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YidC Insertase of *Escherichia coli*: Water Accessibility and Membrane Shaping

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Summary

The YidC/Oxa1/Alb3 family of membrane proteins function to insert proteins into membranes in bacteria, mitochondria, and chloroplasts. Recent x-ray structures of YidC from *Bacillus halodurans* and *Escherichia coli* revealed a hydrophilic groove that is accessible from the lipid bilayer and the cytoplasm. Here, we explore the water accessibility within the conserved core region of the *E. coli* YidC using *in vivo* cysteine-alkylation scanning and molecular dynamics (MD) simulations of YidC in POPE/POPG membranes. As expected from the structure, YidC possesses an aqueous membrane cavity localized to the membrane inner leaflet. Both the scanning data and the MD simulations show that the lipid-exposed TM helices 3, 4 and 5 are short, leading to membrane thinning around YidC. Close examination of the MD data reveals previously unrecognized structural features that are likely important for protein stability and function.

eTOC blurb

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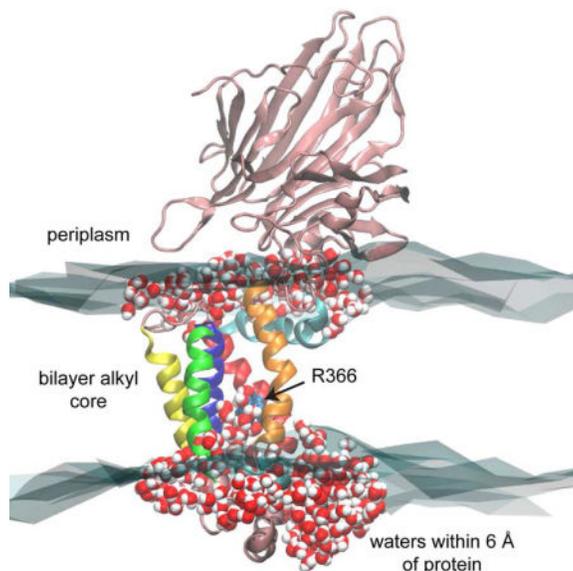
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Author Contributions

R.E.D. and S.H.W. designed the experiments. S.C. carried out the MD simulations under the supervision of S.H.W. Y.C., L. Z. and P.G. performed the cysteine-alkylation studies under the supervision of R.E.D. S.C., J.A.F. and S.H.W. analyzed the molecular dynamics data. S.H.W. and R.E.D. wrote the paper, which was reviewed and approved by all authors. Both S.H.W. and R.E.D. are designated as corresponding authors because of the distinctly different methodologies used in the two labs.



Chen *et al.* have measured the *in vivo* water accessibility of the *E. coli* YidC insertase and carried out MD simulations of YidC in POPE:POPG bilayers. Water accessibilities are similar for the two approaches. The simulations reveal important structural features that stabilize YidC in membranes.

Keywords

YidC; membrane protein insertion; cysteine-scanning mutagenesis; alkylation; aqueous access; membrane thinning; molecular dynamics

INTRODUCTION

In bacteria, the YidC insertase functions in membrane protein insertion and assembly (Dalbey et al., 2014). YidC can promote insertion of membrane proteins independently (Chen et al., 2002; Samuelson et al., 2000; Samuelson et al., 2001; Serek et al., 2004; van Bloois et al., 2004; van der Laan et al., 2004; Yi et al., 2004) or cooperatively with the SecYEG complex (Celebi et al., 2007; Celebi et al., 2006; Yi et al., 2003; Zhu et al., 2012). The single-span pf3 coat protein carrying negative charges in the N-terminal tail is the prototypical substrate for Secindependent insertion (Serek et al., 2004). Structures of YidC were recently determined by x-ray crystallography at high resolution for *Bacillus halodurans* (Kumazaki et al., 2014a) and *E. coli* (Kumazaki et al., 2014b). The crystallographic structure of a YidC-like protein (DUF106) from *Methanocaldococcus jannaschii* demonstrated that YidC-like proteins are present in all branches of life (Borowska et al., 2015).

These YidC structures along with extensive *in vivo* water accessibility studies of the YidC homolog SpoIIIJ in *Bacillus subtilis* (Shimokawa-Chiba et al., 2015) showed that the conserved, membrane-embedded region of YidC contains a U-shaped hydrophilic cavity dominated by a strictly conserved arginine. The cavity is exposed both to the lipid bilayer and cytoplasm, but is inaccessible from the extracytoplasmic side of the membrane. Although this conserved arginine is essential for both bacterial growth and membrane

protein insertion in *Bacillus subtilis*, it is not essential in *E. coli* (Chen et al., 2014). Cysteine cross-linking studies show that the substrate-binding site of the *E. coli* YidC includes TM3 (Klenner and Kuhn, 2012; Klenner et al., 2008; Yu et al., 2008), as well as TM1, TM4, and TM5 (Klenner and Kuhn, 2012). Major contacts between these TM segments of YidC and the hydrophobic segment of a single-span membrane protein substrate occur in the respective YidC/substrate hydrophobic region(s) spanning the membrane (Klenner and Kuhn, 2012). Cryo-EM studies of ribosome-YidC-nascent chain complexes (Kedrov et al., 2016; Wickles et al., 2014) show model substrates bound parallel to TM3 and TM5 of the hydrophilic cavity, consistent with the cross-linking studies.

Here we examine the *in vivo* water accessibility of *E. coli* YidC using Cys-scanning alkylation (Guan and Kaback, 2007; Kimura-Someya et al., 2000; Tamura et al., 2001). In parallel, we carried out a comprehensive 185 ns molecular dynamics (MD) simulation of *E. coli* YidC embedded in a POPE:POPG (75:25) lipid bilayer with the goal of determining water accessibilities for comparison with the *in vivo* measurements. The *in vivo* and simulation results both confirm that the *E. coli* YidC possesses a solvent exposed region that is localized within the inner leaflet of the membrane. Our data reveal significant thinning of the bilayer caused by the short lengths of TM helices 3, 4, and 5. The hydrophilic cavity of YidC and the membrane thinning around it likely reduce the energy barrier of TM helix insertion by decreasing the length of the hydrophobic core of the membrane. The protein appears to be stabilized and tightly linked to the lipid bilayer by interfacial aromatic rings, a cytoplasmic salt-bridge cluster, and a periplasmic interfacial helix enriched in aromatic residues. A cluster of aromatic residues above R366 may provide a polarizable hydrophobic surface for guiding nascent chains across and into the lipid bilayer.

RESULTS

The structure of YidC in a POPE/POPG bilayer becomes more compact compared to the crystallographic structure

Figure 1A shows a snapshot of fully-equilibrated YidC in a 75:25 POPE:POPG lipid bilayer. Interestingly, the key R366 sidechain is located very near the center of mass of the bundle of five helices (TM2 -TM6). Compared to the x-ray structure (Figure 1B), the helix bundle has become more compact. The superposition of the crystal structure with the MD structure (Figure 1C) shows that the main differences are a modest reorientation of Helix CH1 and a very large reorientation of the cytoplasmic helix CH2 (re-labeled here as IF Helix CH2) toward the membrane interface, a likely consequence of the interaction of the C1 region (Figure 1B) with PE and PG headgroups. Residues 480–492—not seen in the crystal structure—have formed a short third cytoplasmic helix CH3. Together, CH1, CH2, and CH3 form what we refer to as the cytoplasmic cap. Importantly, IF helix PH1 is buried in the bilayer hydrocarbon core just beneath the lipid headgroups while IF helix CH2 is located at the interface outside of the hydrophobic core. Importantly, YidC-bilayer interactions have caused the bilayer to thin in the neighborhood of the protein as though riveting the two monolayers together.

Although the crystal structure was determined by the lipidic cubic phase method to produce Type I (lamellar) crystals, the only lipid present was monoölein. We suggest that the absence

of PE and PG headgroups in the crystallization is responsible for the differences between the crystallographic and simulation structures.

