Polarity/Charge as a Determinant of Translocase Requirements for Membrane Protein Insertion

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Abstract

The YidC insertase of *Escherichia coli* inserts membrane proteins with small periplasmic loops (~20 residues). However, it has difficulty transporting loops that contain positively charged residues compared to negatively charged residues and, as a result, increasing the positive charge has an increased requirement for the Sec machinery as compared to negatively charged loops [1, 2]. This suggested that the polarity and charge of the periplasmic regions of membrane proteins determine the YidC and Sec translocase requirements for insertion. Here we tested this polarity/charge hypothesis by showing that insertion of our model substrate protein procoat-Lep can become YidC/Sec dependent when the periplasmic loop was converted to highly polar even in the absence of any charged residues. Moreover, adding a number of hydrophobic amino acids to a highly polar loop can decrease the Sec-dependence of the otherwise strictly Sec-dependent membrane proteins. We also demonstrate that the length of the procoat-Lep loop is indeed a determinant for Sec-dependence by inserting alanine residues that do not markedly change the overall hydrophilicity of the periplasmic loop. Taken together, the results support the polarity/charge hypothesis as a determinant for the translocase requirement for procoat insertion.

Introduction

In bacteria, there are 2 primary specialized devices that catalyze the insertion of proteins into the inner (cytoplasmic) membrane [3-7]. The Sec translocase functions for inserting the majority of proteins into the membrane and YidC mediates membrane protein integration independently and together with the Sec machinery. Remarkably, both the SecYEG and YidC machineries are conserved from bacteria all the way to humans [8]. YidC is an essential protein for bacterial growth, and has homologs in mitochondria and chloroplasts [9-11] as well as in the eukaryotic ER membrane [12-14].

A big breakthrough occurred in 2014 with the determination of the atomic structure of YidC2 from *Bacillus halodurans* and YidC from *Escherichia coli* by X-ray crystallography [15, 16]. The structure revealed that YidC contains a hydrophilic groove that is open to cytoplasm and lipid bilayer, but is closed on the extracellular side of the membrane. The hydrophilic groove contains a strictly conserved positively charged residue that is essential for cell growth and for the translocation of the N-tail region of MifM in *Bacillus subtilis*. The hydrophilic groove resides within the inner leaflet of the membrane and is proposed to function in the recruitment of the hydrophilic peptide chain of the substrate that needs to be translocated across the membrane. At the cytoplasmic entrance of the groove, there is a helical hairpin domain that is believed to be flexible [15, 17] and may be involved in the initial binding of YidC substrates.

The main substrate contacting region of YidC occurs at transmembrane (TM)3 [18-20], and TM5 [19]. From the *B. halodurans* structure, it is readily apparent how a hydrophobic segment of the inserting membrane protein can be accommodated in between TM3 and TM5 by a greasy slide mechanism in the *E. coli* YidC protein (corresponding to TM2 and TM4 of the *B. halodurans* YidC) [21], Binding of the hydrophobic segment to the hydrophobic slide would allow the hydrophilic peptide chain to be accommodated within the hydrophilic groove [21]. Recently, using arrested protein chains of various lengths, the hydrophobic segment of Pf3 coat was shown to first move up the greasy slide followed by bending back the N-tail to be incorporated into the hydrophilic cavity of YidC [22].

One important issue in the membrane protein topogenesis field has to do with the features of membrane proteins that determine whether YidC and/or Sec are required for insertion. Several determinants have been proposed for YidC including negatively charged residues in the TM segment [23] and translocated regions [1]. Recent studies suggest that the low hydrophobicity of the TM segment [1, 24] and the charge and polarity of the translocated region [2] are key determinants for a membrane protein. Identifying translocase determinants is important and informative, as they will allow us to predict the pathway (YidC, or YidC/SecYEG) a protein uses for its insertion process.

In this paper, we have systematically investigated the polarity/ charge hypothesis as a pathway determinant for the M13 procoat-Lep protein. As predicted by this hypothesis, we found generally that procoat-Lep requires only YidC when the polarity of an uncharged periplasmic loop is low but requires YidC and SecYEG for insertion when the polarity of the periplasmic loop was increased. Furthermore, addition of hydrophobic residues in the highly polar uncharged or charged loop can decrease the Sec-dependence of the procoat-Lep mutants. We discuss the charge/polarity hypothesis in relation to the structure of YidC. Overall, the results show that the polarity of the translocated loop region is decisive for the selection of YidC and SecYEG machineries for membrane protein insertion.

