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Role of YidC in folding of polytopic membrane proteins

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YidC of *Echerichia coli*, a member of the conserved Alb3/Oxa1/YidC family, is postulated to be important for biogenesis of membrane proteins. Here, we use as a model the lactose permease (LacY), a membrane transport protein with a known three-dimensional structure, to determine whether YidC plays a role in polytopic membrane protein insertion and/or folding. Experiments in vivo and with an in vitro transcription/translation/insertion system demonstrate that YidC is not necessary for insertion per se, but plays an important role in folding of LacY. By using the in vitro system and two monoclonal antibodies directed against conformational epitopes, LacY is shown to bind the antibodies poorly in YidC-depleted membranes. Moreover, LacY also folds improperly in proteoliposomes prepared without YidC. However, when the proteoliposomes are supplemented with purified YidC, LacY folds correctly. The results indicate that YidC plays a primary role in folding of LacY into its final tertiary conformation via an interaction that likely occurs transiently during insertion into the lipid phase of the membrane.

Introduction

Insertion of polytopic membrane proteins into the membrane and folding into a final tertiary conformation is an important unsolved problem. Most inner membrane proteins of *Echerichia coli* are targeted and insert into the membrane co-translationally using the signal recognition particle and the Sec pathway (Bernstein, 2000; Herskovits et al., 2000; de Gier and Luirink, 2001). The major components of Sec pathway are SecY, SecE, and SecG, which form a complex (SecYEG) homologous to the Sec61 complex in the ER, and both complexes are postulated to function as aqueous channels for protein translocation or insertion (the translocon; Mori and Ito, 2001; Müller et al., 2001; Van den Berg et al., 2004). SecY is the largest component of the Sec complex and essential for viability.

Recently, YidC, which is essential for viability, has been identified as another key component for biogenesis of membrane proteins (Samuelson et al., 2000). YidC is a 60-kD protein with six putative transmembrane helices (Saaf et al., 1998). YidC binds to SecD and SecF, which interact with the SecYEG complex (Nouwen and Driessen, 2002), and homologues are present in the inner membrane of mitochondria (Oxa1) and the thylakoid membrane of chloroplasts (Albino3; de Gier and Luirink, 2003; Kuhn et al., 2003). Like the SecYEG complex, YidC and its homologues are also postulated to play an important role in the biogenesis of membrane proteins. It has been suggested that these proteins function in a similar fashion in each system. For example, Albino3 from the thylakoid membrane of chloroplasts compliments the YidC-depletion strain of *E. coli* (Jiang et al., 2002). Although it is clearly important to understand the function of YidC and its homologues in detail, delineating the precise mechanism of insertion and folding of membrane proteins, particularly polytopic membrane proteins, is an inherently difficult problem.

Lactose permease (LacY) of *E. coli* is a useful model to study insertion and folding of this class of proteins because an in vitro system for transcription, translation, membrane insertion and folding has been developed (Ahrem et al., 1989; Bogdanov and Dowhan, 1998; Nagamori et al., 2003). LacY is a symporter that catalyzes the coupled stoichiometric translocation of a galactoside and a H^+ across the membrane and is one of the most well-studied membrane proteins available (Kaback et al., 2001). LacY belongs to the major facilitator superfamily of membrane transporter proteins (Saier, 2000) which contains >1,000 members many of which are of medical importance. Most importantly, a crystal structure of LacY was solved recently at 3.5 Å (Abramson et

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Key words: LacY; membrane insertion; protein folding; SecYEG; Oxa1/Alb3 protein family

Abbreviations used in this paper: DDM, dodecyl-β-D-maltopyranoside; $DiBAC_4(5)$, *bis*-(1,3-dibutylbarbituric acid)pentamethine oxanol; IPTG, *i*-propyl-1-thio-β-D-galactopyranoside; ISO, inside-out; KP_i, potassium phosphate; LacY, lactose permease; PE, phosphatidylethanolamine.

al., 2003). The molecule is composed of NH_2- and COOH-terminal domains, each with six transmembrane helices, symmetrically positioned within LacY, and the sugar binding site is located at the approximate middle of the membrane in the interface between the two six-helix bundles at the apex of a large hydrophilic cavity facing the cytoplasm.

LacY is inserted into the membrane co-translationally, using signal recognition particle for targeting (Stochaj and Ehring, 1987; Ahrem et al., 1989; MacFarlane and Müller, 1995; Seluanov and Bibi, 1997) and the Sec machinery for insertion (Ito and Akiyama, 1991), but its insertion does not require the H^+ electrochemical gradient (Ahrem et al., 1989). Evidence has also been presented that phosphatidylethanolamine (PE) plays an important role in folding, acting as a molecular chaperon (Bogdanov and Dowhan, 1998, 1999; Bogdanov et al., 1996, 2002). However, in addition to more detail regarding the mechanism of co-translational insertion into the translocon, the mechanism by which LacY exits the SecYEG complex into the lipid bilayer and folds into a final tertiary conformation remains largely enigmatic. In this regard, it has been postulated that YidC assists in the insertion of proteins into the SecYEG complex and lateral transfer into the lipid bilayer (Beck et al., 2001; Urbanus et al., 2001; Houben et al., 2002). However, it has also been suggested (Kuhn et al., 2003) that YidC may be involved primarily in folding. Here, we show directly that YidC likely plays little or no role in membrane insertion per se, but is involved in folding of LacY into its final tertiary conformation in the membrane.

Results

LacY requires SecY for insertion

To study the effect of SecY or YidC on LacY insertion into the membrane in vivo, GFP was attached to the COOH terminus of LacY (LacY-GFP). LacY-GFP is expressed well in *E. coli* T184 (Δ*lacZY*) and catalyzes lactose transport in a manner comparable to wild-type LacY (unpublished data). When wild-type *E. coli* expressing LacY-GFP are examined by fluorescence microscopy, intense fluorescence is observed at the cell surface, particularly at the poles (Fig. 1 A, top right). In contrast, with *secY24*/Syd$^+$cells, which are severely defective in SecY function (Shimoike et al., 1995), fluorescence is markedly decreased and distributed more diffusely throughout the cell (Fig. 1 A, top left). When LacZ-GFP is overexpressed in either wild-type or *secY24*/Syd$^+$cells, intense and diffuse cytoplasmic fluorescence is observed, as expected, because LacZ-GFP is a soluble, cytoplasmic protein (Fig. 1 A, bottom).