Water accessibility of YidC as probed by site-directed alkylation

The structures of YidC from *B. halodurans* and *E. coli* suggested that YidC does not contain a membrane-spanning channel, but rather a U-shaped hydrophilic groove closed on the periplasmic surface but exposed to the cytoplasm and the bilayer (Kumazaki et al., 2014a; Kumazaki et al., 2014b). To examine the water exposure of the groove within TM2-TM6 of the *E. coli* YidC in intact bacteria, we used the Cys alkylation approach of Shimokawa-Chiba et al. (2015), described in detail in Star Methods. Briefly, single Cys residues were introduced one at a time within the TM segments or in the flanking polar regions in a Cys-less YidC, and their accessibility to membrane-permeable NEM determined. Maleimides react with thiolate anions, which require water exposure. They will not react with Cys residues in the low-dielectric (i.e., lipid exposed) membrane environment, because the thiol group is protonated and unreactive. Consequently, NEM reacts with Cys residues within the membrane region only if it has water access. Thus, no change in the position of YidC on a gel when NEM is added prior to the shifting reagent Mal-PEG (M.W.~5000 Da), which labels unreacted Cys residues, indicating a solvent exposed Cys residue (see YidC E24C, Figure 2A, right panel). In contrast, YidC with a TM Cys residue exposed to lipid (see YidC A11C, Figure 2A, middle panel) shows a shift with Mal-PEG even when NEM is added. As a control, we determined that treatment of YidC C24 with Mal-PEG results in a gel shift when NEM is not added before hand. In addition, we verified that a Cys-less YidC does not show a shift with Mal-PEG (Figure 2A, left panel).

To confirm that the YidC Cys mutants were functional, we assayed the mutants for activity using a YidC complementation assay involving the YidC depletion strain JS7131 (Jiang et al., 2003). Figure 2B shows that YidC Cys-less, YidC 24C, and YidC 11C all fully complement the YidC-depletion strain at all the tested dilutions. All the Cys mutants we studied were assayed by this complementation method; except for a few mutants (T362C, M380C, Y412C, N418C, G512C) all were fully active (see Supplementary Table S1). The experimental data for 103 Cys mutants, covering the five conserved TM helices, are presented in summary form in Figure 2C; complete data sets are shown in supplementary Figure S1 and Figure S2. Water exposures were determined from the optical densities of the Mal-PEG-shifted and the non-shifted YidC bands, as described in Star Methods. All experiments were carried out twice.

The mean NEM accessibilities for the Cys mutants are summarized in Figure 3A and Figure 3B. The light-blue bars indicate the NEM accessibility of each mutant. The colored horizontal bars show the locations of the TM helices color-coded as in Figure 1. Broadly, non-helix residues are highly exposed whereas the exposure of helical residues varies from very high to very low. There are only a few residues that have less than 5% exposure (e.g. P431, L436, T503, and L515). Some exposure is expected for all residues, because the *in vivo* experiments probe YidC in a working state that undoubtedly involves significant functional and thermal fluctuations. Cognizant of this fact, we divided exposures into three groups: 30% or less, greater than 30% but less than 75%, and 75% or greater. These results

are similar to those obtained by Shimokawa-Chiba et al. (2015) in their NEM accessibility study of the SpoIIIJ YidC homologue from *B. subtilis*.

Water accessibility of YidC as probed by molecular dynamics

Water accessibilities of the amino acid residues probed using NEM exposures were also determined by counting the average number of waters within 6 Å of each residue during the last 34 ns of the 185 ns equilibrium simulation. Recognizing that large residues inherently have greater exposure than small ones, we normalized the data according to the standard-state accessible surface areas A^0 of fully exposed residues of soluble proteins determined by Rose et al. (1985). A^0 varies between 88 Å² for Gly and 266 Å² for Trp. The normalization allowed accessibility to be expressed as a fraction ranging from 0.0 to 0.85. The fractional accessibilities are shown in Figure 3A and Figure 3B as vertical red bars.

The agreement between the simulation exposures and NEM exposures is quite good, which generally validates the MD simulations. Residues that have low or no exposure according to the simulation correspond to residues with less than 30% NEM exposure. But there are a few significant discrepancies (vertical arrows in panels A and B of Figure 3), specifically L427, I432, L434, and V500. Interestingly, all of these residues are on either Helix 3 (L427, I432, L434) or Helix 5 (V500) that form the entrance to the hydrophilic cavity from the bilayer interior (Figure 3C).

Residues with low water accessibility form a narrow band in the center of YidC

Figure 4A shows a snapshot of the waters within 6 Å of the protein near the midpoint of the equilibrated simulation. Broadly, waters can penetrate to the level of R366, having a funnel-shaped distribution whose narrow end is located at R366 and are highly dynamic (Movie 1). The broad end of the funnel is located roughly at the cytoplasmic lipid headgroup/hydrocarboncore boundary defined by the glycerol C-2 carbon surface. There is little water penetration into the protein at the periplasmic surface.

In Figure 4B and Movie 2, we show the results of the NEM water accessibility of the amino acids in TM segments combined with a snapshot of the residues having waters within 6 Å, as determined in the simulation. The residues are shown in wire-frame surface representations and color-coded according to NEM accessibility. The residues with low accessibility (30% or less) occupy roughly the central one-third of the helical bundle.

YidC thins the bilayer uniformly but asymmetrically in its vicinity

Asymmetric thinning of the POPE:POPG bilayer in the vicinity of YidC is strikingly apparent in Figure 1A. The periplasmic bilayer surface is relatively flat compared to the indented cytoplasmic surface. The general shape of the bilayer within 20 Å of the protein's center of mass at R366 is presented more clearly in Figure 5A. A periplasmic view of the protein and surrounding lipids is presented in Figure 5B. The carbonyl groups of the phospholipids are represented as colored spheres: lipids within 20 Å are colored blue, within 40 Å purple, and greater than 40 Å pink. Phosphates on the opposite (cytoplasmic) monolayer are represented by the same colors, but faded.

We examined the transbilayer distributions of the lipid carbonyl groups along the bilayer normal relative to the protein center of mass, within each of the three zones illustrated in Figure 5B and Figure S6. The carbonyl groups were chosen because they are excellent markers of the headgroup/hydrocarbon boundary of the bilayer (Wiener and White, 1992). The statistical distributions of the carbonyls are shown in Figure 5C. The mean hydrocarbon thickness in the 20-Å shell is about 28 Å whereas in the outer-most shell it increases to about 33 Å (Supplementary Table S3). A striking and significant feature of carbonyl distributions within the 20-Å shell is their asymmetries (arrows), which mirror the flatness of the bilayer in the vicinity of YidC. In addition, the periplasmic peaks are all coincident whereas the cytoplasmic peaks move progressively outward, which also mirrors the flatness of the bilayer in YidC's vicinity. Within the 20 Å zone, the periplasmic carbonyl peak is located 16 Å above the protein center of mass whereas the cytoplasmic peak is located 12 Å below the center of mass, consistent with the dimpling apparent in Figure 1A. These data suggest that YidC and the bilayer in its immediate vicinity are locked together structurally, causing the periplasmic and cytoplasmic surfaces to be remarkably flat.

Rings of aromatic residues and a salt-bridge cluster stabilize YidC in the membrane

Aromatic residues, particularly Tyr and Trp, are found at the membrane interface of virtually all membrane proteins (Killian and von Heijne, 2000; Ulmschneider et al., 2005; Wallin et al., 1997), as first reported for the photosynthetic reaction center (Schiffer et al., 1992). The preference of Trp (and probably Tyr) for interfaces is most likely due to its flat rigid shape that limits access to the hydrocarbon core and its electronic structure and associated quadrupolar moment that stabilize the protein in the bilayer interface by complex electrostatic interactions (Yau et al., 1998). It is thus not surprising that YidC is highly enriched in aromatics at the membrane interfaces (Figure 6 and Movie 3). There are fourteen aromatics (including Phe) at the periplasmic surface. Of these, six located on IF helix PH1 (Figure 6B, Movie 3) likely provide strong stabilization. Two lysine residues (K342 and K345) that snorkel into the interface to interact with lipid phosphates may provide additional stability (Movie 3).

Fewer aromatics are found at the cytoplasmic interface where only seven are found distributed at the cytoplasmic termini of the TM helices except for TM6 (Figure 6B). These seven aromatics and the cluster of salt bridges—the cytoplasmic cap—presumably provide additional stability to YidC (Figure 6A, Figure 6E, Movie 3). The acidic and basic residues involved in salt bridging are heavily concentrated on IF helix CH2 (Figure 6D). As shown in Figure 6E and Movie 4, inter-residue salt-bridges are concentrated in the interfacial extension of TM2 (helix CH1, pink) and IF Helix CH2 (cyan). Some of these also form salt bridges to the phospholipid head groups. Interestingly, there are four basic residues (K416, K382, R384, K401) that are not hydrogen bonded to acidic residues or phosphates. The critical R366 sits above these residues toward the periplasm. We speculate that these residues might serve as guideposts for movement of the negatively charged N-terminal amino acids of substrates (Serek et al., 2004) into the YidC/bilayer interface for passage across the membrane.

