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Cross-Linking-Based Flexibility and Proximity Relationships between the TM Segments of the *Escherichia coli* YidC

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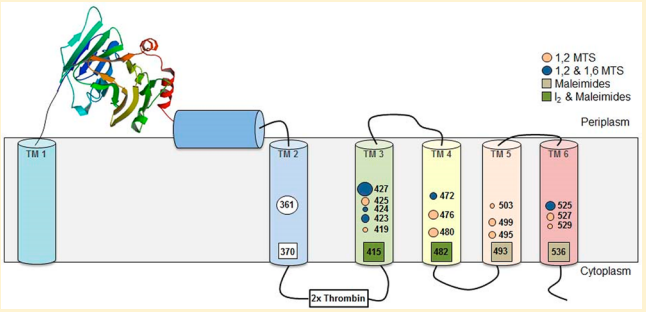
Supporting Information

ABSTRACT: The YidC family members function to insert proteins into membranes in bacteria, chloroplasts, and mitochondria, and they can also act as a platform to fold and assemble proteins into higher-order complexes. Here, we provide information about the proximity relationships and dynamics of the five conserved C-terminal transmembrane (TM) regions within *Escherichia coli* YidC. By using a YidC construct with tandem thrombin protease sites introduced into the cytoplasmic loop C1, cross-linking between paired-Cys residues located within TM segments or in the membrane border regions was studied using thio-specific homobifunctional cross-linking agents with different spanner lengths or by iodine-catalyzed disulfide formation. These *in vivo* cross-linking studies that can detect transient interactions and different conformational states of the protein show that TM3, TM4, TM5, and TM6 each have a face oriented toward TM2 of the *in vivo* expressed YidC. The studies also reveal that YidC is a dynamic protein, as cross-linking was observed between cytoplasmic Cys residues with a variety of cross-linkers. A large number of conserved proline residues on the cytoplasmic side of the five conserved core TM segments could explain the observed flexibility, and the structural fluctuations of the TM segments could provide an explanation for how YidC is able to recognize a variety of different substrates.



The YidC/Alb3/Oxa1 family members promote the insertion, folding, and assembly of proteins into the inner (cytoplasmic) membrane in bacteria as well as the insertion of proteins into the thylakoid membrane of chloroplasts and the inner membrane of mitochondria, respectively.[1] Bacterial YidC can function independently as well as cooperatively with the SecYEG translocase to promote membrane insertion and folding.[2−4] As an independent insertase, *Escherichia coli* YidC has been shown to promote the membrane insertion of the non-native M13 phage procoat[5,6] and Pf3 coat proteins,[7] along with the endogenous substrates subunit c of F_1F_0 ATPase,[8−11] MscL,[12] TssL,[13] and the N-terminal domain of CyoA.[14,15] Working with the SecYEG machinery, YidC catalyzes the insertion of subunits a and b of F_1F_0 ATPase,[8] NuoK,[16] and TatC.[17]

In all YidC homologues, there is a conserved core region that contains five TM segments[18,19] that forms the catalytic insertase domain, facilitating membrane insertion and folding.[20] The most variable regions are the N-terminal domain and C-terminal cytoplasmic tail,[18] possessing, in some cases, a long positively charged segment that functions in ribosome binding.[21,22] In Gram-negative bacteria, YidC contains an extra TM segment at the very amino-terminus and a large translocated region[23] that is known to bind SecDF.[24] X-ray crystallography analysis revealed that the periplasmic domain possesses a super-β-sandwich fold;[25,26] however, the function of this nonessential region in membrane protein biogenesis is not clear.[20] More recently, cryo-electron microscopy studies of YidC ribosome/nascent chain complexes revealed that YidC binds its substrate either as a dimer[27] or monomer.[28] Although YidC obviously can form a dimer under certain conditions, fluorescence correlation spectroscopy experiments show that the functional unit of YidC in the membrane is a monomer.[29]

The YidC substrate contacts have been mapped using disulfide cross-linking to the Pf3 coat. YidC is able to bind substrates by recognizing the substrate TM region during membrane insertion. The YidC substrate contact region includes TM3,[30,31] which may serve as the principal recognition site, as well as TM1, TM4, and TM5.[32] Contacts are observed within the YidC/substrate hydrophobic regions, which span the membrane.[32] The data support a model where YidC functions mainly as a hydrophobic platform to bind the hydrophobic segments of the substrate during membrane protein insertion.[20,33]

In order to better understand the structural features of the membrane-embedded core region of YidC, we have used Cys-based cross-linking studies pioneered by the Kaback lab[34−36] to determine the proximity relationships between TM segments. Our data show that YidC is a dynamic membrane protein on the cytoplasmic side of the membrane and support a model where YidC TM3, TM4, TM5, and TM6 each have a face in

