cryo-EM images of the bicelles and liposomes. In the top bicelle panel (with GlpG and mSA), the particle types were assigned as follows. White arrowheads (circular and ellipsoidal objects): flat or tilted bicellar disk planes; black arrowheads (the dark rod-shaped objects): bicellar rims; blue arrowheads (the dark small particles with diameters of 40 to 50 Å): mSA molecules. The bottom liposome panel (without the proteins) largely contains unilamellar liposomes with a minor fraction of multilamellar liposomes. (Right) The histograms for the diameter size distributions of the bicelles and liposomes. (B) Double cysteine variants employed for the steric trapping of the denatured state of GlpG. In each variant, designated cysteine residues were labeled with a thiol-reactive biotin derivative with a spectroscopic reporter group as shown in C. The regions of the backbone colored in cyan and orange indicate the N- and C-subdomains, respectively. (C) Thiol-reactive biotin derivatives with a paramagnetic spin label (Left, BtnRG–TP) and fluorescent pyrene (Right, BtnPyr–IA). Reproduced with permission from ref. 36. TP: thiopyridine; IA: iodoacetamide.
biotin combinations (Fig. 2D). Native GlpG (23 kDa, the TM domain) was resistant to proteolysis except at the termini due to the protein’s high kinetic stability (SI Appendix, Figs. S4 and S7) (44). In contrast, denatured GlpG was rapidly proteolyzed within 10 min in all hydrophobic environments. However, 20 to 50% of the GlpG sample containing mSA was not proteolyzed, probably due to incomplete biotin labeling (SI Appendix, Figs. S1 and S2C). GlpG doubly bound with mSA was proteolyzed, while unbound or singly bound GlpG was largely protease resistant (SI Appendix, Figs. S7 and S8). Notably, when denatured GlpG was trapped with a biotin pair at the N-subdomain (95N172M), it was extensively proteolyzed into small fragments (<8 kDa). However, when the denatured protein was trapped with the biotin pair at either the C-subdomain (172M267C) or the N and carboxyl termini (95N267C), proteolysis yielded large fragments in micelles and bicelles (∼11, ∼13, or ∼17 kDa). This result supports our previous finding that in micelles, the disruption of the N-subdomain leads to global denaturation, whereas the C-subdomain undergoes subglobal denaturation with the balance of the protein remaining intact (36). In liposomes, however, denatured GlpG was extensively proteolyzed regardless of the position of the biotin pair, implying that the entire chain was less structured, contracted, or buried in liposomes than in micelles and bicelles.

To identify the unstructured and solvent-accessible regions that are sensitive to proteolysis, we employed capillary-zone electrophoresis.

Fig. 2. Reconstitution of denatured GlpG in the lipid bilayers. (A, Top) Schematic description of fluorescence-quenching assay to measure incorporation of native and sterically denatured GlpG into bicelles. GlpG was doubly labeled with fluorescent BlnPyr, while the bicelles contained quencher-labeled lipids, DOPE–dabcyl. Native (“N”) and denatured (“D mSA”) GlpG solubilized in micelles were injected into bicellar solution. Negative control (“Unbound”): water-soluble pyrene-labeled mSA was injected into the bicellar solution. Positive control (“Bound”): native GlpG was first reconstituted in DMPC:DMPG–DOPE–dabcyl liposomes and then solubilized by CHAPS to form bicelles. (Bottom) Assay results for the three doubly biotinylated GlpG variants. Error bars denote ± SEM (n = 3). Based on Student’s t test, significance of the difference between a data pair is marked with single (P < 0.05), double (P < 0.01), and triple asterisks (P < 0.001) or “NS” (not significant, P > 0.05). (B) Liposome-flotation assay in a sucrose gradient indicates a near-complete membrane association of native and denatured GlpG doubly labeled with BlnPyr. Liposomes contained fluorescent DPPE–rhodamine. (C) Proteolytic activity of GlpG as a measure of denaturation and refolding efficiency. GlpG doubly labeled with BlnRG was first denatured upon addition of excess mSA in DDM and then transferred to micelles or liposomes. The addition of DTT, which breaks the disulfide linkage between GlpG and the biotin label bound with mSA, enables refolding (“+DTT”). The activity was normalized relative to that of native GlpG without mSA in each hydrophobic phase. Error bars denote ± SEM (n = 3). Significance of the difference between a data pair is marked as in A. (D, first row) The schematic description of limited proteolysis of native (N) and sterically denatured GlpG (D mSA) by ProK. The time-dependent proteolysis in DDM micelles (second row), bicelles (third row), and liposomes (fourth row) was measured by SDS-PAGE. After quenching of proteolysis at each time point, DTT was added to release bound mSA from GlpG. The extent of GlpG proteolysis was quantified from the GlpG band intensities with and without ProK (asterisks). The proteolytic peptide fragment larger than 10 kDa is marked with a symbol (open circles, 17 kDa; open squares, 13 kDa; open triangles, 11 kDa) on the right side of each band. For mass spectrometry (see Fig. 3), the samples were further solubilized with DDM and the cysteine residues were alkylated with iodoacetamide (IA).
electrophoresis (CZE)-tandem mass spectrometry (MS/MS) or reverse-phase liquid chromatography (RPLC)-MS/MS (SI Appendix, Tables S2–S7). Each method utilizes a distinct principle for peptide separation (electrophoretic mobility for CZE and solubility for RPLC), allowing for complementary peptide identification (45, 46). We chose the double-biotin variant, 172M267C–BtnRG2, because it had a higher biotin-labeling efficiency than the other variants and yielded a larger fraction of denatured GlpG molecules. Overall, RPLC-MS/MS enabled the detection of the larger peptides from the TM regions in the native structure, while the smaller peptides from the loops were identified using CZE-MS/MS (Fig. 3A, Left).