A cluster of aromatic residues shields the critical arginine and may provide additional stability

The aromatic cluster shown in Figure 6A stood out glaringly in our initial examination of membrane-embedded YidC. The cluster in other YidC species, however, is not as dramatic, based upon the sequence alignment of Kumazaki et al. (2014b). Such clusters are known to stabilize soluble proteins and likely play a similar role in stabilizing *E. coli* YidC (Burley and Petsko, 1985; Kannan and Vishveshwara, 1999). The sequence alignment of Kumazaki et al. (2014b) reveals three positions that are invariably occupied by aromatics across species: Y437, Y516, and Y517. The aromatics at positions 516 and 517 are interesting, because they sit directly above R366 toward the periplasmic surface (Figure 7A and Figure 7B) acting as a periplasmic lid over R366. The Tyr residues interact with R366 via H-bonds mediated by water molecules. It is notable that aromatics can interact favorably with positive charges through cation- π interactions (Dougherty, 1996). R366 and its tyrosine lid do not appear to be directly exposed to the TM segments of nascent chains, but rather sit well back from the critical TM3 and TM5 helices that form the so-called greasy slide (Dalbey and Kuhn, 2014) (Figure 7C, Figure 7D, Figure 7E) that have been established as providing the principal interaction zones of incoming nascent chains (Klenner and Kuhn, 2012).

The high sequence conservation of R366, Y516, and Y517 led us to examine the effects of mutating these residues. Surprisingly, Cys substitutions for each of these residues separately complemented the wild-type protein (Table S1) as did Ala and Ser substitutions (Figure S4), which is at odds with the finding that mutation of Y517, but not Y516, to alanine inactivated YidC (Wickles et al., 2014). Because waters are shared between R366, Y516, and Y517 (Figure 4A, Figure 7A, Figure 7B), we determined the NEM accessibilities of the Cys-substituted residues in addition to MD-determined water accessibilities (Table S2). The data show that R366 is 90% exposed whereas Y516 and Y517 are exposed only 8% and 34%, respectively. The accessibilities determined from the molecular dynamics simulation are consistent with the NEM-determined accessibilities.

Discussion

We have used site-directed cysteine alkylation (Figure 2) and molecular dynamics simulations (Figure 3, Figure 4) to examine the water exposure of the hydrophilic groove within the membrane-embedded TM sequences of *E. coli* YidC (Kumazaki et al., 2014a; Kumazaki et al., 2014b). The agreement between the MD data and the alkylation data is striking (Figure 3A and Figure 3B). The only serious disagreement is the water accessibility of residues L427, I432, and L434 on TM3, and V500 on TM5 (Figure 3C). These are quite accessible according to the alkylation data, but inaccessible according to the MD data. We suggest that this reflects in part dynamic motions of TM3 and TM5 that occur *in vivo* in the functioning protein that are not possible in the simulation. A conformational change in the TM2, TM3, and PH1 region was observed in a cryo-electron microscopy study of a Foc substrate complexed with a YidC-nanodisc-ribosome complex (Kedrov et al., 2016). The fully inserted TM segment of Foc was found to be located at the interface of the TM3-TM5 greasy slide region of YidC.

We examined the water accessibility of the protein in the context of the residues on TM3 and TM5 that contact incoming nascent chains (Figure 7F). If we think of the nascent chain-contacting residues on TM5 (F502, F505, P468, and W508) and TM3 (F424, L427, and M430) as marking the insertion pathway, Figure 7F suggests a handoff of the nascent chain from TM3 to TM5, because substrate-interaction sites TM3 are near to the cytoplasmic membrane surface while interaction sites on TM5 are near the periplasmic surface. The aromatic cluster (Figure 6A) shared between TM3 and TM5 might provide an electrically polarizable hydrophobic pathway, allowing it to interact favorably with both non-polar and polar amino acids on the nascent chain.

Compared to the YidC x-ray structure (Figure 1B), the equilibrium structure of YidC in a POPE:POPG (75:25) lipid bilayer in excess water is more compact, involving principally reorientation of the cytoplasmic interfacial helix CH2 (Figure 1C). The missing residues in the crystal structure (480–492), which were modeled into the structure prior to the simulation, form a short helix (CH3) that is part of the cytoplasmic cap and salt-bridge cluster (Figure 6A). Significantly, helix PH1 is buried in the bilayer beneath the lipid headgroups whereas helix CH2, the interfacial extension of TM2 (helix CH1), and helix CH3 are located outside the bilayer hydrocarbon core. These structural rearrangements apparently cause the uniform but asymmetric thinning of the bilayer of about 5 Å that produces the dimpling effect apparent in Figure 1A. The 28-Å thickness of the bilayer surrounding YidC is quite uniform within a 20-Å radius (Figure 5A and Figure 5C). Wickles et al. (2014) reported membrane thinning of up to 8 Å around YidC in an MD simulation, but their simulation was of a clever YidC computational model rather than the native protein. Kedrov et al. (2016) noted a strong evolutionary coupling between PH1 and helix 2, suggesting a tight structural connection. This suggestion is supported by our study in that the geometry of PH1 and helix 2 is essentially identical in the crystal structure and the MD structure (Figure 1C).

We suggest that our MD structure is an accurate representation of the *in vivo* structure, because the packing and orientation of the TM helices and IF helix PH1 are essentially the same as in the crystal structure (Figure 1C). The reorientation of IF helix CH2 in the simulation makes perfect sense in the context of aromatic/membrane interface interactions and lipid/protein salt bridges that are expected to occur at the POPE/POPG interfaces. These interfaces are not available in the crystal, because the bilayer in the crystal lacks phosphate headgroups.

As a further test of our MD structure, we carried out additional NEM accessibility studies of interface-buried IF helix PH1 and non-buried IF helix CH2 (Figure S3). The results are summarized in Figure 1D. As expected for an amphipathic helix at a membrane interface, the residues of PH1 buried in the bilayer hydrocarbon have little water exposure (red highlighting) whereas residues facing away from the bilayer hydrocarbon have high exposure (green highlighting). Helix CH2, on the other hand, is not buried in the membrane but rather is located at the interface where one expects significant water exposure, which we observed. Interestingly, however, the exposure is rated as intermediate (yellow highlighting). This likely reflects the complex environment of CH2 (Figure 6E).

The C1 region of YidC in the crystal structures had rather high crystallographic B factors (Kumazaki et al., 2014b). However, the increased B factor may actually arise in part from looser packing in the crystal due to the absence of the phospholipid bilayer (compare panel B with panel A, Figure 1). Unlike a simulation of the *B. halodurans* YidC in a lipid bilayer (Kumazaki et al., 2014a), the C1 region of our *E. coli* YidC was comparatively stable with RMSFs less than 3 Å (Figure S5D). This might be because the *B. halodurans* YidC simulation was carried out in a pure POPE lipid bilayer. Nevertheless, the C1 region in our simulation did have higher RMSFs compared to the membrane-buried TM helices. Significantly, helix PH1 had an exceptionally low RMSF, consistent with its apparent tight coupling to TM helix 2.

The compact and stable shape of YidC within the bilayer originates in part from impressive rings of aromatic residues on its cytoplasmic and periplasmic ends located at the hydrocarbon-head-group boundaries of the bilayer (Figure 6). Beneath the cytoplasmic aromatic ring, a salt-bridge cluster stabilizes the C1-C2 loop through internal salt bridges and salt bridges to the bilayer phosphate groups (Figure 6E). The NEM water accessibility data are generally consistent with the structure determined in the bilayer environment (Figure 1D, Figure 4B and Figure 7).

Our study clarifies the nature of the U-shaped hydrophilic cavity that penetrates roughly halfway into the membrane from the cytoplasmic surface (Kumazaki et al., 2014a; Kumazaki et al., 2014b; Shimokawa-Chiba et al., 2015). The MD simulation shows that waters penetrate to the level of R366 and are highly dynamic (Movie 1) with little stable water penetration into the protein at the periplasmic surface in the simulation (Figure 4A). This dynamic water penetration allows solvent access observed in both the NEM and MD measurements (Figure 3 and Figure 4B).