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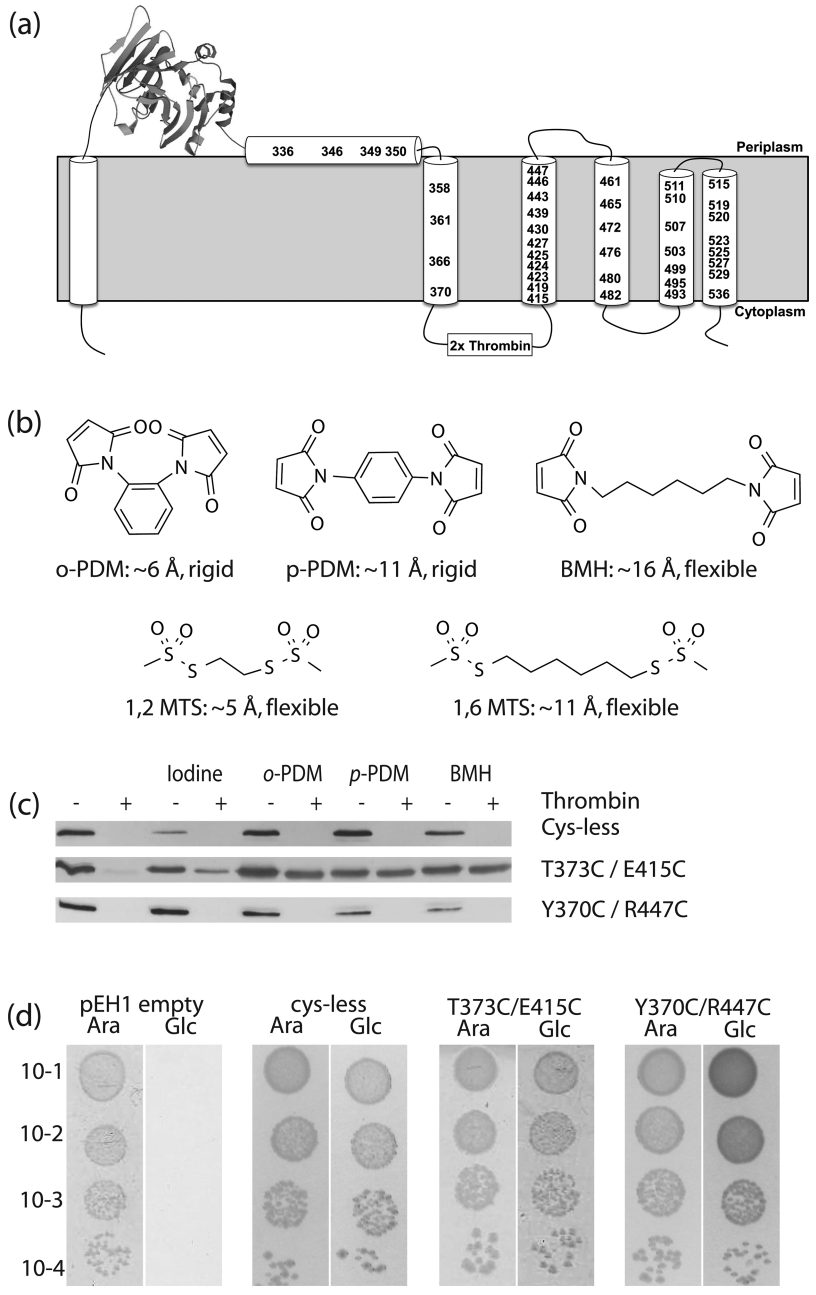

Figure 1. Membrane topology, crosslinking approach, and complementation assay. (A) Approximate TM location of YidC cysteine mutants used in this study with the tandem thrombin site indicated. (B) Structure and spanner length of the bifunctional cross-linking reagents. (C) No cross-linking is observed with the Cys-less YidC or Y370C/R447C control, and they undergo complete thrombin cleavage. Membranes were prepared from BL21 cells expressing the Cys-less YidC or the T373C/E415C and Y370C/R447C constructs and were treated with various bifunctional cross-linking reagents or iodine, catalyzing disulfide bond formation. After treatment with the chemical reagents, membranes containing YidC were solubilized with DDM and incubated with thrombin, as described in the Experimental Procedures. Proteins were resolved on a 15% (w/v) polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and then analyzed with an anti-6×-His antibody. (D) Complementation assay for monitoring the functionality of the YidC mutants. JS7131 bearing the pEH1YidC Cys-less, the T373C/E415C, or the Y370C/R447C mutant was grown under YidC expression (in the presence of arabinose) or YidC depletion (in the presence of glucose) conditions. Cultures were serially diluted and spotted onto an LB agar plate with glucose and 50 μM IPTG in order to express the cysteine mutants. Plates were incubated overnight at 37 °C.

the vicinity of and oriented toward TM2 near the middle of the membrane.

EXPERIMENTAL PROCEDURES

Materials. *N*,*N*′-*p*-Phenylenedimaleimide (*p*-PDM), *N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM), lysozyme, thrombin, and kanamycin were from Sigma-Aldrich. 1,2-Ethanediyl bismethanethiosulfonate (1,2-MTS) and 1,6-hexanediyl bismethanethiosulfonate (1,6-MTS) cross-linkers were purchased from Santa Cruz Biotechnology. *N*-Ethylmaleimide (NEM), 1,6-bis(maleimido)hexane (BMH), and super signal west pico chemiluminescent substrate were from Pierce. Isopropyl 1-thio-β,D-galactopyranoside (IPTG), *n*-dodecyl-β-D-maltopyranoside (DDM), and dithiothreitol were from Anatrace. Pfu Turbo DNA polymerase was obtained from Stratagene. The 6× His tag antibody was from Abcam.

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Plasmids and Strains. Strains BL21(DE3), JS7131 (YidC depletion strain), and C41 were from our lab collection. The *yidC* gene was amplified from *E. coli* strain MC1060 and subcloned into expression vector pEH1 using *Nde*I and *Hin*dIII. A 10×-His tag was introduced into the C-terminus of a Cys-less YidC (C423S) within pEH1-YidC using site-directed mutagenesis.

Construction of YidC with Double Cys Residues. The QuikChange mutagenesis method (Stratagene Inc.) was utilized to make double Cys mutants for the cross-linking studies using the Cys-less YidC expression vector (pEH1-yidC) with a tandem thrombin protease site $(L\text{-}V\text{-}P\text{-}R\text{-}G\text{-}S)_2$ inserted in the C1 loop after 394R.