Furthermore, when LacY is synthesized in vitro in the presence of inside-out (ISO) membrane vesicles from *secY24* cells, only a small amount of labeled protein is observed in membranes from the mutant (Fig. 1 B, lane 1). On the other hand, as demonstrated previously with ISO membrane vesicles from wild-type cells (Nagamori et al., 2003), LacY synthesized in vitro is inserted into the membrane to a much greater extent (Fig. 1 B, lane 2). In addition, whereas LacY synthesized and inserted in vitro remains associated with wild-type membranes after treatment with 5 M urea (Fig. 1 B, lane 4), the small amount of LacY inserted into *secY24* membranes is decreased even further (Fig. 1 B, lane 3), indicating that in the absence of SecY function, LacY insertion into the lipid phase is almost completely defective. Thus, in confirmation of previous studies (Ito and Akiyama, 1991), SecY function is essential for insertion of LacY.

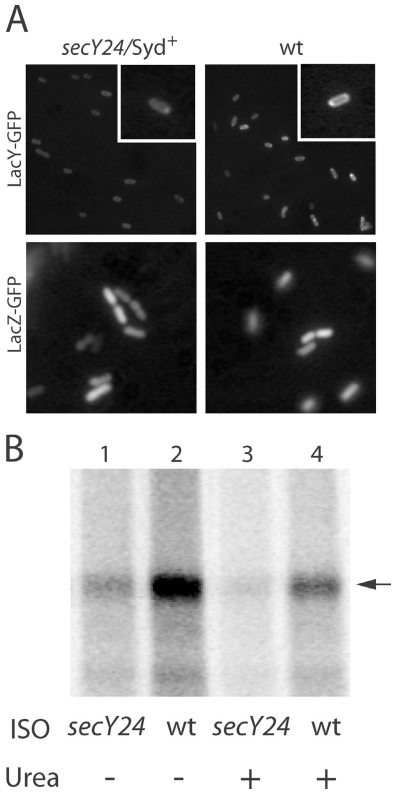

Figure 1. **LacY requires SecY function for insertion.** (A) KI297/pST30 (*secY24*/Syd$^+$) and KI298/pSTV29 (wt) were transformed with plasmids expressing LacY-GFP (top) or LacZ-GFP (bottom) were grown in LB medium with 1% glucose and antibiotics at 30°C overnight. The overnight culture was diluted in 50-fold with fresh medium. After 3 h at 30°C, 1.0 mM IPTG was added, and the sample was incubated for 1 h at 42°C. Cells were harvested, washed with PBS, and inspected under a fluorescence microscope immediately. LacZ-GFP was expressed from pEGFP. (B) The in vitro transcription, translation, and insertion of LacY using *secY24* inside-out (ISO) membrane vesicles. ISO vesicles were prepared from AD202 (MC4100, Δ*ompT*::*kan)* and AD206 (AD202, *secY24*). Reactions were performed at 30°C for 30 min in the presence of [^{35}S]methionine. Reaction mixtures were placed on a 50% sucrose cushion and centrifuged. The inner membrane fraction was collected carefully, diluted and centrifuged, and subjected to 5.0 M urea extraction as specified.

YidC and LacY insertion

When LacY-GFP is expressed in the conditional YidC-depletion strain, *E. coli* JS7131 (Samuelson et al., 2000), fluorescence is preferentially localized to the periphery of cells containing YidC (Fig. 2 A, top right), and little difference is observed in cells depleted of YidC (Fig. 2 A, top left). However, in both cell populations, expression of LacZ-GFP leads to strong, diffuse fluorescence, demonstrating that the YidC-depleted cells are capable of protein synthesis (Fig. 2 A, bottom). In addition, although data are not shown, similar results were obtained with MelY-GFP from *Enterobacter cloacae*.

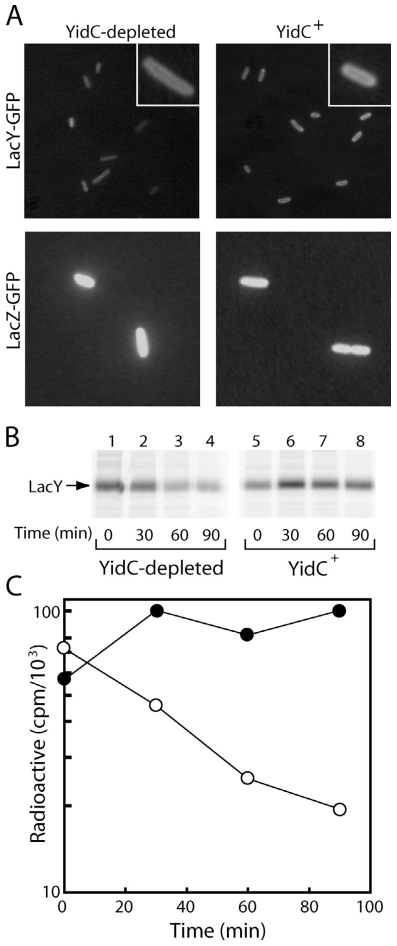

Figure 2. **LacY expression in YidC-depleted cells.** (A) LacY-GFP (top) or LacZ-GFP (bottom) were expressed in the YidC-depletion strain, *E. coli* JS7131. After 3 h of incubation in the presence of 0.2% arabinose (right) or 0.2% glycerol (left) at 37°C, 1.0 mM IPTG was added to express LacY-GFP or LacZ-GFP and cells were incubated for 1 h. Cells were harvested, washed with PBS, and inspected under a fluorescence microscope immediately. (B) Pulse-chase experiments of LacY in conditional YidC-depletion strain JS7131. LacY was expressed in either YidC-depleted (0.2% glycerol) or $YidC^+$ cells (0.2% arabinose). The cells were labeled with [^{35}S]methionine for 30 min and chased with unlabeled methionine for the indicated time period. LacY was purified by using by a metal affinity resin and subjected to SDS/PAGE and autoradiographed as described in Materials and methods. (C) Samples from pulse-chase experiments described above were purified as described in Materials and methods and quantified by the scintillation spectrometry. ○, YidC-depleted cells (0.2% glycerol); ●, $YidC^+$ cells (0.2% arabinose).