The MD simulation reveals novel features associated with the burial of R366 in the membrane environment. This strictly conserved arginine has an aromatic lid on its periplasmic side formed by tyrosines at positions 516 and 517 (Figure 7A, Figure 7B). The Tyr residues interact with R366 via H-bonds mediated by water molecules. Additional stabilization likely arises from cation- π interactions (Dougherty, 1996). We think it is significant that R366 and its tyrosine lid do not appear to be directly exposed to the TM segments of nascent chains, but rather sits well back from the critical TM3 and TM5 helices (Figure 7C, Figure 7D, Figure 7E) that have been established as providing the principal interaction zones of incoming nascent chains (Klenner and Kuhn, 2012). It has been proposed that the hydrophilic region of a YidC substrate is accommodated in the hydrophilic cavity prior to its translocation, meaning that it could approach R366 (Kumazaki et al., 2014b). An alternative hypothesis is that R366 and its tyrosine lid provide a positively charged membrane indentation extending to the center of YidC that shapes the local electric field within the protein. Such shaping of the local electric field might preclude the necessity for a direct approach by the nascent chain. Given the intriguing juxtapositions of R366, Y516, and Y517 and their very high sequence conservation, it is mysterious that each can be mutated to Cys or Ala without affecting *E. coli* viability. We suppose the high conservation results from evolutionary stresses that are not apparent under laboratory conditions.

The water accessibility of the protein in the context of the residues on TM3 and TM5 that contact incoming nascent chains (Figure 7) suggests an insertion pathway in which there is a handoff of nascent chains from TM3 to TM5 via the aromatic cluster situated between TM3 and TM5. These aromatic residues may provide an electrically polarizable hydrophobic pathway that allows it to interact favorably with both non-polar and polar amino acids on the nascent chain on the periplasmic side of the membrane.

CONTACTS FOR REAGENT AND RESOURCE SHARING

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EXPERIMENTAL MODEL AND SUBJECT DETAILS

BL21 (DE3) cells used for Cys alkylation assay were purchased from Agilent Technologies (200131). They were grown in LB media at 37°C with shaking.

JS7131 cells used in complementation assay were from our lab collection. They were grown in LB media supplemented with 0.2% arabinose at 37°C with shaking.

METHOD DETAILS

Bacterial growth, Cys alkylation assay, and Western blots

Single Cys mutants of YidC were made using the pEH1-YidC (C423S, Cys-less) vector as a template. An overnight culture of BL21 (DE3) bearing the single Cys pEH1-YidC expression vector was back-diluted 1:100 into fresh LB media containing kanamycin (50 µg/ml final concentration). Cells were grown to mid-log phase ($OD_{600} \approx 0.55$) and IPTG was added to 1mM final concentration to induce expression of the YidC protein for 30min. The cells were subsequently washed twice with PBS buffer and placed on ice.

For the Cys alkylation assay, the cell density was adjusted to $OD_{600} \approx 2.0$ and split into 3 aliquots (0.2ml for each tube). The first aliquot was treated with NEM (0.5 mM final concentration) while the second and third aliquots were left untreated. These aliquots were then held at 30°C for 30min and subsequently washed twice with PBS buffer (with 5mM DTT) and three times with PBS buffer (without DTT). To remove YidC aggregates, cells were harvested, resuspended in 20 µl buffer A and incubated for 30 min on ice. Buffer B (380 µl) was then added, followed by sonication with Sonic Dismembrator Model 500 (Fisher Scientific) (microtip, 40% duty, eight cycles) while cooling. Intact cells and aggregates were removed by centrifugation at 16,000 g for 30 min at 4°C. Super natants were collected and precipitated with 10% (final concentration) ice-cold trichloroacetic acid for 1h on ice. The pellets were washed with ice-cold acetone and solubilized with 100µl Tris-SDS-Urea buffer [15 mM Tris-HCl, 6M Urea, 2% SDS, pH 7.5]. The first and third aliquots was treated with Mal-PEG (M.W.~5000 Da, 5mM final concentration) for 40 min at 37 °C, while aliquot 2 was mock-treated with 5mM PEG6000. The protein samples were then analyzed by 10% SDS-PAGE followed by Western blotting. The YidC proteins with a

C-terminal His-tag were detected using anti-6His antibody (HRP conjugated, from Abcam) and a Chemiluminescent detection kit (Pierce).

Complementation assay

The YidC depletion strain, JS7131 bearing pEH1-YidC encoding the single cysteine mutants were cultured in LB media containing 0.2% arabinose and 50 μ g/ml Kanamycin at 37°C. Overnight cultures were washed twice with fresh LB media and back diluted 1:100 into LB media without arabinose. After 2h of growth, the cells were serially diluted (1:10, 1:100, 1:1000 and 1:10,000 fold) in LB medium. Aliquots of the diluted cells were then spotted on LB plates containing 50 μ g/ml Kanamycin, and 0.2% arabinose or 0.2% glucose, respectively. Where indicated, 50 μ M IPTG was added and the plates were incubated overnight at 37 °C.

Quantification of NEM modification of YidC single cysteine mutants

The optical density of the Mal-PEG-shifted and the non-shifted YidC bands were quantified using ImageJ software developed at the National Institutes of Health (<https://imagej.nih.gov/ij/>) using modification efficiency $E = (\text{Intensity of non-shifted YidC protein}) / [(\text{Intensity of non-shifted YidC protein}) + (\text{Intensity of shifted YidC protein})]$.

Molecular Dynamics Simulations

We carried out all-atom molecular dynamics simulation of YidC embedded in a lipid bilayer using NAMD 2.9 software (Kalé et al., 1999; Phillips et al., 2005) with CHARMM 36 (Klauda et al., 2010) for the lipids and CHARMM 22 with the CMAP correction (MacKerell et al., 1998; MacKerell et al., 2004) for the protein. TIP3P model (Jorgensen et al., 1983) was used to describe water molecules. To start the simulation, we used the coordinates from the *E. coli* YidC crystal structure (Kumazaki et al., 2014b) (PDB code: 3WVF). Missing residues 480–492 between TM4 and TM5 were modeled using Phyre2 software (Kelley et al., 2015). We modeled all the His amino acids in the N δ 1 tautomeric state and maintained positive charge for Arg/Lys and negative charge for Asp/Glu. This complete YidC structure was then minimized in vacuum and used as a starting configuration to set up the atomistic simulation. Using VMD software (Humphrey et al., 1996), we aligned the principal axis of YidC with the x, y, and z space directions. We then embedded YidC in a bilayer formed by 500 lipid molecules (250 each leaflet) constructed with CHARMM-GUI software (Wu et al., 2014). The bilayer was formed with 75% of POPE and 25% of POPG lipid molecules, meaning 186 POPE and 64 POPG in each leaflet. We used these percentages of lipid species in order to mimic the real membrane environment in which YidC is found. We solvated the system with 89 waters per lipid and added 116 potassium ions in order to conserve electrical neutrality. Before starting the minimization and equilibration of the system, we ensured that steric clashes were avoided. The simulation was carried out for ~185 ns, as described in detail in Supplementary Information.

We minimized the system energy using a conjugate gradient algorithm for 8000 steps heated it through a temperature ramp made of steps of 25 K starting from 25 K up to 300 K. In order to equilibrate the positions of the lipids, water molecules, ions, and the protein, we used harmonic restraints, which have been switched off in a series of consecutive runs of 1

ns length. Once the equilibration procedure was completed, we started the production run in the *NPT* (constant number of particle *N*, pressure *P*, and temperature *T*) ensemble. We used a time step of 1 fs and a reversible multiple time-step algorithm (Grubmüller et al., 1991) to integrate the equations of motion with time steps of 1 fs for the bonded forces, 2 fs for the short-range non-bonded forces, and 4 fs for the long-range electrostatic interactions. These last interactions were computed using the smooth particle mesh Ewald summation (Darden et al., 1993; Essmann et al., 1995). The short-range interactions were cut off at 12 Å by means of a switching function between 10 and 12 Å. The SHAKE algorithm (Ryckaert et al., 1977) was used to maintain the length of the bonds involving hydrogen atoms. The temperature was maintained at 300 K using a Langevin dynamics scheme, and a flexible cell in association with the Nosé-Hoover Langevin piston to keep the pressure constant at 1 atm (Feller et al., 1995; Martyna et al., 1994). The simulation was carried out for ~185 ns; trajectories were saved every 10 ps. We monitored the secondary structure by using STRIDE plugin of VMD (Frishman and Argos, 1995). We calculated the solvent exposure of the YidC TM domain by monitoring over the full length of the simulation the residence time of the water molecules within 6 Å of each residue. Then, we normalized each value using the standard accessible surface areas (Rose et al., 1985).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TM	transmembrane
NEM	N-ethylmaleimide
Cys	cysteine
TCA	Trichloroacetic acid
Mal-PEG	Methoxypolyethylene glycol maleimide
DTT	Dithiothreitol
MD	molecular dynamics
POPE	palmitoyloleoylphosphatidylethanolamine
POPG	palmitoyloleoylphosphatidylglycerol
RMSD	root mean-squared deviation
RMSF	root meansquared fluctuation