Expression of YidC Cys Pairs and Membrane Preparation for Cross-Linking Studies. Overnight cell cultures bearing the pEH1 plasmid expressing the YidC Cys pairs were back diluted 1:100, grown at 37 °C until OD_{600} ~ 0.6, and induced with 1 mM IPTG for 2 h. The cell cultures were harvested, washed once with PBS buffer, pH 7.2, and then resuspended in PBS buffer. Membrane vesicles were prepared by adding 0.1 mM EDTA and 0.1 mg/mL lysozyme to the culture followed by incubation on ice for 10 min. The membrane preparations were then sonicated, and unbroken cells were pelleted by centrifugation at 5000*g* for 5 min.

Site-Directed Cross-Linking. Membrane samples with the YidC double Cys mutants possessing a tandem thrombin protease site in the C1 loop were incubated with 0.5 mM aqueous iodine for 10 min at room temperature to catalyze disulfide bond formation. To quench the disulfide reaction, NEM (10 mM final concentration) was added to modify any unreacted Cys residues. For Cys pairs located in the cytoplasmic or periplasmic membrane border regions, cross-linking reactions were initiated by incubation with various homobifunctional cysteine specific cross-linkers, namely, *o*-PDM, *p*-PDM, and BMH, to a final concentration of 0.5 mM at room temperature for 10 min. DTT was added at a final concentration of 10 mM to quench the reactions. Chemical cross-linking of Cys pairs in transmembrane regions was carried out for 10 min with two homobifunctional MTS reagents, namely, 1,2-MTS and 1,6-MTS, at a final concentration of 0.5 mM as well as with iodine (0.5 mM final concentration) for 10 min. The MTS reactions were quenched by adding NEM at a final concentration of 10 mM to react with unmodified Cys residues. After cross-linking, the membranes were pelleted using ultracentrifugation, and the supernatant was removed. The membrane pellet was resuspended in thrombin reaction buffer (50 mM Tris-HCl, 150 mM NaCl, 2.5 mM $CaCl_2$, 1% DDM, pH 8.0), and the samples were then centrifuged at 18 000*g* for 5 min in order to remove unsolubilized or aggregated proteins and other debris. After centrifugation, 0.2 units of thrombin were added to the supernatant, and the protease reaction was incubated overnight at room temperature. Gel sample buffer was added to the samples, and the proteins were resolved on 15% (w/v) polyacrylamide gels. Following electroblotting onto nitrocellulose membranes, they were probed for YidC using the 6×-His antibody against the C-terminal His tag.

Complementation Assay. The YidC depletion strain, JS7131, bearing the pEH1-YidC vector encoding the Cys pairs was grown in LB media containing 0.2% arabinose and 50 μg/mL kanamycin at 37 °C. After washing twice with fresh LB media, the overnight culture was back-diluted 1:100 into LB media without arabinose. The culture was grown for 2 h and then serially diluted (1:10, 1:100, 1:1000, and 1:10 000) in LB medium. A portion of the diluted cells was then spotted on LB plates containing 50 μg/mL kanamycin and 0.2% arabinose or 0.2% glucose plus 50 μM IPTG to express the double cysteine mutants. The plates were then incubated at 37 °C overnight.

RESULTS

Topology and Cross-Linking Approach. The *E. coli* YidC spans the inner membrane six times, with its N- and C-termini in the cytoplasm,[23] and contains a membrane-associated region that is predicted to link the large periplasmic folded region to TM2 (Figure 1A). To help define the TM arrangement within the conserved C-terminal core of YidC, we used a Cys cross-linking method to determine the proximity of engineered Cys residues located in different TM regions. A Cys was introduced into TM2 or its flanking region, and a second Cys was then introduced in TM3, TM4, TM5, or TM6. To examine cross-linking between the two Cys residues, we introduced tandem thrombin protease sites into the cytoplasmic loop C1 in between TM2 and TM3 (Figure 1A). Without the addition of cross-linking reagents, the thrombin protease completely cleaved YidC. If cross-linking occurs, then the two segments of YidC remain together as a full-length protein after thrombin cleavage. In our cross-linking studies, we have defined four categories based on the amount of cross-linking observed during western blot analysis: strong (above 50%), moderate (21−50%), weak (6−20%), or none (5% or less). Table S2 summarizes all of our cross-linking results in this article, which were performed in duplicate.

Cross-linking in the membrane was initiated by adding iodine or two homobifunctional cross-linking reagents: 1,2-MTS and 1,6-MTS (see Figure 1B for the structure of the reagents). Iodine catalyzes the formation of a disulfide bond (~2 Å), whereas 1,2-MTS has a flexible spanner length of ~5 Å and 1,6-MTS flexibly spans ~11 Å between the two MTS groups. Maleimide cross-linkers are used along the cytoplasmic and periplasmic border regions because they are known to react in a water-exposed environment more so than in the nonpolar membrane interior. The maleimide reagents were *o*-PDM and *p*-PDM (see Figure 1B for their chemical structures), which had fixed 6 and 11 Å spanner lengths, respectively, and BMH, which has a flexible 16 Å spanner length.

Despite treatment with various cross-linking reagents, Figure 1C shows that a Cys-less control was entirely cleaved by thrombin. Formation of a disulfide bond with iodine or cross-linking with a bifunctional reagent would result in the two fragments remaining together. The protein runs at a similar position as that of the full-length parent YidC protein on a SDS-PAGE gel, as seen with the T373C/E415C mutant (Figure 1C). As a negative control, we examined cross-linking with Y370C/R447C, a mutant containing cysteines on opposite sides of the membrane.