Results

The charge/polarity hypothesis predicts that if the polarity of the translocated protein region is below a certain threshold then insertion can use a YidC only mechanism, while further increases in the polarity of the translocated region would go by the YidC/Sec mechanism [2]. If the polarity is too high, insertion is blocked even with the assistance of the translocases. In contrast, the protein can insert by an unassisted mechanism if the polarity of the translocated region is very low. To test this hypothesis we used the M13 coat protein. The coat protein is synthesized in a precursor form (termed procoat) with a cleavable leader peptide and a mature region with a 20 amino acid periplasmic loop and a membrane anchor (TM2). In our studies, we use procoat-Lep (PCLep) in which we have extended the cytoplasmic region of the M13 procoat protein with 103 amino acids of the SP1 (also called Lep, [25]). By adding the extension, we can immunoprecipitate the protein using Lep antiserum.

To determine the YidC dependence of insertion, the YidC depletion strain JS7131 was employed. JS7131, which is arabinose-dependent for growth, has the *yidC* gene under the control of the araBAD promoter at the lambda attachment site and it also has its endogenous *yidC* gene inactivated by a deletion [26]. Membrane insertion was examined under YidC depletion conditions by growing the cells in media supplemented with glucose. JS7131 cells expressing various PCLep mutants were labeled with [^{35}S]-methionine for 1 min under YidC expression conditions (0.2%

arabinose) or YidC depletion conditions (0.2% glucose), respectively. Protease accessibility was used to examine membrane insertion. If PCLep inserts into the membrane, it is cleaved by signal peptidase 1 and converted to the mature coat protein. Proteinase K (PK) cleaves the translocated loop of PCLep, resulting in an additional shift on the gel (Fig. 1). When YidC is depleted, the precursor form of M13 PCLep accumulates in the cytoplasm and is resistant to PK digestion (Fig. 1A, left panel). CM124, the SecE depletion strain, was employed to examine the SecYEG dependence of insertion [27]. Proteins that require the SecYEG machinery are strongly inhibited under SecE depletion conditions, while Sec-independent proteins are not affected. Like the YidC depletion strain, CM124 is arabinose dependent for growth. It has the *secE* gene under the control of the araBAD promoter. Depletion of SecE leads to a decrease in the level of the entire SecYEG complex [28], as SecE is required for the stability of SecY [29]. CM124 cells expressing different PCLep mutants were labeled with [^{35}S]-methionine for 1 min under SecE expression conditions (0.2% arabinose and 0.4% arabinose) and SecE depletion conditions (0.4% glucose), and proteinase K mapping was used to evaluate membrane insertion as described for JS7131.

For all the experiments, we analyzed OmpA as a positive control for SecYEG inhibition/depletion respectively. OmpA accumulates in a cytoplasmic precursor form when SecE is depleted. Also, we used OmpA as a negative control for YidC depletion. OmpA (a YidC independent protein) should not accumulate in a

precursor form under YidC depletion conditions. In most cases, one representative OmpA data set is shown.

Increasing the polarity of the uncharged PCLep loop causes the protein to become YidC/Sec dependent for insertion

If simply the polarity of the loop determines the translocase requirements, an extremely polar loop without any charged residues should also require both YidC and the Sec translocase for insertion. Starting with the Sec-independent ANGNN mutant (+188 kJ/mol GES) we used asparagine residues to increase the hydrophilicity of the periplasmic loop. First, we substituted the less polar Ala residues within Ala7-Lys8-Ala9-Ala10 with asparagines to increase the hydrophilicity and substituted the Lys to Asn to keep the residue hydrophilic but uncharged. Shown in Figure 1B, this 7N PCLep with a GES of 251 kJ/mol inserted across the membrane in a YidC dependent and slightly Sec-dependent manner. [^{35}S]-labeled 7N PCLep mutant accumulates after 1 min in the precursor form when YidC is depleted and is mostly processed when SecE is depleted. The PCLep WT (A) and ANGNN PCLep (data not shown; see [2]) are completely blocked under YidC depletion conditions but unaffected under SecE depletion conditions. 8N PCLep (237 kJ/mol GES) with 1 additional asparagine substituting the glutamyl residue in the loop at position +20 resulted in YidC-dependent and mostly Sec dependent insertion (Fig. 1C). Further substitution with two asparagine residues at +16 and

+18 (Fig 1A) gave rise to a strictly YidC and Sec-dependent protein (Fig. 1D, see PCLep 10N with a 264 kJ/mol GES).

Addition of hydrophobic residues to the highly polar loop decreases the Sec-dependence of insertion

The results shown in Fig. 1 indicate as the polarity of the PCLep periplasmic domain is increased, the protein becomes dependent on the Sec machinery for insertion, in addition to YidC. However, there is a limit that can be translocated even with the help of Sec. No insertion is observed with the 11N PCLep protein (316 kJ/mol GES) that has one more asparagine added (at +19)(Fig. 2A), suggesting there is a polarity threshold by which the protein can translocate a loop even with both the YidC and Sec machineries.