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Highlights

- * *In vivo* water accessibility of *E. coli* YidC was determined using NEM labeling
- * Accessibility of YidC determined from all-atom MD simulations agrees with NEM results
- * Compared to crystal structure, YidC structure in POPE:POPG is much more compact
- * YidC thins the bilayer locally due to interface aromatic residues and salt bridges

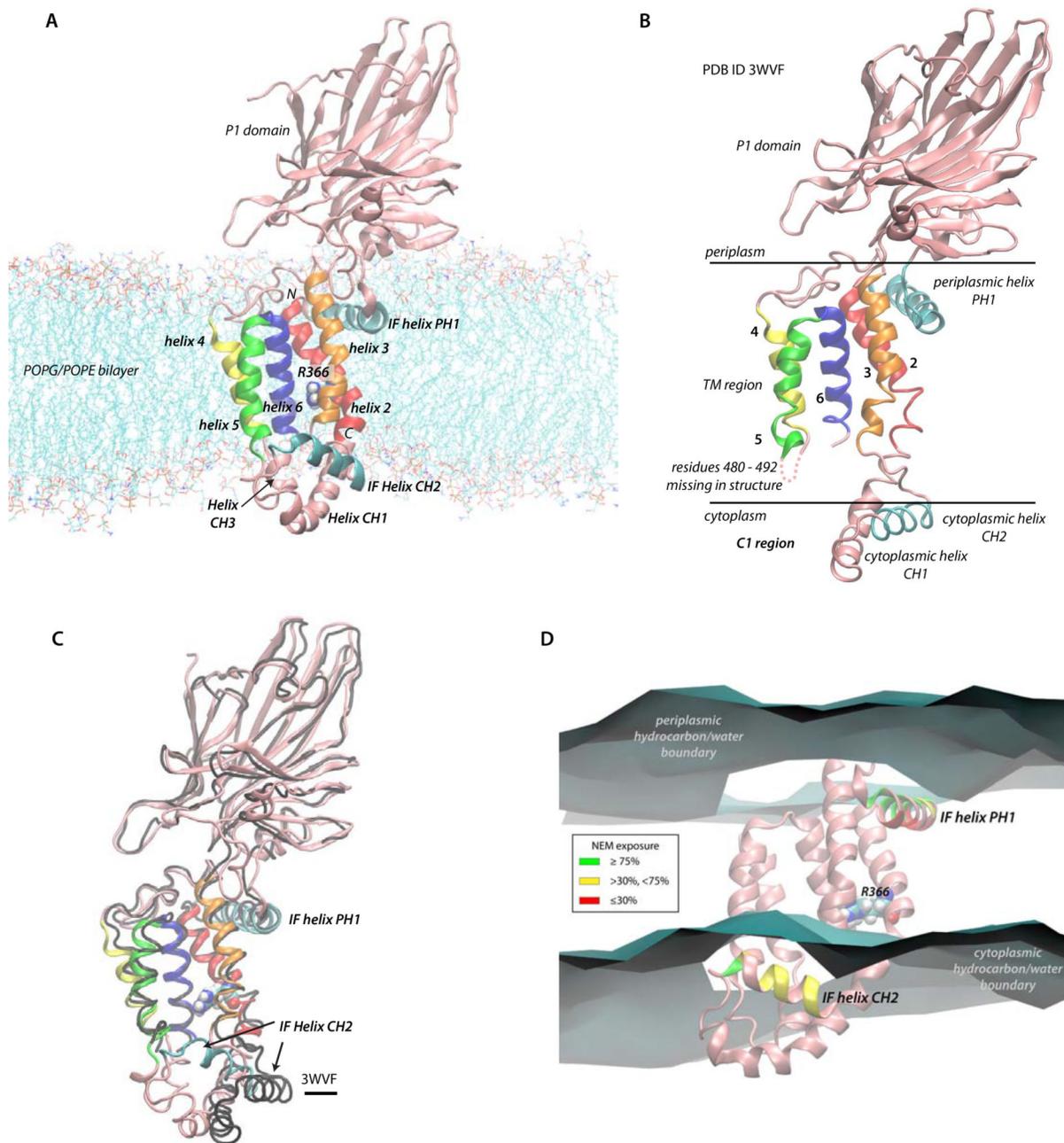
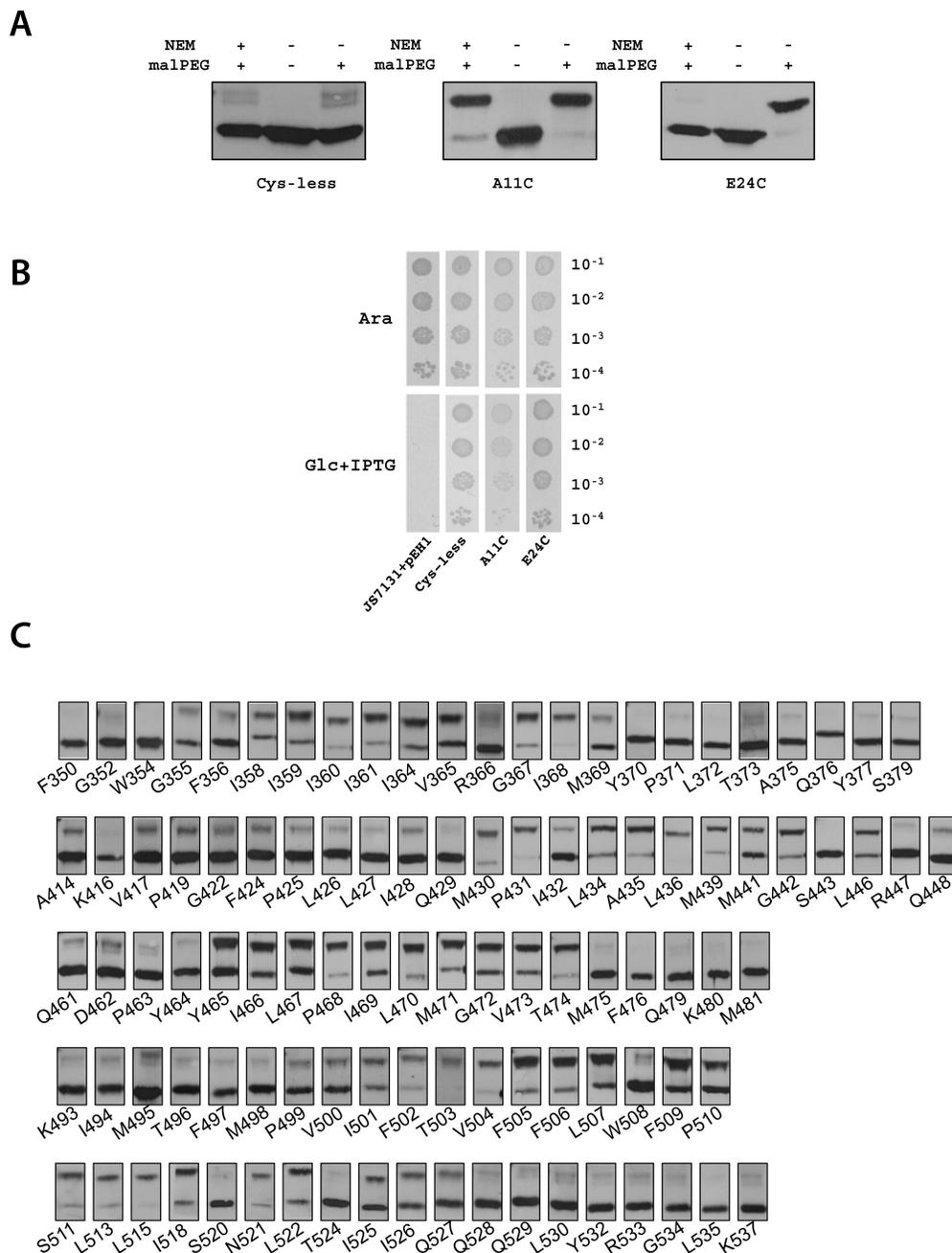


Figure 1.