We confirmed that the Cys-less YidC (and all pairs of Cys mutants) was functional by performing a complementation assay (Figure 1D and Supporting Information Table S1). We assayed the mutant for activity using the YidC depletion strain, JS7131,[20] in which YidC expression is controlled by the *araBAD* promoter.[5] Whereas growth in the presence of glucose represses the endogenous YidC expression and leads to depletion of YidC in growing cells, growth in arabinose allows for YidC expression. The overnight culture of JS7131 bearing YidC mutants was back-diluted 1:100 and grown in LB media for 2 h. The culture was then serially diluted and spotted on the

LB agar plate containing arabinose or glucose plus IPTG. Figure 1D shows that the Cys-less, T373C/E415C, and Y370C/R447C YidC fully complement the YidC depletion strain. As a negative control, we confirmed that JS7131 containing the pEH1 empty vector could not support cell growth on a glucose plus IPTG plate because YidC is required for cell viability.

Cross-Linking of Paired Cys Residues in Helices 2 and 3. We defined the proximity of Cys pairs located in TM2 and TM3 or their flanking regions. A Y370C/E415C Cys pair was used to determine the distance between the cytoplasmic ends of TM2 and TM3 (Figure 2A). The TM segments appear to be very dynamic because iodine (forming a disulfide) and all of the maleimide cross-linkers ranging from 6 to 16 Å were able to cross-link at the cytoplasmic border. We switched to the MTS cross-linkers to analyze Cys pairs in the membrane because they are considerably more reactive than maleimides, so they can react with lipid-exposed Cys (ref 37 and also see Supporting Information Figure S3, where we compare the efficiency of cross-linking of all six reagents for a Cys pair in hydrophilic (Y370C/E415C) and in hydrophobic (I361C/L427C) environments). Figure 2B shows that YidC becomes less flexible moving toward the membrane interior from the cytoplasmic border because 1,2-MTS and 1,6-MTS were still able to cross-link the R366C/P419C mutant, but no cross-linking was observed with iodine. The trend was the same for the majority of the 361C mutants at various positions; 1,2- and 1,6-MTS were able to cross-link I361C/C423, I361C/F424C, and I361C/L427C. Only 1,2-MTS cross-linking was seen for I361C/P419C and I361C/P425C, which suggests that the distances between the two helices are constricting toward the membrane interior. No cross-linking was observed moving toward the periplasmic side of the TMs, as evidenced by I358C/L427C. However, cross-linking, as well as flexibility, returned with the I358C/M430C mutant, which was evident in the 1,2- and 1,6-MTS cross-links. Interestingly, cross-linking was also observed between the membrane-associated periplasmic loop before TM2 and the periplasmic portion of TM3 (Figure 2B). The F350C/M430C mutant was able to form a weak disulfide bond using iodine along with strong cross-links using the 1,2-MTS reagent and moderate cross-linking with 1,6-MTS. A similar pattern was seen with F350C/M439C except that the iodine cross-linking faded away. Interactions between the C1 loop and TM3 become more constricted at the periplasmic end of TM3; both iodine and 1,2-MTS were able to cross-link W346C/A435C and W346C/S443C. For W346C/M439C, 1,2-MTS was the only reagent that was able to cross-link moderately. However, no cross-linking was observed for W346C/R447C (Figure 2B) and F350C/R447C (Figure 2C), and weak cross-linking was observed with BMH for W346C/L446C (Figure 2C).

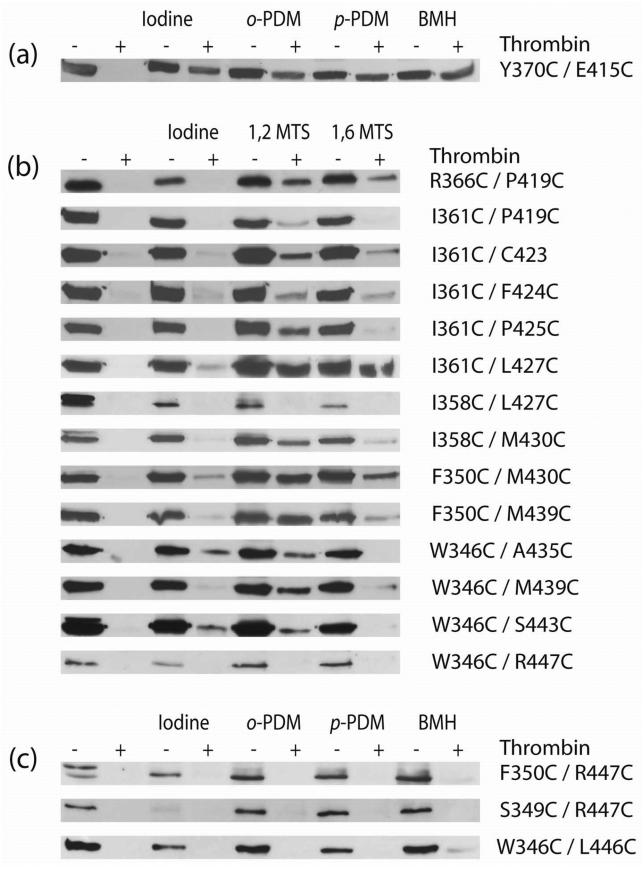

Figure 2. Cross-linking of paired Cys residues in TM2 and TM3. Membranes were prepared from BL21 cells expressing various Cys pairs and treated with various bifunctional cross-linking reagents or iodine, which catalyzes disulfide bond formation. The membranes were treated with thrombin, as described in the Experimental Procedures. Samples were analyzed by SDS-PAGE followed by western blotting with anti-6×-His antibody. The cross-linked YidC runs at a position close to that of native YidC. (A) Cross-linking between residues at or near the cytoplasmic border of the TM segments was performed using maleimides and iodine. (B) Residues thought to be located within the membrane were cross-linked using two MTS reagents and iodine. (C) For residues exposed to the periplasm, cross-linking was performed using the maleimides and iodine.