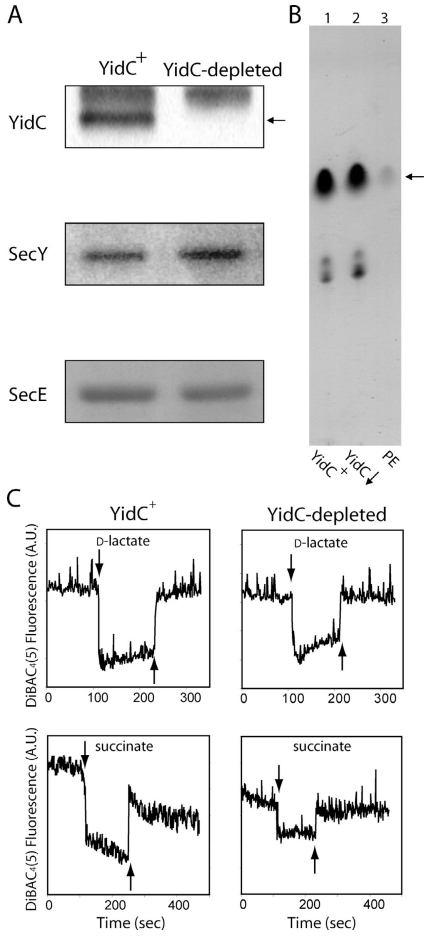

Figure 3. **YidC, SecY, SecE, PE, and generation of $\Delta\Psi$ (interior positive) in ISO membrane vesicles from $YidC^+$ and YidC-depleted cells.** (A) ISO vesicles were prepared from JS7131 $YidC^+$ or YidC-depleted as described in Materials and methods, and the amount of YidC (arrow), SecY or SecE in 10 μg of protein from a given preparation was analyzed by immunoblotting using the appropriate antibody. (B) Phospholipid composition in $YidC^+$ or YidC-depleted membranes. Arrow indicates the position of PE. Thin layer chromatography was performed with $CHCl_3$/MeOH/H_2O as a solvent (65:25:4, vol/vol). 2.5 mg/ml of $YidC^+$ or YidC-depleted membrane vesicles were mixed with 3× volumes of MeOH and same volume of $CHCl_3$. After 5 min at RT, 2× volumes of H_2O was added, and samples were centrifuged at 2,000 *g* for 20 min. Lipids were in the $CHCl_3$ fraction. Lipid extracts from 75 μg of membrane vesicles and 10 μg of PE (Avanti polar lipid) in $CHCl_3$ were spotted on Slica gel 60 F_{254} plate (Merck) as indicated. (C) Generation of $\Delta\Psi$ (interior negative) in ISO membrane vesicles from $YidC^+$ and YidC-depleted cells. Where indicated, 20 mM D-lactate or 5 mM succinate was added to generate $\Delta\Psi$ (first arrow), as indicated by quenching of $DiBAC_4(5)$ fluorescence. Where indicated (second arrow), 20 μM carbonylcyanide-*m*-chlorophenylhydrazone was then added to collapse $\Delta\Psi$.

To examine expression and stability of LacY in YidC-depleted cells, pulse-chase experiments were performed (Fig. 2, B and C). Interestingly, radioactive bands corresponding to LacY are observed in either YidC-depleted cells or $YidC^+$ cells at similar intensities in the membrane after a 30-min incubation with [^{35}S]methionine (Fig. 2 B, lanes 1 and 5; Fig. 2 C). Addition of excess unlabeled methionine causes the intensity of the LacY band in YidC-depleted cells to decrease in a time-dependent fashion (Fig. 2 B, lanes 1–4; Fig. 2 C), whereas the intensity of the LacY band in $YidC^+$ cells remains essentially constant (Fig. 2 B, lanes 5–8; Fig. 2 C). The data suggest strongly that LacY is inserted into the membrane normally without YidC, but is unstable and subjected to proteolysis.

To pursue the role of YidC, in vitro transcription, translation, and insertion was studied with ISO vesicles prepared from $YidC^+$ or YidC-depleted *E. coli* JS7131. Quantities of YidC, as well as SecY and SecE, in the two membrane preparations were analyzed by immunoblotting with appropriate antibodies (Fig. 3 A). YidC is not detected in YidC-depleted membranes, whereas normal levels of both SecY and SecE are

observed. Because PE, the major phospholipid in the *E. coli* membrane, is known to play a role in LacY folding (Bogdanov et al., 1996, 2002; Bogdanov and Dowhan, 1998, 1999), the PE content of ISO membrane vesicles from YidC$^+$ or YidC-depleted cells was analyzed by thin layer chromatography (Fig. 3 B). Clearly, the PE content in both vesicles preparations is comparable. Furthermore, the effect of YidC-depletion on generation of the H^+ electrochemical gradient was tested by using *bis*-(1,3-dibutylbarbituric acid)pentamethine oxanol ($DiBAC_4[5]$), an anionic fluorophore that exhibits quenching when accumulation occurs in response to a membrane potential ($\Delta\Psi$, interior positive; Matsushita et al., 1987). Generation of $\Delta\Psi$ was monitored during oxidation of either D-lactate or succinate (Fig. 3 C). In conformation of the findings of van der Laan et al. (2003), YidC-depleted vesicles exhibit a relatively small decrease in $\Delta\Psi$ relative to control vesicles (from 124 to 114 mV with D-lactate or from 106 to 89 mV with succinate), indicating that YidC plays a role in membrane biogenesis of other polytopic membrane proteins involved in generation of $\Delta\Psi$. Together, the findings demonstrate that with the exception of depletion of YidC and a small decrease in $\Delta\Psi$, YidC$^+$, and YidC-depleted membranes appear to be quite similar.

In vitro transcription, translation, and insertion with ISO membrane vesicles from either YidC$^+$ or YidC-depleted cells demonstrate that LacY is inserted into the membrane similarly in both preparations (Fig. 4 A). LacY insertion into YidC-depleted ISO membrane vesicles is comparable to that observed in YidC$^+$ vesicles without urea extraction (Fig. 4 A, lanes 1 and 2), and the intensity of the band in YidC-depleted membranes is only mildly diminished by urea extraction relative to YidC$^+$ membranes (Fig. 4 A, lanes 3 and 4). Although insertion of LacY into the control and YidC-depleted membranes is comparable qualitatively, when insertion is studied as a function of time, rates of insertion are similar over the initial 15 min, but by 30 min, the YidC-depleted vesicles exhibit about half the amount of LacY (Fig. 4 B). In all likelihood, the decrease observed at 30 min reflects a degree of instability of the LacY inserted into the YidC-depleted membranes and residual proteolytic activity remaining in the vesicles. In any case, the results indicate that insertion of LacY into the membrane per se is only mildly diminished, if at all, in YidC-depleted membranes.