The native state ensemble (NSE) of GlpG in the bilayer likely undergoes functional motions that permit cleavage by ProK. We identified several peptide fragments from native GlpG in liposomes (Fig. 3A, Top). These sequences were mapped onto the kinked cytoplasmic end of TM2 and the middles of TM4 and TM6. Notably, these regions are directly involved in the proteolytic mechanism of GlpG: The interface between TM2 and the gating helix TM5 forms the substrate binding site (47). TM4 and TM6 are packed through the conserved glycine-zipper motif harboring the catalytic dyad Ser201–His254 (48). Upon denaturation, the entire length of TM4 and TM6 became susceptible to proteolysis in addition to the periplasmic half of TM3 and the flanking loops of TM5 (Fig. 3A, Bottom). The middle regions of TM3, TM4, and TM6 also were highly susceptible to proteolysis. These three helices are the least hydrophobic in GlpG (Fig. 3B), suggesting that they can transiently become unstructured and solvent exposed in the DSE.

In micelles, the overall proteolysis pattern was similar to that in the bilayers for native and denatured GlpG, although in micelles, cleavage was more pronounced in the C-subdomain of GlpG.

Fig. 3. Mapping the flexible regions in native and denatured GlpG using limited proteolysis by ProK and mass spectrometry. (A, Left) The proteolysis products of native and denatured GlpG (the double biotin variant 172M267C) in liposomes (Top) and micelles (Bottom) were analyzed using CZE- (cyan vertical bars) and RPLC- (red vertical bars) MS/MS. The peptide identification number (peptide ID, lower x-axes) denotes a unique fragment identified under each designated condition in the ascending order of the N-terminal residue number of the fragment. Each vertical bar spans the range from the N- to the carboxyl-terminal residue number of each fragment and is mapped onto the primary and secondary structures (y-axes). The traces in green represent both the accuracy and frequency of each cleavage site (y-axes) (SI Appendix, Supplementary Text). (Right) The cumulative score of each site was mapped onto the tertiary structure and color coded according to the heat map. The catalytic dyad (Ser201–His254) is shown in spheres in each structure. (B) Hydrophathy plot of the TM domain of GlpG using the Wimley-White (WW) water–octanol (88), Hessavon Heijne (HvH) translocon–membrane (17), Tian-Lin-Liang (TLL) membrane depth-dependent (44, 89), and Moon-Fleming (MF) water–membrane (74) hydrophobicity scales. For the WW and HvH scales, a 19-residue window was slid along the sequence of GlpG summing ΔGtransfer of each residue. For the TLL and MF scales, the ΔGtransfer Values only for the residues in each TM helix from the structure were summed. (C) Possible modes of the membrane-topology dynamics and unfolding in the DSE of GlpG in E. coli liposomes. The models were based on limited proteolysis, MS/MS, MD simulations, and the charge distributions in the membrane-water interfacial regions of GlpG.
than in the N-subdomain (Fig. 3A, Bottom). The extensive proteolysis in the C-subdomain in both bilayers and micelles points to higher dynamics at this end of the protein, being an intrinsic property of the protein rather than stemming from steric repulsion in this subdomain. Supporting this reasoning, denatured GlpG trapped using the biotin pair (95N172M) in the N-subdomain still underwent extensive proteolysis in the C-subdomain (SI Appendix, Fig. S9).