Cross-Linking of Paired Cys Residues in Helices 2 and 4. Flexibility was also seen at the cytoplasmic border of TM2 and TM4, as iodine and the maleimide cross-linkers all showed cross-linking between the Y370C/S482C Cys pair (Figure 3A). However, no cross-linking was seen toward the interior of the membrane (Figure 3B) with R366C/F476C until the I361C/K480C mutant, which reacted with 1,2-MTS, along with the I361C/F476C mutant. This latter mutant, like three additional TM2 and TM4 mutants that were studied, was only partially active in complementation studies using JS7131 (Table S1). The cross-linking then continues for the I361C/G472C and I358C/G472C mutants until F350C/Y465C is reached and iodine is able to form a strong disulfide bond along with weak 1,2-MTS cross-linking. Located beyond the TM borders and within the periplasmic region (Figure 3C), the W346C/Q461C mutant was able to cross-link with *p*-PDM and BMH. This suggests that part of the membrane-associated region of P1 is within 11 Å of the periplasmic portion of TM4.

Cross-Linking of Paired Cys Residues in Helices 2 and 5. A slightly less dynamic cytoplasmic border (Figure 4A) was observed using the Y370C/K493C mutant; the maleimides were able to cross-link, but a disulfide bond was not seen with iodine. Moderate-to-weak cross-linking with 1,2-MTS was seen throughout most of the tested TM Cys pairs between the TM2 and TM5 segments (Figure 4B). This pattern was seen with R366C/P499C, I361C/M495C, and I361C/P499C and weakly with I361/T503C in addition to weak 1,6-MTS cross-linking. Weak 1,2-MTS cross-linking was observed with I358C/T503C; the I358C/507C mutant also showed cross-linking with the 1,2-MTS reagent, which confirms the relationship between

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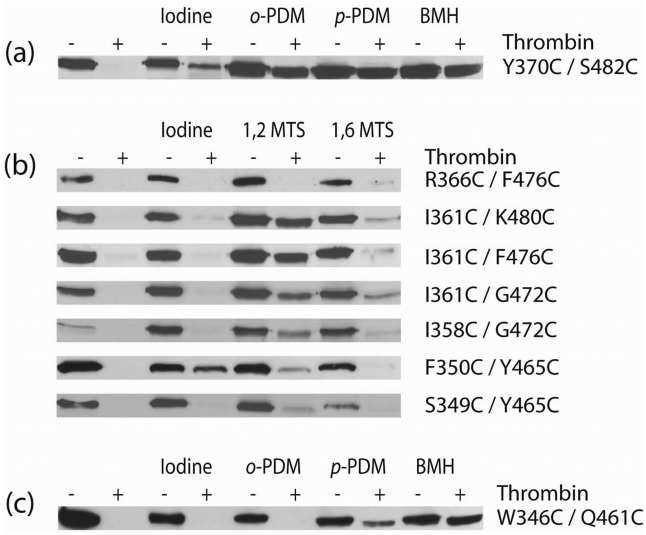

Figure 3. Cross-linking of paired Cys residues in TM2 and TM4. Membranes prepared from BL21 cells expressing the double Cys mutants were prepared, treated with cross-linking reagents, and digested with thrombin as described in the Experimental Procedures. Samples were subjected to SDS-PAGE followed by western blotting with anti-6×-His antibody. (A) Cross-linking between residues near the cytoplasmic border of the TM segments was performed using maleimides and iodine. (B) Residues thought to be located within the membrane were cross-linked using two MTS reagents and iodine. (C) For residues exposed to the periplasm, cross-linking was performed using the maleimides and iodine.

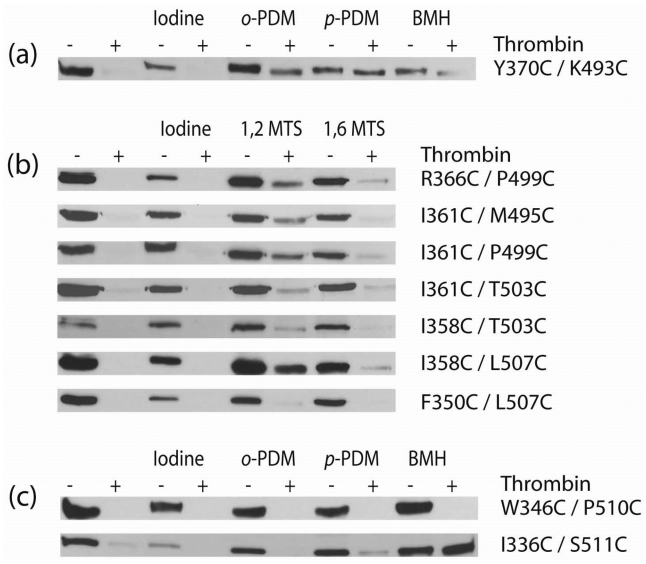

Figure 4. Cross-linking of paired Cys residues in TM2 and TM5. Conditions for membrane preparation and cross-linking are described in the Experimental Procedures. Samples were analyzed by SDS-PAGE and western blotting with anti-6×-His antibody. (A) Cross-linking between residues near the cytoplasmic border of the TM segments was performed using maleimides and iodine. (B) Residues thought to be located within the membrane were cross-linked using two MTS reagents and iodine. (C) For residues exposed to the periplasm, cross-linking was performed using the maleimides and iodine.

these two areas of the protein. Similarly, the N-terminal portion of the membrane-associated periplasmic segment before TM2 is predicted to be farther away from the periplasmic side of TM5, as we see cross-linking of the I336C/S511C mutant with BMH, which has the 16 Å spanner length (Figure 4C), and weakly with *p*-PDM. No other cross-linking was seen on the periplasmic side of the transmembrane segment, as identified with the F350C/L507C and W346C/P510C mutants.