When LacY is unable to insert into the bilayer, the protein can be extracted with urea (Roepe and Kaback, 1989; Nagamori et al., 2003). Moreover, LacY synthesized in vitro is not detected with SecY mutant membranes (Fig. 1 B). Therefore, according to this criterion, LacY synthesized and inserted into YidC-depleted membranes is largely inserted into the bilayer. To establish this point more definitively, LacY synthesized in vitro and inserted into the YidC-depleted membranes was digested first with proteinase K (Fig. 5 A). LacY translated in the absence of ISO membrane vesicles is resistant to degradation by proteinase K relative to LacY translated in the presence of vesicles (Ahrem et al., 1989). Proteinase K digestion of LacY in YidC-depleted membrane yields a pattern similar to that observed in membranes, and the results are essentially identical with YidC$^+$ and YidC-depleted membranes. Thus, in neither membrane does LacY appear to be aggregated to a significant extent.

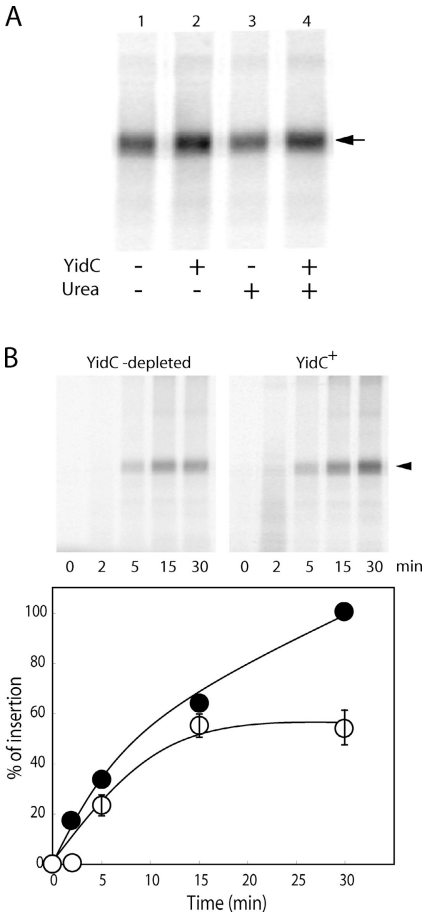

Figure 4. **In vitro transcription, translation and insertion in the presence and absence of YidC.** (A) Reactions were performed at 30°C for 30 min in the presence of YidC-depleted ISO vesicles (lanes 1 and 3) or YidC$^+$ ISO vesicles (lanes 2 and 4) as described in Materials and methods. Reaction mixture was placed on a 50% sucrose cushion and centrifuged. The inner membrane fraction was collected carefully, diluted and centrifuged, and subjected to 4.0 M urea treatment on ice for 20 min as specified (lanes 3 and 4). ISO vesicles were pelleted by ultracentrifugation and analyzed by SDS-PAGE. Arrow denotes position of LacY. (B) Time course of in vitro insertion of LacY into ISO vesicles in the presence and absence of YidC. Reactions were performed at 30°C, terminated by addition of 0.2 mg/ml chloramphenicol and put on ice at various time points as specified. ISO membrane vesicle fractions were isolated on the 50% sucrose cushion and subjected to 4.0 M urea wash on ice for 20 min. Arrowhead indicates the position of LacY. Quantitative data are presented as a percentage of the amount of LacY inserted into YidC$^+$ ISO vesicles at 30 min. ○, YidC-depleted vesicles; ●, YidC$^+$ vesicles. Error bars represent the SD from three independent experiments.

Moreover, LacY with tandem engineered factor Xa sites in periplasmic loop VII/VIII was translated in vitro with YidC$^+$ or YidC-depleted ISO membrane vesicles (Fig. 5 B). Bands corresponding to full-length LacY are observed in absence of detergent and only faint digestion products are observed in YidC$^+$ or YidC-depleted membranes (Fig. 5 B, lanes 3 and 7). However, when digestion is performed in the presence of dodecyl-β-D-maltopyranoside (DDM), loop VII/VIII clearly becomes accessible to factor Xa protease (Fig. 5 B, lanes 4 and 8). The data are clearly consistent with the interpretation that loop VII/VIII is inserted into the ISO

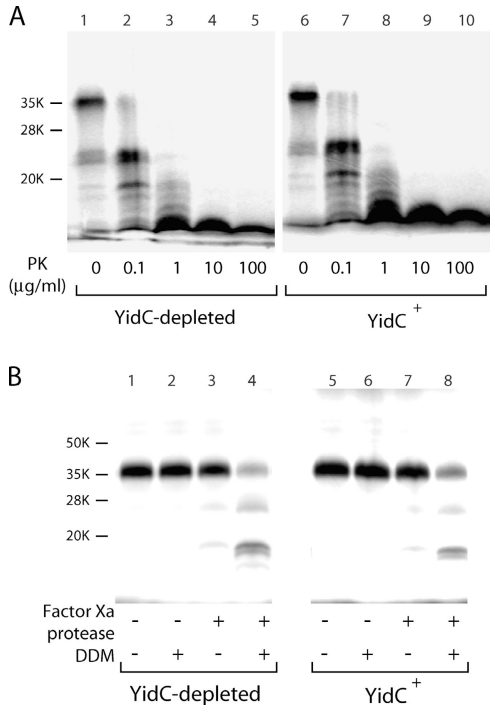

Figure 5. **Protease digestions of LacY translated and inserted in vitro with YidC$^+$ and YidC-depleted ISO vesicles.** (A) Proteinase K digestion of YidC$^+$ and YidC-depleted ISO vesicles. In vitro reactions was performed as described in Materials and methods. Urea-washed ISO membrane vesicles with LacY synthesized in vitro were resuspended in 50 mM Tris-HCl, pH 7.5, and incubated with proteinase K at a specified concentration on ice for 30 min. Digestion was terminated by addition of 2 mg/ml of Pefabloc SC (Pentapharm). (B) Factor Xa protease cleavage of LacY with three tandem Xa sites in periplasmic loop VII/VIII synthesized and inserted in vitro in YidC$^+$ or YidC-depleted ISO vesicles. The experiment was performed as described previously (Nagamori et al., 2003).