In summary, our proteolysis study reveals dynamic features of the DSE under native conditions. The DSE involves various modes of membrane-topology changes including the unfolding of TM segments due to their low hydrophobicity and, probably, the charge distribution in the flanking regions (Fig. 3C). Although we minimized the duration of proteolysis (5 to 7 min) to capture unstructured regions in the DSE of full-length GlpG, it is likely that a certain fraction of the identified peptides were obtained from the polypeptides already proteolized (“double hit”). Even so, the proteolysis patterns nonetheless would reflect intrinsic conformational features of individual structural segments in their isolated forms.

**Contraction of the DSE Measured with Double Electron–Electron Resonance Spectroscopy.** We next examined the compactness of the DSE in micelles, bicelles, and liposomes using double electron–electron resonance spectroscopy (DEER) suitable for measuring distances between 18 to 60 Å (49). Specifically, we measured the interspin distances between a pair of paramagnetic biotin labels (BtNRG) in native and sterically denatured GlpG for the three constructs, 95N172M–BtNRG2, 172M267C–BtNRG2, and 95N267C–BtNRG2. The probability distribution of interspin distances was obtained from the time-dependent dipolar evolution data fitted with the model-free, nonnegative Tikhonov regularization algorithm (Fig. 4) (49). To minimize unwanted intermolecular dipolar coupling between multiple spin-labeled GlpG molecules in a single liposome, we increased the lipid-to-protein molar ratio sixfold (from 2,000 to 12,000) as compared to our activity and proteolysis measurements and incorporated unlabeled GlpG at a three- or six-times molar excess relative to spin-labeled GlpG (SI Appendix, Figs. S10 and S11).

The probability distributions of interspin distances, P(r), were similar for the native proteins in micelles, bicelles, and liposomes (Fig. 4B). Their distributions were narrow centered at a short mean distance (r_{\text{mean}}) of 29 to 30 Å for 95N172M (the SD of distribution, σ = 6 to 9 Å), 25 to 33 Å for 172M267C (σ = 4 to 11 Å), and 37 to 38 Å for 95N267C (σ = 6 to 9 Å) (SI Appendix, Table S8). For the DSE, however, distributions of the three variants were highly heterogeneous with multiple local maxima spanning the distance range detectable by DEER. We have previously shown that upon denaturation by steric trapping in DDM micelles, r_{\text{mean}} values increase by factors of 1.52 ± 0.02 and 2.03 ± 0.01 for 95N172M and 172M267C, respectively (36). For the new 95N267C variant, the r_{\text{mean}} between the termini increased by a factor of 1.35 ± 0.01 upon denaturation (Fig. 4B). Thus, steric trapping overall induced expansion of the DSE in micelles.

We expected that relative to micelles, which are less organized assemblies, the quasi-2D lipid bilayer would confine the movements of the TM segments in the DSE, leading to its compaction. Indeed, the DSEs in bicelles were less expanded than in micelles by factors of 1.23 ± 0.02, 1.37 ± 0.03, and 1.29 ± 0.01 for 95N172M, 172M267C, and 95N267C, respectively. Nevertheless, the DSE in liposomes was more expanded than in bicelles despite both being quasi-2D bilayers by factors of 1.38 ± 0.01, 1.65 ± 0.03, and 1.45 ± 0.04 for 95N172M, 172M267C, and 95N267C, respectively. This difference may be attributed to the use of DMPC:DMPG:CHAPS in the bicelles versus E. coli phospholipids in the liposomes. Overall, the expansion of the DSE depended on the position of the steric repulsion as well as the lipid composition.