Cross-Linking of Paired Cys Residues in Helices 2 and 6. Flexibility was once again seen at the cytoplasmic border of helices 2 and 6 using the Cys pair Y370C/E536C as cross-linking was observed for all of the maleimide cross-linkers (Figure 5A). Upon moving farther into the membrane, I361C/

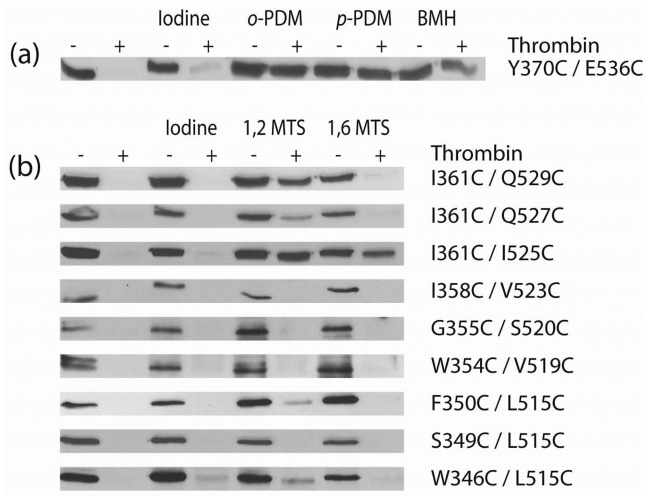

Figure 5. Cross-linking of paired Cys residues in TM2 and TM6. Conditions for cross-linking and western blotting are described in the Experimental Procedures. (A) Cross-linking between residues near the cytoplasmic border of the TM segments was performed using maleimides and iodine. (B) Residues thought to be located within the membrane were cross-linked using two MTS reagents and iodine.

Q529C was able to cross-link using the 1,2-MTS reagent, as was I361C/Q527C, but with less efficiency (Figure 5B). These two mutants would be on opposite sides of the helix, assuming the transmembrane segments adopt a helical conformation, which could explain the difference in reactivity. Flexibility and strong cross-linking are again seen with I361C/I525C using the 1,2- and 1,6-MTS cross-linkers. The remaining mutants located within the periplasmic TM region do not cross-link, as shown with the I358C/V523C, G355/S520C, and W354C/V519C mutants except for F350C/L515C, which shows weak 1,2-MTS cross-linking. The periplasmic border of helix 6 shows some cross-linking, albeit weak cross-linking, to the membrane-associated helix before TM2, as evidenced by the weak cross-linking by iodine and moderate cross-linking by 1,2-MTS with the W346C/L515C mutant.

■ DISCUSSION

In this work, we exploited Cys cross-linking to begin to define the proximity relationships and flexibility among the TM segments in the evolutionarily conserved core domain of YidC. Cross-linking is a very powerful approach to define the general structural features of a membrane protein, including interhelical distances and helix tilting.[34−36,38,39] In our studies, various cross-linking reagents with different spanner lengths were used to probe a wide range of distances between Cys pairs within YidC's TM segments. Cross-linking between Cys pairs can provide information regarding average distances as well as distances that only occur transiently within the YidC protein

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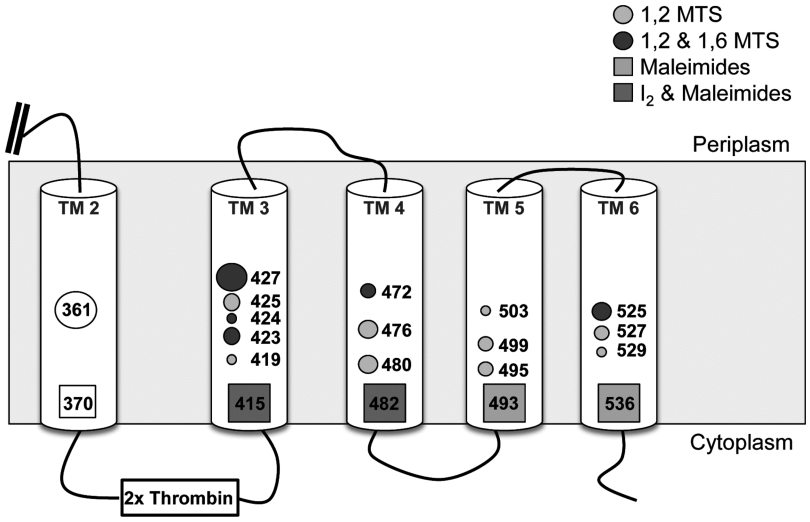

Figure 6. Cartoon showing one possible arrangement of TM2, TM3, TM4, TM5, and TM6 of YidC based on the cross-linking analysis. In this schematic of the flexibility observed, we assume that there is only one population of YidC. For simplicity, the N-terminal portion of YidC was not shown and is represented by the hash marks. The light circles depict residues that cross-link to 361C (membrane) with only 1,2-MTS; the dark circles are mutants that cross-link with 1,2- and 1,6-MTS. The light squares depict residues that cross-link to 370C (cytoplasm) with all of the maleimides; the dark squares are mutants that cross-link with iodine and all of the maleimides. The size of the symbol represents an approximation of the total cross-linking observed for that pair of residues.

because of natural protein dynamics and breathing motions. Moreover, cross-linking with multiple reagents can provide insight into the conformational changes that YidC undergoes. Another advantage of our study includes the utilization of membrane vesicles for our cross-linking experiments instead of protein solubilized in detergent. The membrane vesicles allow the protein to be probed in its natural membrane under conditions where protein synthesis and membrane protein insertion are not occurring. However, a complication in studying cellular membranes is that multiple states of the protein (i.e., YidC by itself or YidC bound to SecYEG or substrate) may occur, which may make interpretation more challenging.