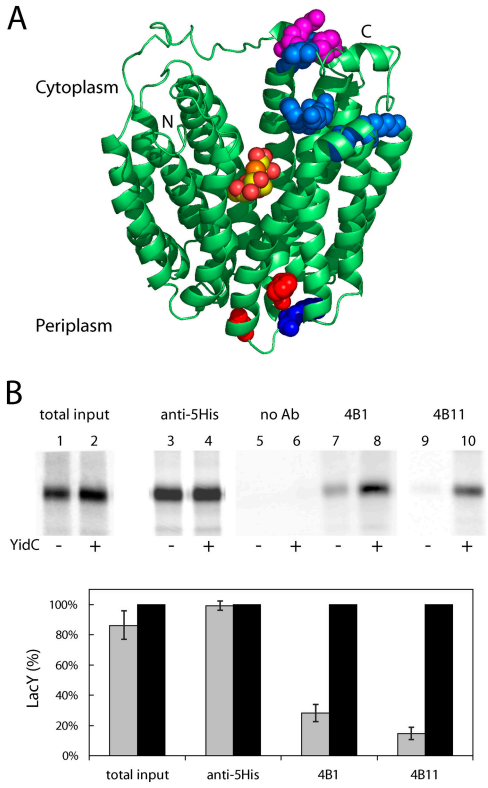

Figure 6. **YidC is important for LacY folding.** (A) mAbs 4B1 and 4b11 epitopes. The ribbon diagram shown is based on the structure of C154G LacY with bound β,D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG; Abramson et al., 2003). Red spheres indicate side chains that are the primary determinants and the dark blue spheres indicate the side chain that is a secondary determinant in the 4B1 epitope (Sun et al., 1996). Magenta and light blue spheres indicate side chains that are the primary and secondary determinants in the 4B11 epitope, respectively (Sun et al., 1997). TDG is represented by multi color spheres (yellow, orange, and red) in the middle of the twofold plane of pseudo-symmetry in LacY. (B) mAbs that recognize conformational epitopes in the LacY, bind to LacY translated and inserted in vitro with YidC$^+$ ISO vesicles, but not with YidC-depleted ISO vesicles. YidC$^+$ ISO vesicles were used in even-numbered lanes; YidC-depleted ISO vesicles were used in odd-numbered lanes. mAb 4B1 or 4B11 were used as indicated (lanes 7–10, respectively). Anti–penta-His antibody (QIAGEN) was used as positive control (lanes 3 and 4). For negative control indicated as "no Ab," experiments were done without any antibody (lanes 5 and 6). "Total input" indicates the total amount of products from in vitro synthesis before immunoprecipitation (lanes 1 and 2). Histogram presentation of average data from at least three independent immunoprecipitation experiments performed as shown in the top panels. Gray bars indicate amounts of LacY from YidC-depleted ISO vesicles. Black bars indicate amounts of LacY from YidC$^+$ ISO vesicles. Error bars represent the SD.

vesicles with the proper topology (i.e., a periplasmic loop should be exposed on the inner surface of ISO vesicles and therefore inaccessible to protease).

YidC is important for LacY folding

mAb 4B1 binds specifically to periplasmic loop VII/VIII (Sun et al., 1996), whereas mAb 4B11 recognizes a discontinuous epitope that contains determinants from cytoplasmic loops VIII/IX and X/XI (Sun et al., 1997; Fig. 6 A). Binding of these mAbs to LacY synthesized and inserted in vitro in ISO membrane vesicles from YidC$^+$ or YidC-depleted cells exhibit dramatic differences when analyzed by immunoprecipitation (Fig. 6 B). In vitro transcription, translation, and insertion were carried for 15 min at 30°C in order to obtain similar amounts of LacY as starting material (Fig. 6 B, lanes 1 and 2; Fig. 4 B). As a positive control, anti–penta-His antibody was used, and the quantity of immunoprecipitated LacY is similar for YidC$^+$ and YidC-depleted ISO vesicles (Fig. 6 B, lanes 3 and 4). LacY synthesized and inserted into ISO YidC$^+$ vesicles is immunoprecipitated well by both mAbs 4B1 and 4B11 (Fig. 6 B, lanes 8 and 10, respectively). In contrast, LacY inserted into YidC-depleted ISO vesicles is not immunoprecipitated well by either mAbs 4B1 or 4B11 (Fig. 6 B, lanes 7 and 9, respectively). Thus, it is clear that LacY synthesized in vitro and inserted into YidC-depleted ISO vesicles does not fold into a normal tertiary conformation. The immunoprecipitation results with 4B11 shown in Fig. 6 were performed in detergent; however the same results were also obtained when the mAb was incubated with the ISO vesicles before detergent solubilization (not depicted).

To examine the requirement of YidC for LacY folding more directly, His-tagged YidC (YidC-His) was constructed and purified (Fig. 7 A). Expression of YidC-His comple-

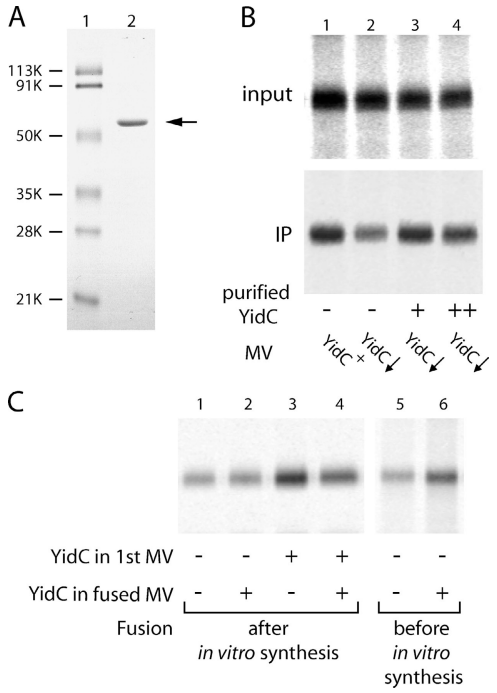

Figure 7. **The effect of YidC is co-translational.** (A) Molecular weight maker (lane 1) and 10 μg of YidC purified as described in Materials and methods were subjected to SDS-PAGE and Coomassie brilliant blue staining. Arrow indicates YidC-His. (B) Effect of YidC on immunoprecipitation of LacY with mAb 4B11. In vitro transcription, translation, and insertion into reconstituted proteoliposome were performed as described in Materials and methods (top, lanes 1–4). Reconstituted proteoliposomes were prepared from solubilized YidC$^+$ membranes (lane 1) or YidC-depleted membrane (lanes 2–4), and purified YidC were included in the reconstitution mixture as indicated (lane 3 and 4). Proteoliposomes with LacY synthesized and inserted in vitro (lanes 1–4) were solubilized and immunoprecipitated with mAb 4B11 as described in Materials and methods (bottom, lanes 1–4, respectively). (C) The 4B1 epitope does not form unless YidC is present during translation and insertion. LacY was synthesized and inserted in vitro with ISO membrane vesicles (1st MV) from either YidC-depleted vesicles (lanes 1 and 2) or YidC$^+$ vesicles (lanes 3 and 4). ISO membrane vesicles from YidC$^+$ cells (YidC in fused MV) were fused to ISO membrane vesicles containing LacY synthesized and inserted in vitro. In lanes 5 and 6, in vitro translation and insertion reactions were performed after the vesicles were fused. Each sample was then solubilized and immunoprecipitated mAb 4B1 as described in Materials and methods.