To test the effect of doubly bound mSA molecules on the compactness of the DSE, we measured the interspin distances in the SDS-induced DSE of a double-biotin variant with and without mSA (SI Appendix, Fig. S12). In SDS, mSA is resistant to denaturation and can still bind to the biotin labels (SI Appendix, Fig. S1). SDS molecules are expected to bind the surface of mSA (50). The resulting negatively charged mSA–SDS complexes may repel each other when doubly bound to GlpG, inducing further expansion of the DSE. Nonetheless, binding of mSA in SDS did not noticeably affect the distance distribution. Also, the distances measured with bound mSA in a high concentration of SDS (~4% wt/vol) were similar to those in neutral DDM micelles (SI Appendix, Fig. S12). Thus, repulsive or attractive interactions between bound mSA molecules, even if they exist, do not significantly affect the compactness of sterically denatured GlpG. This result supports our modeling of mSA bound as hard spheres in the DSE simulations (see DSE Simulations).

**DSE Simulations.** To generate models of compact and expanded DSEs and provide references for comparison to the DEER data, we used our Upside MD simulations modified for membrane proteins. This recently created algorithm can fold small proteins with accuracy comparable to all-atom methods (51, 52). Upside employs six interaction centers per residue: the three backbone atoms (N, Cα, and C), the carbonyl oxygen and amide proton, and a side chain represented by a direction-dependent bead. After every MD step, the side chains are globally repacked to have the lowest global free energy. Our coarse graining, the use of an implicit solvent, and the global side-chain packing step explain much of the 10³- to 10⁴-fold speed up compared to standard all-atom simulations (51, 52).

Our membrane-burial potential includes knowledge-based, depth- and lipid exposure–dependent energies for side-chain burial and backbone H bonding within the bilayer (53, 54). Energies are determined from the statistics of a large training set of proteins, which accounts for both the depth Z in the membrane and the level of side-chain exposure to the lipid: $E_{\text{Energy}}(Z, \text{exposure}) \propto \ln(\text{frequency})$. We incorporate depth-dependent energies for unsatisfied backbone H-bond donors and acceptors within the bilayer and allow helices to fold and unfold during the simulations.

At every MD step, side-chain burial is recalculated to account for the exchange of protein–lipid interactions for protein–protein interactions as helices come into contact. This recalculation avoids overestimating the energetics of helix association. Through these and the careful treatment of the bilayer boundary position, our potential can optimally predict the bilayer thickness and protein location, as compared to the OPM (orientations of Proteins in Membranes) method (55). Our force-induced unfolding studies using the membrane potential for GlpG and bacteriorhodopsin agree well with the experiment (53).

A total of 20 4-ms simulations were run for native GlpG and the three doubly mSA-bound versions (95N172M, 172M267C, and 95N267C) at T = 274, 308, 343, 377, and 411 K (400 simulations total, SI Appendix, Fig. S13 A–F). Each mSA was held in its native conformation using a set of stiff restraint springs and linked with a stiff 4-A spring at Asn49, a position near the biotin-binding pocket, to each of the two biotinylation sites on GlpG (Fig. 5A). To eliminate steric overlap, only short-range repulsive interactions were employed between the two bound mSA molecules with each other and with GlpG (a sigmoid potential with ~1 Å width and a drop-off at 8 Å and the repulsive energy of 2kT, where R is a gas constant). To inhibit the
mSA molecules from entering the bilayer, they were subject to the same membrane-burial potential as GlpG.

During the simulations, GlpG and the three doubly mSA-bound versions maintained a native and near-native fold at the lowest two temperatures, 274 K and 308 K, respectively (SI Appendix, Fig. S13 A and B and Table S9). The $C_p$-RMSD for mSA-free GlpG was $5 \pm 1$ Å and $9 \pm 1$ Å at 274 K and 308 K, respectively (Fig. 5B). In support of our steric trapping strategy, the RMSDs of the three mSA-bound GlpG versions were larger ($C_p$-RMSD = 7 to 9 Å and 10 to 12 Å at 274 K and 308 K, respectively) with the average $R_g$ within 1.2 and 2.5 Å of the native value of 16 Å. Based on these RMSD and $R_g$ values, we selected the 274 K and 308 K simulations to represent the NSE (“native-like274K”) and a compact DSE (“collapsed308K”), respectively.