The transmembrane region of the YidC insertase becomes more compact and ordered in POPE/POPG bilayers. *E. coli* YidC has six TM helices, but TM1 near the N-terminus, which is not required for normal functioning, was disordered and not observed in the structure. Consequently, the observable TM helices in the structure are numbered 2 through 6. The colors of the helices are preserved for all figures in the paper (TM2, red; TM3, orange; TM4, yellow; TM5, green; TM6, blue). **A**. A snapshot of the equilibrated structure of *E. coli* YidC in a POPE:POPG (75:25) bilayer. The front half of the bilayer facing the viewer has been removed to show more clearly the arrangement of the YidC helices. **B**. The crystal structure

of *E. coli* YidC determined by Kumazaki et al. (2014b); structural regions designated by Kumazaki et al. are shown. Because of crystal disorder, residues 480–492 in the TM4-TM5 loop were missing in the structure (dashed pink line). For the MD simulations, the missing loop residues were modeled into the protein using Phyre2 (Kelley et al., 2015). **C.** The crystal structure in panel B, shown in this panel in black, has been overlaid on the simulation structure shown in panel A to reveal that the most significant differences between the two structures occur in the C1 region (panel B). We attribute the compaction of the C1 region to interactions between POPE and POPG that are missing in the crystals, which contain only glycerol monoolein. **D.** Summary of NEM exposure of residues in helices PH1 and CH2. We arbitrarily define inaccessibility as 30% or less modified by NEM, partially accessible by 30%–75% labeling, and fully accessible by greater than 75% labeling. Notice that the amphipathic character of PH1 buried in the membrane interface is clearly revealed by the accessibilities (unexposed on the surface facing the bilayer hydrocarbon core; highly exposed on the surface facing the periplasm). The labeled residues on the CH2 helix reveal only partial exposure for all labeled residues. High exposures would be expected based on the crystal structure alone, because CH2 is not able to engage a membrane interface.

**Figure 2.**

Cys-based alkylation method to map the lipid and solvent exposed membrane regions of YidC. **A.** The gel-shift assay employed to examine whether a Cys residue is in a solvent- or lipid-exposed environment. The solvent-exposed YidC 24C and lipid exposed 11C, as well as Cys-less control were analyzed in this example. BL21 cells expressing the indicated YidC mutants were treated with or without NEM, washed and analyzed as described in the Experimental Procedures. Where indicated, the samples were treated with Mal-PEG, which modifies unreacted Cys residues, and then analyzed by SDS-PAGE and Western blot. Solvent exposures were quantitated using optical scans of the blots (STAR Methods). **B.**

Complementation assay for monitoring the activity of the YidC mutants. JS7131 cells harboring the indicated pEH1 YidC Cys mutant were grown under YidC expression condition (top panel) and YidC depletion (bottom panel). For controls, JS7131 cells with vector encoding the Cys-less YidC (C423S) mutant or with the empty vector (pEH1) were analyzed. C. Summary of the accessibility of Cys residues introduced into helices TM2 through TM6 to map the solvent and lipid exposed residues following the procedures summarized in Panel A. In all cases, the samples were treated with NEM followed by treatment with Mal-PEG, as discussed for panel A. The entire data set is shown in Figures S1 and S2.

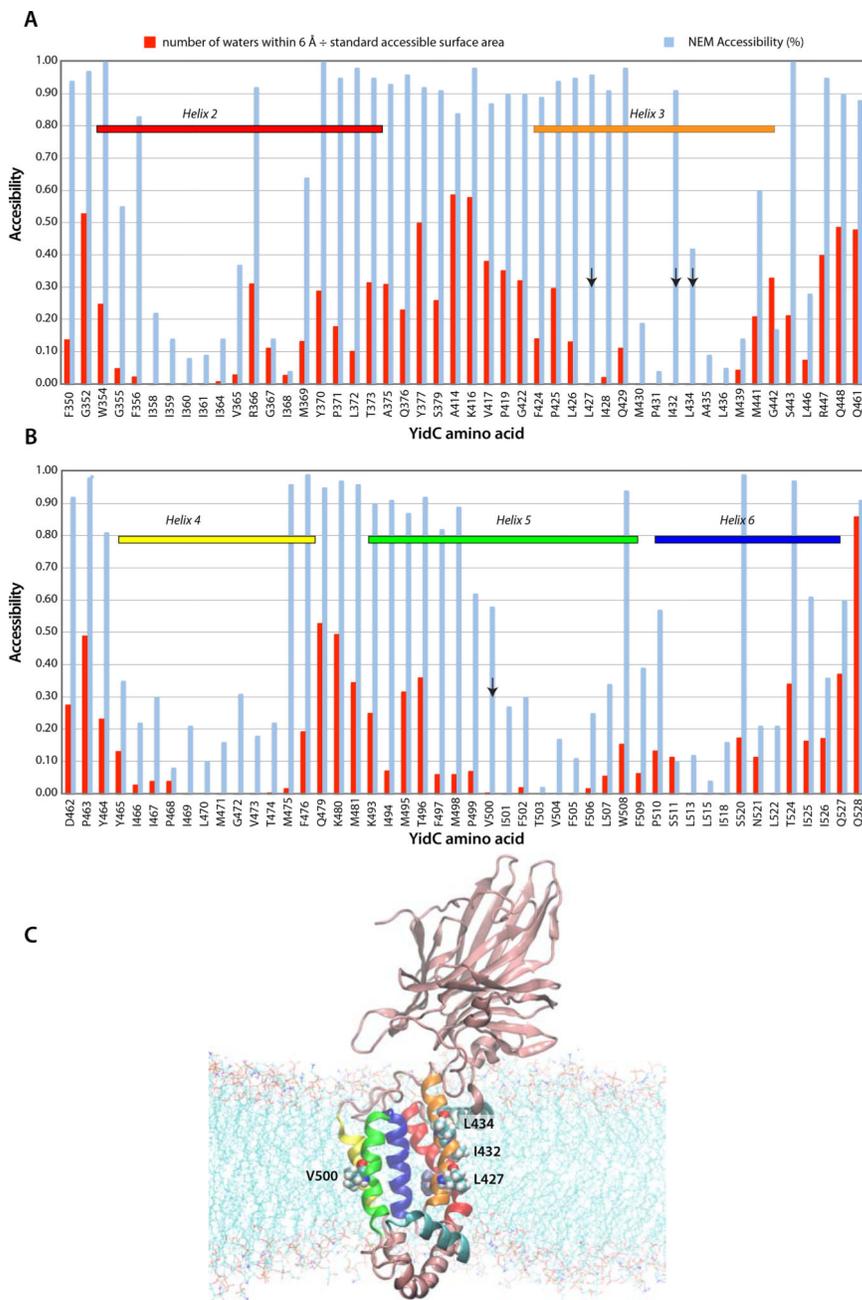


Figure 3. The water accessibilities of YidC residues determined by the NEM assays generally agree with water accessibilities determined from the MD simulations of YidC in POPE/POPG bilayers. Accessibilities determined by the NEM assays are shown as light blue bars; accessibilities determined from the MD simulation are shown as red bars. The vertical arrows indicate four residues for which significant discrepancies exist between the NEM and MD data. We arbitrarily define inaccessibility as 30% or less modified by NEM, partially accessible by 30%–75% labeling, and fully accessible by greater than 75% labeling. Vertical arrows indicate residues with high NEM exposure but low MD-determined accessibility. **A.**

Accessibility results for residues 350–461. **B.** Accessibility results for residues 462–528. **C.** Structure of YidC in the lipid bilayer showing the four residues (vertical arrows, panels A and B) for which significant discrepancies exist between the NEM and MD results. L427, I432, L434, and V500 have significant exposure (greater than 30%) according to NEM labeling but virtually no exposure according to the MD results.