ments the conditional YidC depletion strain JS7131 when YidC is depleted, demonstrating that YidC-His is functional in vivo (unpublished data). YidC is estimated to constitute ~2% of the total membrane protein (Urbanus et al., 2002). Therefore, purified YidC was added to solubilized YidC-depleted membrane vesicles at a ratio of YidC/total proteins of 1:100 (1%) or 1:50 (2%), and the mixtures were reconstituted into proteoliposomes (Fig. 7 B, lanes 3 and 4, respectively). The reconstituted proteoliposomes were then used for in vitro translation and insertion, followed by immunoprecipitation with mAb 4B11 (Fig. 7 B). LacY synthesis in vitro and insertion into the proteoliposomes is almost the same whether purified YidC is added to the reconstitution mixture or not (Fig. 7 B, top, lanes 1–4). However, LacY immunoprecipitation by mAb 4B11 is increased when the reconstituted proteoliposomes contain YidC (Fig. 7 B, bottom, lanes 1–4). Similar results were also observed when LacY was immunoprecipitated by mAb 4B1 (unpublished data). Thus, YidC is clearly required for proper folding of these epitopes.

To examine when YidC is required for folding (i.e., co-translationally or posttranslationally), YidC$^+$ ISO membrane vesicles were fused with YidC-depleted ISO vesicles after or before in vitro translation and insertion of LacY by using PEG3350-induced fusion (Akiyama and Ito, 2003). The fused vesicles were then solubilized and immunoprecipitated with mAb 4B1 (Fig. 7 C). Immunoprecipitation of LacY synthesized in vitro and inserted into YidC-depleted or YidC$^+$ membrane vesicles does not increase when YidC is presented by fusion after translation and insertion (Fig. 7 C, lanes 1–4, respectively). In contrast, when YidC is presented by fusion before in vitro translation and insertion, a highly significant increase in 4B1 immunoprecipitation is observed (Fig. 7 C, lanes 5 and 6). The results indicate that YidC must be present during translation and insertion in order for LacY to fold into a final tertiary conformation.

Discussion

This paper focuses on LacY, a polytopic membrane transport protein with a known structure (Abramson et al., 2003), in order to examine the role of YidC in membrane insertion and/or folding of polytopic membrane proteins in *E. coli*. First, by using both in vivo and in vitro approaches, it is demonstrated that SecY, a well-known component of the translocon in *E. coli*, is required for LacY insertion into the membrane, thereby confirming previous findings of Ito and Akiyama (1991). Having verified that expression and membrane localization of LacY-GFP is dependent on SecY function, the same approach was used to study the role of YidC in vivo. Unlike the findings with the SecY mutant, there is little difference in the fluorescence intensity of LacY-GFP in the membrane of YidC$^+$ or YidC-depleted cells. Moreover, pulse-chase experiments indicate that although LacY synthesized in vivo is localized at membrane essentially normally in YidC-depleted cells, the protein is unstable and proteolyzed rapidly. As suggested by cross-linking experiments (Houben et al., 2000; Beck et al., 2001; Urbanus et al., 2001), YidC functions downstream of SecY during translation and insertion, which is consistent with the results of the pulse-chase experiments presented here. This functional sequence is also supported by in vitro experiments with YidC-depleted membrane vesicles. Thus, although the rate of insertion of LacY in vitro in YidC$^+$ and YidC-depleted ISO vesicles is similar, there is a significant difference in levels at 30 min (Fig. 4 B), which may also be due to incomplete folding and proteolysis by residual proteases in the vesicle preparations.

It is difficult to distinguish clearly between insertion and folding of membrane proteins, because folding appears to start during insertion (Nagamori et al., 2003). However, LacY synthesized in vitro with either YidC$^+$ or YidC-depleted ISO vesicles resistant to urea extraction. In addition, periplasmic loop VII/VIII, which should be inaccessible, is protected from external Xa protease (Fig. 5). Thus, YidC does not appear to be required for targeting of LacY to the

membrane or for insertion. Moreover, immunoprecipitation experiments with two mAbs, which recognize structural epitopes on either the outer or inner surface of LacY (Fig. 6 A), reveal that LacY synthesized in vitro and inserted into $YidC^+$ membranes binds both mAbs well, whereas the same experiment performed with YidC-depleted membranes yields much less immunoprecipitated LacY (Fig. 6 B). Together, the results indicate that LacY can insert normally into membranes depleted of YidC; however, YidC is required for LacY to fold correctly, at least with respect to the 4B1 and 4B11 epitopes. This conclusion may explain most of the results reported thus far for YidC depletion in vivo. Unfolded proteins such as the NH_2- or COOH-terminal fragments of LacY synthesized and inserted independently (Bibi and Kaback, 1990; Wrubel et al., 1990; Zen et al., 1994; Nagamori et al., 2003) or certain LacY mutants (Roepe et al., 1989; Jung et al., 1995; Weinglass and Kaback, 2000) are inserted normally in vivo, but rapidly proteolyzed. It has also been reported that Oxa1 is specifically required for the stability of the membrane subunits of cytochrome *c* oxidase or F_1/F_o-ATPase complex (Lemaire et al., 2000). Thus, YidC and its homologues likely play a general role in folding and perhaps assembly of polytopic membrane proteins.

Possibly, YidC interacts with another protein(s) that is (are) involved in membrane insertion and/or folding. The reconstitution experiments presented here with purified YidC and YidC-depleted membranes (Fig. 7 B) do not resolve the question, although coreconstitution of purified YidC and solubilized YidC-depleted membranes markedly increases immunoprecipitation of LacY by mAb 4B1 and 4B11. In any case, it is noteworthy that YidC function increases, as judged by binding of 4B1, only when present during translation and insertion of LacY (Fig. 7 C). It is known that a portion of YidC forms a complex with the Sec machinery (Scotti et al., 2000; Nouwen and Driessen, 2002), and it has been suggested that YidC forms a homo-oligomer (van der Laan et al., 2001). Therefore, it is interesting to speculate that YidC may assist movement of LacY from a hydrophilic environment in the translocon complex to the hydrophobic environment of the bilayer and provide a local environment for folding before these proteins enter the hydrophobic environment of the bilayer. Indeed, it has been suggested that YidC may function as an assembly site for polytopic membrane proteins mediating the formation of helix bundles before their release into the lipid bilayer (Beck et al., 2001). It appears that LacY cannot achieve its final tertiary structure without YidC because the newly synthesized protein cannot transfer into the bilayer sufficiently well to fold properly in the absence of YidC. Recently, it is reported that large amounts of YidC inhibit insertion of a membrane protein with single transmembrane domain via Sec machinery (van der Laan et al., 2004). This result is also consistent with the notion that YidC may arrest insertion of transmembrane domains of polytopic membrane proteins.