We ran simulations at higher temperatures in hopes of creating an expanded DSE. At 343 K, the protein displayed significantly larger dynamics than at 308 K (Fig. 5B). However, it remained compact ($R_g < 24$ Å) even though the $C_p$-RMSD was often above 10 Å (SI Appendix, Fig. S13C), indicating that the helices remained in contact but no longer retained their native arrangement. Notably, some trajectories underwent large excursions in which TM6 unfolded and remained outside the bilayer, allowing the mSA molecules to sample a much larger region of space (Fig. 5 C and D). This observation agrees with the experimental result that TM6 and the flanking loops of TM5 are prone to proteolysis (Fig. 3). Nonetheless, higher temperatures increased the separation between the TM segments and broadened the corresponding distance distributions. Likewise, the attachment of doubly bound mSA preferentially increased the distance between the structural segments to which their steric repulsion was directly exerted. Based on these conformational features, we referred to this simulated model as “partially expanded DSE343K”.

Given the persistence of helix–helix contacts at 343 K, we adopted an alternative strategy to create a more expanded DSE (Fig. 5 B–D and SI Appendix, Fig. S13F). A total of 20 additional 4-ms simulations were run retaining H bonding and the membrane potential but with the attractive side-chain terms turned off, leaving only a repulsive term to prevent steric overlap. Without interhelical interactions, the temperature was reduced to 240 K to help maintain the helices within the bilayer. Under this protocol, the helices TM1 through TM5 independently diffused around in the membrane, with their separation restricted by interhelical loops. As observed at 343 K, TM6 occasionally unfolded and partitioned into the surface of the membrane. The two small helices in the loop between TM1 and TM2 occasionally unfolded, which allowed TM1 to separate more than the other helices. The overall $C_p$-RMSD and $R_g$ values were large, 23 Å and 30 Å, respectively. We selected this DSE for use as our reference “fully expanded DSEOFF.”

Comparison between DEER and Simulations. Of primary interest is the comparison between the DEER and the simulated distance distributions for sterically denatured GlpG in bilayers.
To quantitatively describe the expansion of the DSE relative to the collapsed and fully expanded states, we defined the normalized expansion ratio, $R_{\text{Expansion}}$ (Eq. 6B):

$$R_{\text{Expansion}} = \frac{r_{\text{Mean,DEER}} - r_{\text{Mean,collapsed}}}{r_{\text{Mean,expanded}} - r_{\text{Mean,collapsed}}},$$

where $r_{\text{Mean,DEER}}$ is the mean distance between a specific residue pair in denatured GlpG determined by DEER, while $r_{\text{Mean,collapsed}}$ and $r_{\text{Mean,expanded}}$ denote the mean distances between the same pair in the simulated collapsed and fully expanded DSE, respectively. When the DSE was trapped using mSA at the more stable N-subdomain (95N172m) which has more extensive intraprotein contacts in the native state than the C-subdomain (SI Appendix, Fig. S14), $R_{\text{Expansion}}$ was 0.05 ± 0.04 in bicelles; that is, this region in the DSE was as compact as the simulated collapsed state. In contrast, when the DSE was trapped using mSA at the less stable C-subdomain (172m267c), $R_{\text{Expansion}}$ reached 0.49 ± 0.08; that is, the DSE expanded close to the simulated partially expanded state. In the DSE trapped at the termini (95n267c), $R_{\text{Expansion}}$ was intermediate (0.26 ± 0.01) which may be an outcome of the collapse in the N-subdomain being offset by the expansion in the C-subdomain.

As noted, the DSE was more expanded in the liposomes than in the bicelles (Figs. 4 and 6A and B). Upon denaturation, the $R_{\text{Expansion}}$ values in liposomes increased from the corresponding values in bicelles by $\Delta R_{\text{Expansion}} = 0.18 ± 0.04, 0.22 ± 0.12, and 0.17 ± 0.03$ for the N-subdomain, C-subdomain, and termini, respectively. This result is consistent with the DSE...
having higher proteolysis levels in liposomes than in bicelles irrespective of the position of the biotin pair (Fig. 2D).

Since the diameters of the bicelles (190 ± 40 Å) and liposomes (510 ± 220 Å) are larger than the maximal N-to-C distances (∼100 Å) in the simulated fully expanded DSE, it is unlikely that the dimensions of the lipid environment affected the compactness of the experimentally obtained DSE. Given the general trends, we infer that for the DSE of GlpG, bicelles and liposomes can qualitatively behave as solvents somewhere between poor and good solvent limits. The precise behavior depends on the specific region in the protein and the type of bilayer, suggesting an inherent degree of variability to solvent quality in different combinations of proteins and bilayers.