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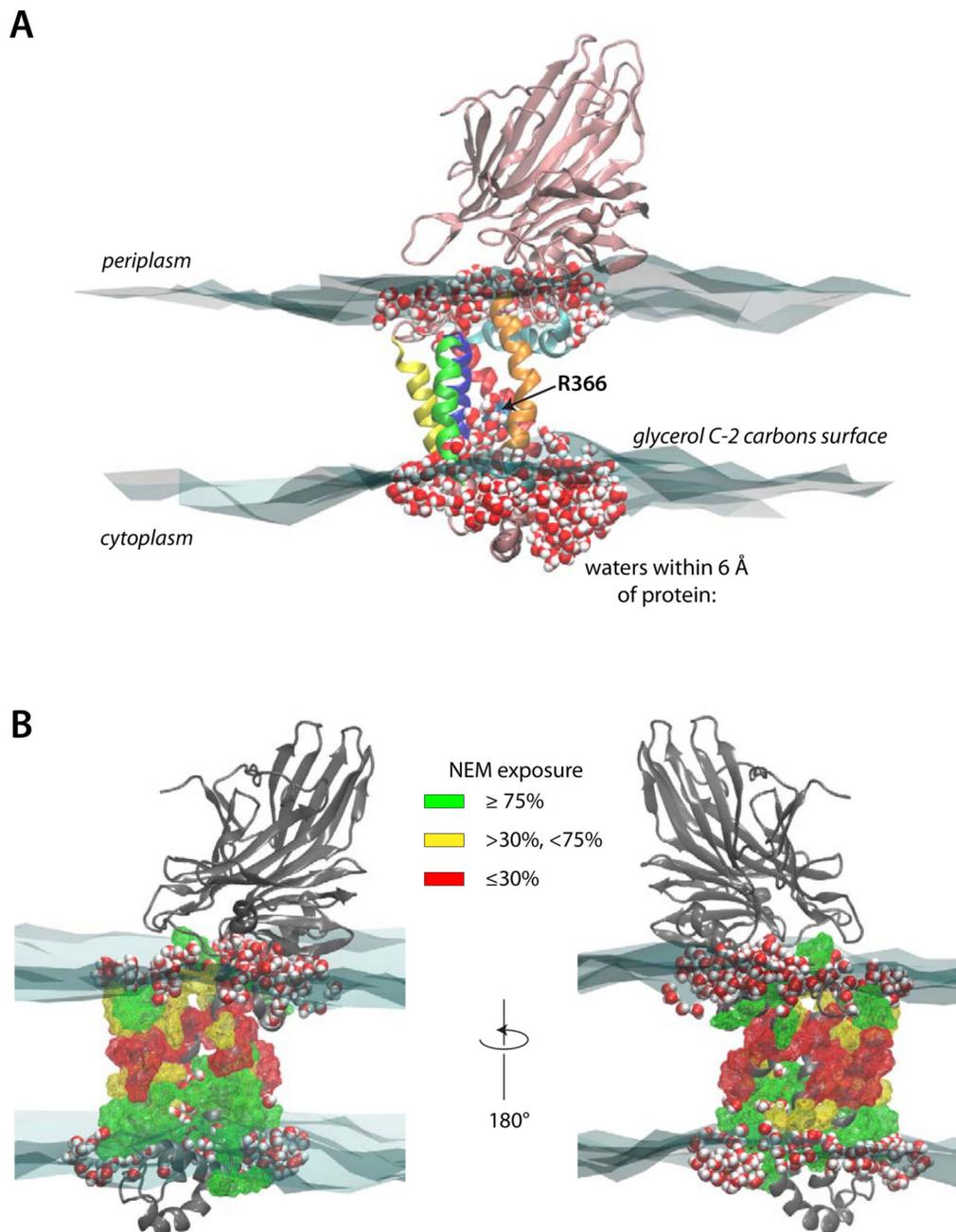


Figure 4.

Water accessibilities in the context of the in-bilayer YidC structure reveal a narrow band of inaccessibility near the bilayer midplane. **A.** A snapshot from the MD simulation showing the waters within 6 Å of the protein. The headgroup/hydrocarbon boundary defined by the glycerol C-2 carbons (Wiener and White, 1992) is shown as a transparent blue surface constructed using Delaunay triangulation. Waters can penetrate roughly to the middle of the protein due to hydration of the strictly conserved Arg366 residue. **B.** NEM water accessibility of TM-segment amino acids represented by wire-frame surfaces colored according to NEM exposure.

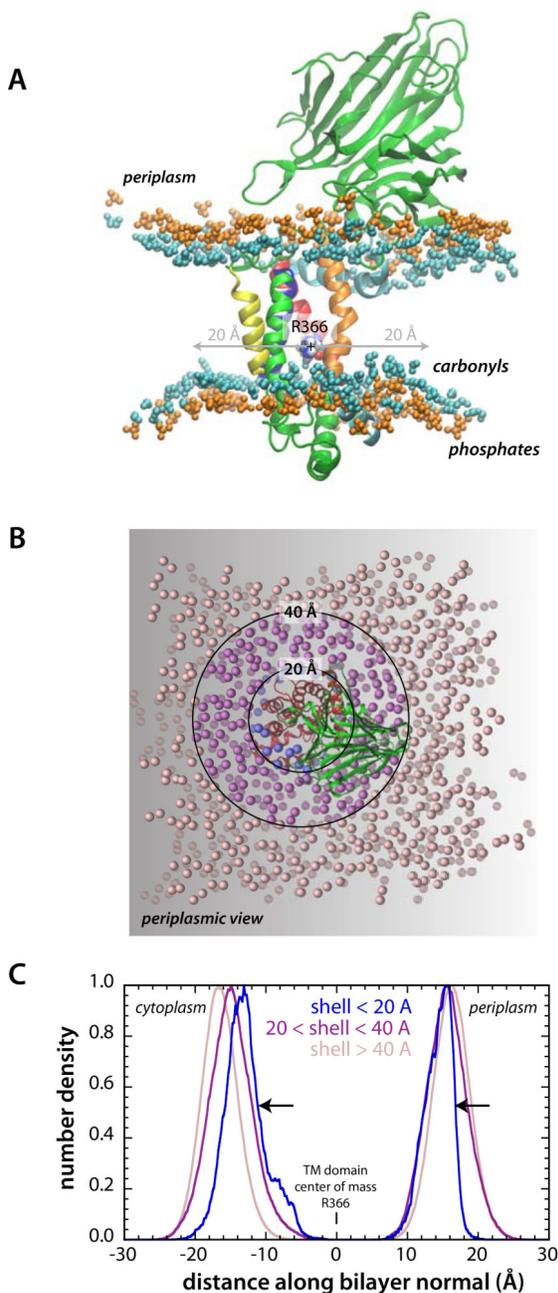


Figure 5.

YidC thins and flattens the membrane in its vicinity. **A.** A snapshot of *E. coli* embedded in the POPE:POPG membrane. The lipid carboxyl (cyan) and phosphate (orange) groups are drawn as van der Waals spheres. Except for the interfacial and transmembrane helices colored as in Figure 1, the protein is colored green. The image shows that the membrane is thinnest and quite flat within a radius of 20 Å of R366 due to interactions described in Figure 6. R366 is located at the center of mass of the TM helices and is about 11 Å above the cytoplasmic lipid carbonyl plane. **B.** View of the membrane-embedded protein from the periplasm along an axis normal to the membrane plane. The spheres represent the lipid

carbonyl groups located within 20 Å (blue), between 20 and 40 Å (purple), and greater than 40 Å (pink) from R366 as projected onto the bilayer plane. The faded spheres are on the cytoplasmic surface. **C.** Distributions of lipid carbonyl positions projected onto the bilayer normal as a function of distance from the protein center of mass at R366. Within the 20-Å shell, bilayer thickness is about 28 Å whereas in the outer shell the average thickness is about 33 Å; the change is asymmetric with respect to R366. Of the approximate 5-Å increase in thickness, about 3 Å can be attributed to cytoplasmic half and 2 Å to the periplasmic half. The distributions were determined over the last 80 ns of the simulation (Figure S5A, Figure S5D, and Table S3).