Absence of PE in membranes also causes incorrect folding in manner similar to that observed with YidC-depleted membranes. Thus, as observed with YidC-depleted membranes, LacY inserted into PE-deficient membranes does not bind mAb 4B1 (Bogdanov and Dowhan, 1998). As shown in Fig. 3 B, YidC-depleted membranes contain the same large amount of PE as $YidC^+$ membranes. Therefore, it is unlikely that deficiency of PE in YidC-depleted membranes causes improper folding of LacY. However, it is possible that newly synthesized LacY in YidC-depleted membranes cannot interact with PE and that it is the latter interaction which is directly involved in the formation of the 4B1 mAb epitope. On the other hand, LacY in PE-deficient membranes binds mAb 4B11 (Bogdanov and Dowhan, 1998) unlike as LacY in YidC-depleted membranes (Fig. 6 B). Thus, it seems more likely that the role of YidC is independent of PE.

In addition to LacY, subunit II of cytochrome *o* oxidase and the a and c subunits of the F_o portion of F_1/F_o ATPase; van der Laan et al., 2003), as well as SecE (Yi et al., 2003), also require YidC, and it is likely that many more polytopic membrane will be found to require YidC for folding into a final tertiary conformation.

Materials and methods

Materials

[^{35}S]Methionine was obtained from Amersham Biosciences. mAbs 4B1 and 4B11 were prepared as described previously (Carrasco et al., 1982). ImmunoPure immobilized protein A was purchased from Pierce Chemical Co., and $DiBAC_4(5)$ was obtained from Molecular Probes. Anti-YidC antibody (Samuelson et al., 2000) was the gift of R. Dalbey (Ohio State University, Columbus, OH), whereas anti-SecY antibody (Nishiyama et al., 1991) and anti-SecE antibody (Matsuyama et al., 1993) were provided by H. Tokuda (The University of Tokyo, Tokyo, Japan). All other materials were obtained from commercial sources.

Strains and plasmids

E. coli KI297 (MC4100, *secY24 zhd-33*::Tn*10* F` *lac*$^+$ *lacI*$^q$)/pST30 (Shimoike et al., 1995) and *E. coli* KI298 (MC4100, *zhd-33*::Tn*10* F` *lac*$^+$ *lacI*^{q})/pSTV29 (Shimoike et al., 1995) were used for experiments involving fluorescence microscopy. Plasmid pSTV29 is the parent of pST30, which carries the *syd* gene under the *lac* promoter. ISO membrane vesicles for SecY experiments were prepared from *E. coli* AD202 (MC4100, Δ*ompT*::*kan*) or *E. coli* AD206 (AD202, *secY24*; Homma et al., 1997). All of the strains and plasmids described above were the gift of K. Ito (Kyoto University, Kyoto, Japan). *E. coli* JS7131 was used for YidC-depletion experiments. The chromosomal *yidC* gene of this strain is disrupted and an intact *yidC* gene under control of the *araBAD* promoter/operator is present (Samuelson et al., 2000). An expression plasmid for LacY-GFP was constructed from plasmids pT7-5 lacY/CXB (Consler et al., 1993) and pEGFP (CLONTECH Laboratories, Inc.). A new XbaI site was introduced at position 1,830 bps in pT7-5 lacY/CXB. The XbaI fragment from pEGFP was inserted into the new XbaI site of pT7-5 lacY/CXB. pTYidCH was constructed for expression of YidC-His in this work. The *yidC* gene was amplified by PCR from *E. coli* MC4100 chromosomal DNA. The PCR products were generated with a new BamHI site immediately before the initiation codon and six histidine codons followed by a HindIII site at COOH terminus. The PCR products were digested with BamHI and then with HindIII. BamHI was used at a relatively low concentration relative to DNA in order to obtain DNA fragments encoding YidC with six histidine residues at the COOH terminus. The appropriate fragments were cloned into pT7-5 lacY/CXB between the BamHI and HindIII sites. The plasmid was sequenced and tested for complementation of the YidC-depleted strain.

Microscopic observation of GFP

LacY-GFP or LacZ-GFP was expressed in the temperature-sensitive *secY24*/Syd$^+$ strain or the conditional YidC-depletion strain. Cells were grown as described in the figures and harvested by centrifugation. GFP fluorescence was visualized with an Axiovert 25 fluorescence microscope (Carl Zeiss MicroImaging, Inc.). Images were captured with a high performance color CCD camera (Cohu) and Scion Image software.

Pulse-chase assays

Plasmid pT7-5 LacY/6-His (Weinglass and Kaback, 2000) encoding wild-type LacY with six histidine residues at the COOH terminus was trans-

formed into *E. coli* JS7131, and the cells were grown in LB medium with 0.2% arabinose and 50 μg/ml ampicillin at 37°C overnight. Cells from overnight cultures were tested for viability on LB plate with or without arabinose. The cells were harvested by centrifugation and washed twice with LB medium. After resuspension in same volume of LB medium, the samples were diluted 50-fold in LB medium with 0.2% arabinose or 0.2% glycerol, and grown for 2.5 h at 37°C. Cells were then harvested by centrifugation, washed twice with M9 minimal media (with glycerol in place of glucose), and resuspended M9 minimal media containing 20 μg/ml of each amino acid except methionine in presence or absence of 0.2% arabinose. After 30 min at 37°C, 1.0 mM *i*-propyl-1-thio-β-D-galactopyranoside (IPTG) was added to induce expression of LacY/6-His and the cells were grown for an additional 5 min. Labeling was initiated by addition of [^{35}S]methionine to final concentration of 150 μCi/ml. After a 30-min incubation, cold methionine was added at 0.4 mg/ml. 1.0-ml aliquots were removed at 30-min intervals and placed on ice, followed by addition of 34 μg/ml chloramphenicol. The cells were harvested by centrifugation and flash frozen in liquid N_2. Membranes were prepared by sonification as described previously (Weinglass and Kaback, 2000) and resuspended in 100 μl of 50 mM potassium phosphate (KP_i), pH 7.5/10% (wt/vol) glycerol/10 mM imidazole/2% DDM. Supernatants were obtained by centrifugation and mixed with same volume of Talon cobalt affinity resin (CLONTECH Laboratories, Inc.), which had been equilibrated with same buffer, for 2 h at 4°C. Resins were collected by a brief centrifugation (1 min, 5,000 *g*) and washed with 3.5 ml of 50 mM KP_i, pH 7.5/10% glycerol/10 mM imidazole/0.2% DDM. Purified proteins were eluted with 50 μl of 50 mM KP_i, pH 7.5/500 mM imidazole/0.2% DDM, and treated as described previously (Weinglass and Kaback, 2000).