Discussion

By combining experiment and simulation, we provided a molecular-level description of the DSE of a membrane protein in a native-like lipid bilayer. The overall picture is a partially collapsed DSE with considerable dynamics and susceptibility to proteolytic cleavage. TM helices can transiently dock against each other but also can unfold. The comparison of the interhelical distance distributions from DEER measurements and the simulations suggests that bicelles and liposomes behave as solvents somewhere between the poor and good solvent limits depending on the bilayer composition and the region of the protein being considered.

Conformational Diversity of the DSE. The two-stage folding model has served as a useful framework to describe the folding of helical membrane proteins (16). Our experimental data and simulations indicate that GlpG’s DSE is more diverse than what is expected from this canonical model where formation of TM helices occurs before their association. Whereas individual TM segments undergo restricted motions within the lipid bilayer, less hydrophobic segments can become solvent exposed after undergoing membrane-topology changes and even unfolding, suggesting that the hydrophobicity of the TM segments is a key determinant of the conformational distribution in the DSE.

Diversity in membrane topology has also been observed locally (e.g., the N-terminal TM helix of multidrug transporter EmrE) (56) and globally (e.g., EmrE and a lactose permease LacY) (57–59) depending on the hydrophobicity of the TM as well as the distribution of charged residues in the flanking regions. Notably, a hydropathy analysis of helical membrane proteins in E. coli predicts that about half of the TM segments have a low tendency to insert into the membrane (ΔGapp,insertion > 0) (56). This observation, combined with our current findings, suggests that in general, multiple segments that are TM helices may not always remain so in the DSEs of the membrane proteome.

We find that the DSE of GlpG expands more in E. coli liposomes than in bicelles despite both having a similar quasi-2D physical constraint. Although multiple factors are operational, the difference could be explained by differing lipid composition. Bicelles are composed of the saturated lipids, neutral diC14:0–PC (75 mol-%) and negatively charged diC14:0–PG (25%), and CHAPS, while E. coli lipids contain neutral PE (∼75 wt/wt-%) and negatively charged phosphatidylglycerol (PG, ∼20%) and cardiolipin (∼5%) headgroups with 16:0 (∼40%), 16:1c9 (∼30%), and 18:1c11 (∼15%) fatty acyl chains (60). Each monolayer leaflet in the bilayer containing the small headgroup and unsaturated PE or cardiolipin tends to bend outward (negative spontaneous curvature) compared to that containing the larger headgroup and saturated PC or PG lipids (61–64). Hence, the E. coli lipid bilayer is “frustrated” by the difference between the spontaneous and actual curvatures with an increased repulsive force between the hydrocarbon chains (lateral tension) in the bilayer core. The increase in lateral tension may facilitate the separation of TM segments within the bilayer or their exclusion to the water-membrane interface, inducing increased expansion and conformational diversity in the DSE.

Contraction of the DSE in the lipid bilayer reflects a balance between the strengths of protein–protein, protein–lipid, and lipid–lipid interactions. Contraction of the DSE can be driven by the increased chemical potential for intramolecular van der Waals packing and the burial of polar residues. For example, the N-subdomain of GlpG, which is more contracted in the DSE, contains more intraprotein contacts than the C-subdomain in the native state (SI Appendix, Fig. S14). Also, an all-atom MD simulation suggests that in the folding of the

Fig. 6. Comparison of the dimensions of the DSEs measured by DEER and Upside simulations. (A) Comparison of the distance distributions obtained from DEER and the simulated DSEs doubly bound with msA. To mimic the interspin distances measured from DEER, the distances from the simulations were monitored between the Asn49 Cαs on bound msA molecules, which were the attachment sites to GlpG. The $r_{\mathrm{mean}}$ values for the simulated “collapsed308K” (green), the experimentally obtained “DSE (DEER)” (black), and the simulated “fully expanded DSEoff” (red) are shown as vertical lines. (B) Expansion ratio ($R_{\exp}$, Eq. 1) of the DSE measured between each residue pair in the micelles, bicelles, and liposomes. The $R_{\exp}$ values of the simulated DSEs at an increasing temperature are overlaid.