After cross-linking and isolating membrane pellets, we used the detergent DDM to extract YidC from the membrane. The YidC solubilized in detergent (after the 18 000*g* spin) is considered to be a protein that is folded properly, whereas the unextracted protein is aggregated.[40,41] Under our experimental conditions using BL21 pEH1 YidC cells, most of the YidC is extracted using DDM and found in the supernatant after centrifugation in order to remove the unsolubilized and aggregated protein (Figure S1; ∼90%, left panel, lane 1). No additional YidC is solubilized and found in the supernatant by repeating the DDM extraction of the membranes (Figure S1, lane 2); however, there is a small amount of YidC that remains in the pellet (∼10%, left panel, lane 3). Similarly, we saw excellent detergent extraction using the Walker strain C41 (DE3) that allows production of many membrane proteins to high levels.[42] In C41 pEH1YidC, all of the YidC was found in the first DDM extraction (Figure S1, right panel, lane 1), with no visible YidC remaining in the membrane fraction (right panel, lane 3). Also, no detectable YidC is seen in the supernatant from the second DDM extraction with C41 (lane 2). For additional confirmation, a representative handful of double cysteine mutants was expressed in C41, allowing for optimal insertion and folding of YidC.[43] Figure S2 shows that similar cross-linking results were observed in these studies when compared to that in BL21. This provides increased confidence that the proximity relationships and dynamics that we observed in our cross-linking studies using BL21 pEH1YidC reflect the properly folded YidC and not aggregated material.

Cys cross-linking between TM segments indicates that TM3, TM4, TM5, and TM6 come in close contact to TM2 near the membrane interior. For instance, we found that I361C in TM2 can be cross-linked either to L427C in TM3, F476C in TM4, P499C in TM5, or I525C in TM6. Furthermore, this TM arrangement may show that there is a site between them where substrate binds, which is consistent with the previously reported substrate contacts.[32] In our limited Cys cross-linking studies, TM2 and TM3 form the most contacts of the Cys pairs tested and also seem to be closely associated. This close association of TM2 and TM3 is supported by the fact that a mutation in TM2 (T362E) suppresses the cold-sensitive phenotype of YidC in TM3 (C423R).[44] The number of contacts between TM2 and TM3 may also arise because of the flexibility of the N-terminal half of TM3 due, in part, to the large number of helix-breaking residues (P419, G421, G422, P425, P431, and G442). A GXXP motif (similar to the one present in the TM3 segment of YidC) has been shown to be important to increase the flexibility of a channel protein.[45] The membrane-associated periplasmic loop region preceding TM2 was able to cross-link efficiently with short spanner lengths to multiple upstream TM segments (TM3 and TM4 segments) (i.e., W346C/M439C, F350/M439C, F350/M430C, W346C/Q461C, and F350C/Y465C) close to the periplasmic side of the membrane (Figures 2 and 3). Either a portion or the entire surface of the P1 membrane-associated loop (residues 325−352) interacting with TM3 and TM4 could form the extracellular region of YidC and help to maintain the permeability barrier when YidC is inserting substrate. This periplasmic region in between the large periplasmic domain and TM2 has been shown to be important for the function of YidC.[20]

Our results are consistent with a model in which TM2−TM6 face each other in the ground state and the substrate-binding site is toward the TM2−TM6 center.[32] Indeed, Klenner and

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Kuhn proposed, based on YidC/Pf3 coat disulfide cross-linking studies, a model where the substrate TM segment binds in among TM1, TM3, TM4, and TM5. They found the substrate TM segment makes contact to TM1, TM3, TM4, and TM5 across the entire length of the membrane.[32] Notably, Kohler et al.[27] proposed that a YidC dimer is bound to the translating ribosome at the exit channel by cryo-electron microscopy (cryo-EM). They hypothesized that a pore is formed between TM2 and TM3 of one subunit and TM2 and TM3 of the other subunit. However, another cryo-EM study of a nascent chain complex bound to a YidC chimera showed the ribosome made contact with a YidC monomer,[28] and spectroscopic studies showed that monomeric YidC in nanodiscs is sufficient to bind a substrate.[29] In our Cys cross-linking studies, we did not observe any dimers of YidC, despite placing double cysteines over the entire conserved core region.

Our experiments also reveal that YidC is a very dynamic protein within cellular membranes. The majority of the tested Cys pairs in the cytoplasmic border region between TM2 and the downstream TM helices showed cross-linking between a range of both flexible and rigid cross-linkers with different spanner lengths. For example, Cys pairs between TM2 and TM3 showed cross-linking with the three bifunctional cross-linking maleimides with spanner lengths varying from 5 to 16 Å and iodine, which forms a disulfide bond. Also reflecting the dynamic nature of YidC is the fact that Cys361 in TM2 can cross-link to a range of residues in TM3, TM4, TM5, and TM6. For instance, Cys361 can be cross-linked to residues spanning Cys419 to Cys427 in TM3, Cys472 to Cys480 in TM4, Cys495 to Cys503 in TM5, and Cys525 to Cys529 in TM6, all with the same 1,2-MTS bifunctional reagent that has a flexible spanner length of ~5 Å. Figure 6 shows a cartoon view of the flexibility observed in the cytoplasmic half of the YidC transmembrane segments. The residues cross-linked to TM2 Cys361 are indicated by circles, and the residues cross-linked to Cys370 in the TM2 border region are indicated by squares. Residue 361 in TM2 is highlighted, and the cross-linking to residues in other TM segments is indicated. We also show that the cytoplasmic region of YidC is flexible, as indicated by the cross-linking with multiple reagents of different spanner lengths. Clearly, YidC is in different protein states, such as YidC by itself or YidC associated with other proteins (e.g., SecYEG and/or substrate). This could be the reason for the varying distances in the cytoplasmic border regions as well as the flexibility of cross-linking observed with Cys361 paired with Cys in other tested TM helices. One drawback of the cross-linking technique employed is that the residues have to be in proximity only for a fraction of a second for cross-linking to occur and this could also explain the flexibility that we observed.