Preparation of ISO membrane vesicles and in vitro transcription/translation/insertion

ISO membrane vesicles were prepared as described previously (Yamada et al., 1989) with minor modifications (Nagamori et al., 2000). YidC-depleted ISO vesicles were isolated from YidC-depleted cells. The YidC conditional depletion strain *E. coli* JS7131 was grown at 37°C in LB medium with 0.2% arabinose from a single isolated colony. At an OD_{600} of 0.9–1.0, cells were harvested by centrifugation, washed with LB medium and diluted 50-fold in LB with 0.2% glucose. The cells were also tested for arabinose dependence on LB plates. 0.2% arabinose was used for $YidC^+$ cells in place of glucose. After a 3-h incubation at 37°C, cells were collected and ISO vesicles were prepared. In vitro transcription/translation/insertion was performed as described previously (Nagamori et al., 2003). Reaction mixtures were placed on a 50% sucrose cushion and centrifuged. The inner membrane fraction was collected carefully, diluted and centrifuged, and subjected to 4.0 M urea wash, as specified. ISO vesicles were pelleted by ultracentrifugation and analyzed by SDS/PAGE. To stop the in vitro reaction at the times indicated, 0.2 mg/ml of chloramphenicol was added.

Measurement of $\Delta\Psi$

Generation of $\Delta\Psi$ (interior positive) in ISO membrane vesicles was monitored by measuring fluorescence quenching of $DiBAC_4(5)$ (Matsushita et al., 1987). The reaction mixtures contained 1 μM $DiBAC_4(5)$ and ISO membrane vesicles (0.3 mg of protein) in 1 ml of 50 mM KP_i, pH 7.5/5 mM $MgSO_4$. Fluorescence at 613 nm was recorded in a spectrofluorimeter (excitation at 607 nm; model 8100; SLM-Aminco).

Immunoprecipitation of in vitro synthesized LacY

mAbs were purified with ImmunoPure immobilized protein A AffinityPak columns (Pierce Chemical Co.) and concentrated by using a Microcon concentrator (Millipore). In vitro transcription, translation, and insertion reactions were performed at 30°C for 15 min and washed with urea as described above. ISO membrane vesicles were washed with 50 mM Tris-HCl, pH 7.5, and incubated with 10–50 μg/ml of purified mAb as specified in 50 mM Tris-HCl, pH 7.5/150 mM NaCl/1.0 mM EDTA/2% DDM overnight at 4°C. Supernatants obtained by ultracentrifugation were treated with protein A–Sepharose beads (20% of the volume of the supernatants) for 90 min at 4°C. The beads were collected by a brief centrifugation (30 s, 10,000 *g*), washed four times with 100-fold protein A volumes of wash buffer (50 mM Tris-HCl, pH 7.5/150 mM NaCl/1 mM EDTA/0.2% DDM), and resuspended in 1% DDM and 1.0 mM DTT. After the addition of sample buffer, the supernatant obtained from a brief centrifugation were analyzed by SDS/PAGE.

Purification of YidC

Plasmid pTYidCH was transformed into *E. coli* BL21 DE3 pLysS cells (Novagen) and cultured overnight in LB medium at 37°C with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. The overnight culture was diluted 50-fold into fresh LB medium. YidC-His was expressed by the adding 0.5 mM IPTG at OD_{600} = 0.6 and incubated for another 2 h. Purification was performed as described previously (van der Laan et al., 2001) with modification. Cytoplasmic membranes were isolated and solubilized at 5 mg/ml in 10 mM Tris-HCl, pH 8.0/20% (vol/vol) glycerol/5.0 mM imidazole/2% DDM. After 1 h at 4°C, the supernatant was isolated by centrifugation (30 min, 320,000 *g*) and applied to a column packed with Talon cobalt affinity resin (CLONTECH Laboratories, Inc.) that had been equilibrated with 10 mM Tris-HCl, pH 8.0/20% glycerol/5 mM imidazole/0.1% DDM (buffer A). The column was washed with five column volumes of buffer A plus 45 mM imidazole (50 mM imidazole, final concentration). Bound protein was eluted with buffer A containing 300 mM imidazole. YidC eluted from the Talon column was further fractionated on a Mono-S column (Amersham Biosciences) using a 0–1.0 M NaCl gradient. YidC-enriched fractions were combined, dialyzed against 10 mM Tris-HCl, pH 8.0/20% glycerol/0.01% DDM, and concentrated.

Reconstitution

Proteoliposomes were reconstituted as described previously (Akimaru et al., 1991). ISO membrane vesicles were mixed at 1 mg/ml with 2.5 mg/ml *E. coli* phospholipids in 10% (wt/vol) glycerol/1.0 mM DTT/150 mM NaCl and 2.5% octyl-β-D-glucopyranoside were added. Where specified, purified YidC (10 μg/mg or 20 μg/mg of membrane proteins) was also added to the mixture. The reconstituted proteoliposomes were suspended in 50 mM KP_i, pH 7.5, and used for in vitro translation and insertion assays. Extraction with 4.0 M urea after in vitro synthesis was performed in the presence of 150 mM NaCl.

PEG3350-induced fusion of ISO membrane vesicles

Fusion of membrane vesicles was performed as described previously (Akiyama and Ito, 2003) with minor modification. After in vitro synthesis and insertion, ISO membrane vesicles were isolated as described above. The vesicles were resuspended in 50 mM MOPS, pH 7.0/0.5 M KCl. An equivalent amount (0.2 mg/ml) of vesicles in the in vitro synthesis and insertion reaction was added to the suspension, followed by an equal volume of 50 mM MOPS, pH 7.0/0.5 M /25% PEG3350. After incubation at 37°C for 5 min, the reaction mixture was diluted in four volumes of 50 mM KP_i, pH 7.5/150 mM NaCl and the membranes were harvested by ultra centrifugation (15 min, 350,000 *g*). The pellet was resuspended in buffer containing 50 mM KP_i, pH 7.5/150 mM NaCl/2.0 mM $MgSO_4$/1.0 mM DTT with briefly sonification and incubated at RT for 30 min. Membranes were collected by centrifugation and subjected to immunoprecipitation with a given antibody as described above.

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