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Lipid bilayer induces contraction of the denatured state ensemble of a helical-bundle membrane protein

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CLC-ec1 Cl/H\(^+\) antiporter monomer, the association of the N- and C-domains is promoted by the burial of polar residues and the release of water molecules (22).

A hydrophobic mismatch between the bilayer and TM helices can cause lipid deformation at the TM helix–lipid interface facilitating helix–helix association (27). In the dynamic monomer–dimer equilibrium of CLC-ec1, a coarse-grained MD simulation finds that dimerization is driven by the local lipid deformation induced by the hydrophobic mismatch between the bilayer and the dimer interface (28). A recent folding and assembly study of the tetrameric ion channel KcsA finds that helical monomers rapidly associate into a protein-dense region in asolectin liposomes, indicating that this class of bilayer serves as a poor solvent for these monomers (65). For the isolated monomers, however, the two TM helices are often separated due to the difference in their length, which results in different tilt angles. In general, multiple lipid- and protein-dependent factors can influence the effective solvent quality (65).

Lipid composition can also influence the compactness of the DSE by modulating the properties of the bulk lipid bilayer including “force-from-lipids” (66). For example, TM helix association is affected by lipid composition with negligible changes in the bilayer thickness for various types of single membrane-spanning helices, including the TM domain of glycoporphin A (26, 67), a proton channel M2 (68), a transcriptional regulator Mga2 (29), and an unfolded protein stress sensor Ire1α (69). The origin of such lipid dependence has been attributed to the changes in lateral tension profile (26), lipid packing density (29), membrane fluidity (69), and electrostatic interactions between negatively charged membranes and positively charged residues flanking the TM helices (26).

**Challenges in Quantifying the Solvent Quality of Lipid Bilayers.** Solvent quality has been quantified for disordered soluble proteins using the Flory scaling relationship, \( R_g \propto N^\nu \) (9, 10, 12, 14). This powerful formalism, however, is not readily adaptable to DSEs of membrane proteins. Although a tempting 2D counterpart is a disordered chain in a quasi-2D slab (70), this is a poor solvent for these monomers (65). For the isolated monomers, however, the two TM helices are often separated due to the difference in their length, which results in different tilt angles. In general, multiple lipid- and protein-dependent factors can influence the effective solvent quality (65).

This property can present both benefits and challenges. TM helices largely remain intact in the DSE, but they are dynamic with variable levels of interhelical contacts. The DSEs of other membrane proteins include helical ensembles in SDS, which trade native tertiary contacts for protein–detergent interactions (40, 71), an extended chain in solution when force is applied normal to the bilayer at one end using atomic force microscopy (AFM) (72), and a “zig-zag” state with intact but noncontacting TM helices in the membrane when force is applied to both termini parallel to the bilayer plane using magnetic tweezers (37). In the AFM measurements, the penalty for removing the hydrophobic residues from the lipid bilayer contributes to the stability, whereas this factor is less relevant in the tweezers measurements, as the helices largely remain within the bilayer (37, 72). In contrast, in some folding studies of β-barrel membrane proteins using chemical denaturants, the reference state is a disordered chain in solution (73, 74).

Because the mode of denaturation alters which factors change upon folding, comparisons of the stability (∆\(\Delta G\)) obtained from different modes should be made judiciously. This issue also applies to mutational studies; for example, a change in helical propensity will not affect the change in stability (∆\(\Delta G\)) if the site remains helical in the DSE. Hence, meaningful interpretation of thermodynamic parameters requires the characterization of the DSE. With this extra challenge, along with the inherent difficulties of quantifying the energetics of protein–lipid interactions, it is not surprising that membrane protein thermodynamics remains an extremely challenging area of research.