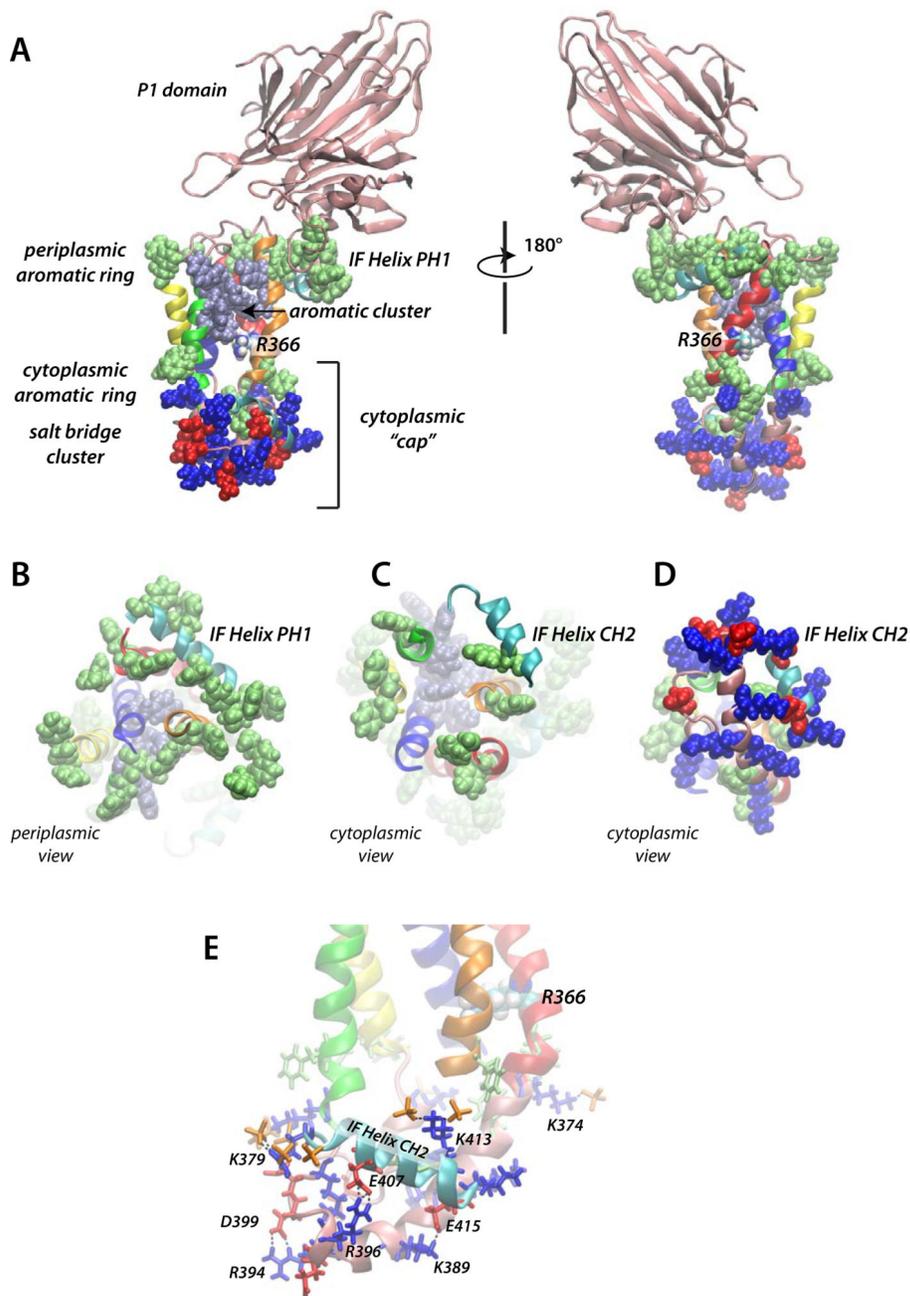


Figure 6. Several significant structural features of YidC stabilize it in the POPE:POPG bilayer. **A.** Rings of aromatic residues on both the periplasmic and cytoplasmic surfaces stabilize the protein in the bilayer interfaces. Beneath the cytoplasmic aromatic ring is a salt-bridge cluster that, together with the aromatic ring, forms a tightly packed cytoplasmic cap. A striking feature is the 9-residue aromatic cluster (F433, Y437, F502, F505, F506, W508, F509, Y516, Y517) located near the periplasmic surface. Notice that R366 is situated toward the cytoplasm directly beneath the aromatic cluster. **B.** The periplasmic aromatic ring, represented by van der Waals spheres. The amphipathic aromatic-rich interfacial helix PH1

on the periplasmic surface should provide strong anchoring in the periplasmic membrane surface (Schibli et al., 2002; Yau et al., 1998). **C.** The cytoplasmic aromatic ring is composed of seven aromatic residues. **D.** Cytoplasmic view of the protein that includes the salt-bridge cluster sitting above the cytoplasmic aromatic ring. Basic residues are colored blue and acidic residues red. **E.** The cytoplasmic cap viewed parallel to the membrane, including H-bonds (black dashes). Lipid phosphates (orange), basic residues (blue), and acidic residues (red) are represented in stick format. The salt bridges are shown more clearly in Movie 4. The significant salt-bridge pairs are R394-D399, K389-E415, and R396-E407. The residues that H-bond to lipid phosphates include K374, R403, K413, and K493.

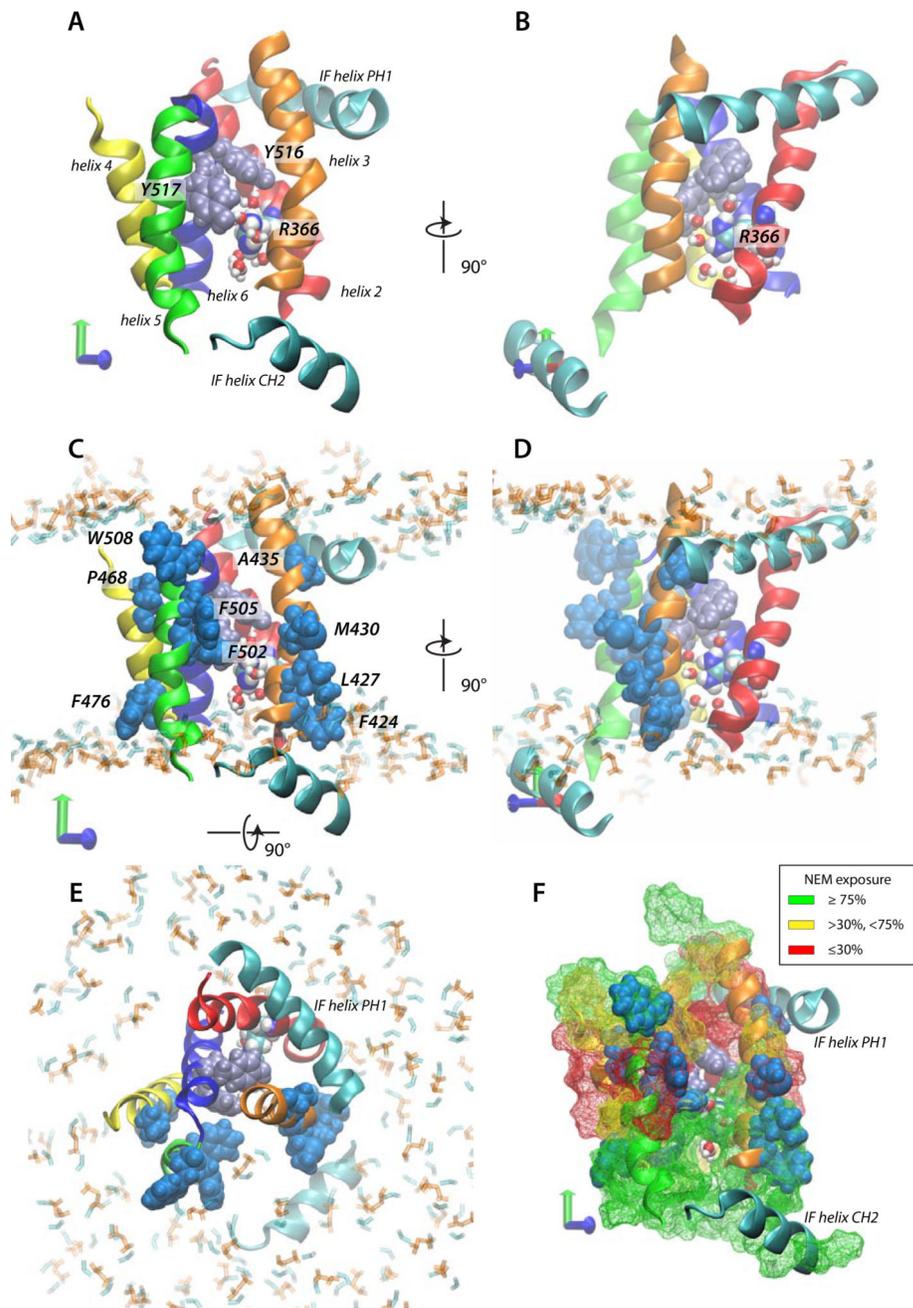


Figure 7.

Two highly conserved tyrosine residues, Y516 and Y517, form a hydrophobic lid facing toward the periplasm above the critical arginine residue (R366). **A.** The tyrosines are hydrogen bonded to R366 via intervening waters. We suggest that these tyrosines play two important roles: they stabilize the position of R366 and they provide a hydrophobic transition to the hydrophobic core of the protein, which in the case of YidC is comprised of the aromatic cluster (Figure 6A). **B.** The stabilizing tyrosine-arginine hydrogen bonding causes R366 on TM helix 2 to be set well back into the protein toward TM helices 5 and 6. **C, D.** Residues, represented by blue van der Waals spheres, that have been shown to interact

strongly with the single-span capsid protein pf3 during YidC-mediated insertion (Klenner and Kuhn, 2012) have been added to the Panel A and Panel B structures. Transparent stick models of the carbonyl (cyan) and phosphate (orange) groups define the bilayer surfaces. The structure is consistent with the idea that TM helices 3 and 5 act as a 'greasy slide' for nascent single-span TM helices (Dalbey and Kuhn, 2014). **E.** This periplasmic view shows that all of the strongly acting residues are located on the membrane-facing surfaces of TM helices 3 and 5, well away from R366. **F.** The water accessibility (Figure 4) is shown superimposed on the structure of Panel C. Waters apparently can penetrate from the cytoplasmic side into the protein only up to F505 and M430.

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