Although flexibility of proteins in the membrane is not novel,[46] a protein with multiple highly flexible TM segments is quite interesting. YidC has many conserved proline residues in the cytoplasmic half of the TM segments (425P and 431P in TM3, 468P in TM4, and 499P in TM5), which could explain the observed flexibility in our study. This flexibility may explain why YidC is able to act as an insertase and assembly site for a wide range of membrane proteins despite it not requiring an energy source for activity.

During the course of this work, the structure of *Bacillus halodurans* YidC2 containing five TM segments was solved by X-ray crystallography at 2.4 Å resolution.[47] Briefly, the structure revealed that *B. halodurans* YidC2 has a novel protein fold with the five TM regions forming a hydrophilic groove that is open to both the cytoplasm and to the membrane interior. At the entrance of the hydrophilic groove, YidC2 has a hairpin structure in the cytoplasm, which is parallel to the membrane surface and might be involved in substrate binding. Intriguingly, Kumazaki et al. proposed that the C1 region containing the helical hairpin is very flexible;[47] this is consistent with our data. First, the flexibility is supported by the fact that, in the structure of the two *B. halodurans* YidC2 variants, C1 has two very different orientations with respect to the membrane-embedded region. Second, the high crystallographic B factors of the C1 region, as well as the cytoplasmic side of the TM segments (in comparison to other regions of the protein), support the flexibility of these regions. Third, molecular dynamic simulations of *B. halodurans* YidC2 show that the hairpin structure within the cytoplasm is very dynamic and flexible. Thus, the C1 region and TM cytoplasmic side are flexible both in *E. coli* and *B. halodurans* YidC.

The *B. halodurans* structure reveals that TM1 is in proximity to TM2 and TM5 and that TM5 is also in proximity to TM3 and TM4. Because *E. coli* YidC has an extra TM segment at its N-terminus, TM2, TM3, TM4, TM5, and TM6 of *E. coli* YidC correspond to TM1, TM2, TM3, TM4, and TM 5 of *B. halodurans* YidC. Therefore, at first glance, the structure is consistent with our *E. coli* YidC model where TM2 faces TM3 and TM6. Although TM1 does not contact TM3 and TM4 in the case of the crystal structure, *E. coli* TM2 could come close to the corresponding TM segments with breathing, such that it could be cross-linked with the 5 Å MTS cross-linking agent. Indeed, our Cys361 cross-linking data with Cys residues introduced into the TM3, TM4, TM5, or TM6 segment show that the Cys361 can come in close proximity to a wide variety of Cys residues, even within a TM segment that is predicted to be far away from TM2 (Figure 6).

The deviation of the observed distances between the structure and our cross-linking data could be due to divergence of functional homologues, inherent flexibility and breathing of the membrane embedded domain, structural variances between lipid and detergent solubilization, or conformational changes induced by substrate[48,49] and/or partner protein binding.[50] The flexibility or different conformational states of YidC with substrate bound may account for the large proximity differences we observe from cross-linking versus those obtained from the crystal structure of *B. halodurans* YidC without substrate.

In summary, this article on *E. coli* YidC shows that the cytoplasmic border region and the cytoplasmic half of the YidC conserved TM segments are flexible. The flexibility may be important for changing conformational states of YidC upon capturing substrate. We also show that the loop that links the large P1 domain to TM2 can be cross-linked efficiently to the TM3 and TM4 segments. Our studies point to the TM2 segment being in close proximity to TM3, TM4, TM5, and TM6 in at least one, but possibly more, conformational states.

■ ASSOCIATED CONTENT

Supporting Information

Various experiments were performed to rule out the possibility that our results were due to protein aggregation because of overexpression in our studies. The amount of aggregate was compared between expression for 2 h in BL21 and expression for 2h in C41 (Figure S1). The C41 strain is known to limit protein aggregation because of overexpression of membrane proteins. A handful of experiments were repeated in C41 in order to confirm that the results were not due to aggregation

(Figure S2). All of the reagents were used to cross-link an aqueous (Y370C/E415C) and membrane (I361C/L427C)-exposed cysteine pair so that the reactivity in each environment could be compared (Figure S3). The complementation efficiency of the various mutants utilized in our studies is summarized in Table S1. Table S2 provides a summary of the average, from duplicate experiments, cross-linking efficiency for the mutants in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS USED

TM, transmembrane; Cys, cysteine; *p*-PDM, *N*,*N*′-*p*-phenylenedimaleimide; *o*-PDM, *N*,*N*′-*o*-phenylenedimaleimide; BMH, 1,6-bis(maleimido)hexane; 1,2-MTS, 1,2-ethanediyl bismethanethiosulfonate; 1,6-MTS, 1,6-hexanediyl bismethanethiosulfonate; NEM, *N*-ethylmaleimide; IPTG, isopropyl 1-thio-β,D-galactopyranoside; DDM, *n*-dodecyl-β-D-maltopyranoside

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