**Broader Implications.** The solvent quality of the lipid bilayer for membrane proteins is relevant to a variety of biological processes including folding, association, and proteostasis. Our results imply that the lipid bilayer generally facilitates contraction of DSEs and promotes intraprotein interactions. This property can present both benefits and challenges. TM segments make both native and nonnative contacts with each other during the cotranslational insertion (75, 76), while the folding efficiency and maturation of newly synthesized membrane proteins is surprisingly small, 20 to 50% (77). In liposomes, the affinity of specific TM helix–helix interactions dramatically decreases in the presence of total membrane protein extracts, probably due to enhanced nonspecific interactions (26). In addition, various types of TM chaperones are either components of the translocon complex or function independently (77–80). These observations imply that cellular membranes are not necessarily optimal environments for efficient assembly of membrane proteins. The bilayer may promote nonspecific compaction and misfolding, resulting in species that become targets for protein quality control mechanisms in the cells (81–83). On the other hand, enhanced intraprotein interactions can facilitate the formation of membrane protein complexes and the clustering of receptors for effective cellular signaling (84, 85). Presumably, the interaction strength is tuned by varying lipid composition in the different bilayers in the cell to achieve an appropriate compromise between folding efficiency and function in each one.

**Materials and Methods**

Detailed information on materials, preparation and biotinylation of GlpG, activity determination, mass spectrometry, and Upside MD simulation can be found in the SI Appendix.

**Preparation of Biotinylated GlpG.** Double cysteine variants (95C172C, 172C267C, and 95C267C) of E. coli GlpG with an N-terminal His\(_6\)-tag were expressed in E. coli BL21(DE3)RPlp cells, purified in DDM (Anatrace), and labeled with thiol-reactive BtnPyr or BtnRG (36).

**Activity Assay of GlpG.** To measure GlpG activity in micelles and bicolices, the model substrate SN (staphylococcal nuclease)-LYTM2-His\(_6\) fusion in DDM was labeled with the thiol-reactive environment-sensitive fluorophore, iodoacetyl-7-nitrobenz-2-oxa-1,3-diazol (IA-NBD, Setareh Biotech) (36). Time-dependent decrease of NBD fluorescence was monitored with \( \lambda_{ex} = 485 \text{ nm} \) and \( \lambda_{em} = 535 \text{ nm} \). To measure GlpG activity in liposomes, SN-LYTM2-His\(_6\) was separately labeled with fluorescein–iodoaacetamide (Sigma-Aldrich) or dabcyl–maleimide...
Denaturation of GlpG Using Steric Trapping. GlpG labeled with BtnPyr or BtnRG was incubated with a 5-times molar excess of mSA in DDM at 25 °C until maximum denaturation was reached (up to 48 h). The extent of denaturation was monitored using GlpG activity for LTM2.

Reconstitution of GlpG. For reconstitution in bicelles, native or sterically denatured GlpG in DDM was injected into 3 wt/vol-% bicelles (DMPC:DMPG:CHAPS, maximum denaturation was reached (up to 48 h). The extent of denaturation in liposomes, native or sterically denatured GlpG in DDM was added to the extruded liposomes (10 mM E. coli polar extracts, Avanti Polar Lipids) equilibrated with 10 mM DDM. DDM was removed using Bio-Beads (Bio-Rad) followed by additional extrusion. The final GlpG concentration was 5 μM.

Cryo-EM. The bicellar (15 wt/vol-%) sample was prepared with GlpG (95,267–BtnRG, 2 μM) and mSA (25 μM). The lipidosomal (50 mM E. coli lipids) sample was prepared using the same method as for reconstituting denatured GlpG but without the proteins. Cryo-EM grids were frozen using a Vitrobot Mark IV (Thermo Fisher). Briefly, 3.5 μL of each sample was applied to a glow-discharged Quantifoil Cu 1.2/1.3 holey carbon 200-mesh grid. Images were recorded in counting mode using EPU software at a nominal magnification of 92,000× (1.12 Å per pixel), with a defocus of ~3.0 μm. Micrographs were collected as single-frame images with a total exposure time of 1.5 s and a total dose of 30 electrons/Å².

ProK Digestion. Native or sterically denatured GlpG prepared in 10 mM DDM, 3% bicelles, or 10 mM liposomes was proteolysed by ProK (0.14 μg/mL, Sigma-Aldrich) and quenched by permethylation (0.1 mM).

Mass Spectrometry. For CZE-MS/MS, the proteolysis products of free mSA, native GlpG, and denatured GlpG were obtained using the solid phase–enhanced method (86). After washing with 90% acetonitrile and elution with 100 mM NH₄HCO₃ buffer (pH 8), recovered peptides were separated by nanoCZE, using a 12 cm long column with 75 μm inner diameter and 0.075 mm wall thickness as well as the necessary parameter repository (https://github.com/sosnicklab/upside-md). Previously published data were used for this work (36).

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