If the translocase requirements are dictated by the polarity of the loop, then adding hydrophobic residues to the neutral apolar loop should decrease the translocase requirements for translocation. Starting with the 11N mutant, we substituted either 2 or 3 residues to hydrophobic phenylalanines (PCLep11N+2F and PCLep11N+3F). As documented in Fig. 2, the periplasmic region of these 11N mutants can cross the membrane. Interestingly, while the PCLep11N+2F mutant is dependent on the SecYEG for efficient insertion, the PCLep11N+3F inserts independent of Sec (Fig. 2B, C). This clearly demonstrates that the introduced

hydrophobicity in the periplasmic loop supports the translocation of the highly polar loop containing 11 asparagines.

Similarly, adding hydrophobic residues to a highly charged loop decreases the Sec-requirement for membrane insertion. Here we substituted three residues to phenylalanines in the periplasmic loop of the strictly Sec-dependent 3R and -5 PCLep mutants (See schematic in Fig 2A). Remarkably, these PCLep mutants insert in a YidC dependent and Sec-independent manner (Fig. 2E and 2G). Taken together, these results support the polarity/charge hypothesis that it is the overall polarity of the periplasmic loop that dictates the translocase requirement for protein insertion.

Increasing the hydrophobicity of TM2 rescues membrane insertion of PCLep mutants with highly polar periplasmic loops

Our hypothesis is that increasing the hydrophilicity of the loop beyond a certain limit will prevent translocation even with both the YidC and SecYEG cooperating in membrane insertion. This explains why the 11N (Fig. 2A), the -7 (Fig. 3C; [30]) and 4R (Fig. 3A) are blocked in membrane insertion. We asked whether we could promote translocation of these highly polar periplasmic loops if we increase the driving force for insertion by introducing 4 leucines into TM2. As shown in Fig. 3, translocation of the 4R PCLep was completely rescued by the 4L mutation in TM2 with both YidC and Sec promoting translocation (Fig. 3, compare panels A and B). In addition, translocation of the -7 PCLep and PCLep 11N was restored to some extent

by the substituting the 4 leucines into TM2 (Fig. 3, see panels D-E). The results show that increasing the hydrophobicity of TM2 of PCLep increases the capacity of PCLep to translocate the highly polar periplasmic loops.

Increasing the length of the translocated region increases the Sec dependence of membrane insertion

Based on the polarity/charge hypothesis, the reason why proteins that go by the YidC only pathway possess short translocated region is due to the fact that, when the length of the periplasmic loop increases, the polarity of the loop is too high and exceeds the energy threshold to be inserted by the YidC insertase (i.e. larger translocated loops have higher polarity and therefore would require the Sec machinery). The YidC insertase by itself can insert the 20-residue loop of procoat [31] and the 29-residue loop of MscL [32]. The MscL loop is the longest loop identified to date to insert by the YidC only pathway. We tested whether increasing the loop size of procoat (PCLep) by the insertion of 5, 10, and 15 alanine residues after the phenylalanine at position +11 changes the insertion pathway of the protein (see Fig. 4). The alanine insertion should not increase the overall hydrophilicity of the loop significantly because alanine has a GES of -1.7 kJ/mol. As shown in Figure 4, PCLep with the addition of the 5 alanine residues, inserts Sec-independently but still in a strictly YidC-dependent manner (Fig. 4A). Increasing the loop length further by adding 10 alanine residues resulted in membrane insertion that is markedly dependent on the Sec machinery, in addition to YidC (Fig. 4B).

Interestingly, when increasing the driving force for membrane insertion by the addition of 4 leucine residues to TM2, results in very efficient insertion under SecE-depletion conditions (Fig. 4B). Insertion of this 4 leucine PC mutant is YidC dependent (Fig. S1). Further increases in the loop size by addition of a total of 15 alanine residues made insertion very inefficient, suggesting there is a size limit even with SecYEG (Fig. 4C). The addition of 4 leucines to the TM segment did not improve insertion (Fig. 4C, right panel). In conclusion, a loop length of 30 residues seems to be the maximum that can be translocated.

The Sec-dependent PCLep inserts at the interface of YidC and SecYEG

In support of our hypothesis of membrane protein insertion occurring at the interface of YidC and SecYEG, we examined the interaction of the substrate PCLep with YidC and SecYEG using an *in vivo* site-directed cross-linking approach. Single cysteine was incorporated at 430 in the hydrophobic slide region of YidC or at residue 69 in in the lateral gate of SecY, respectively. These constructs were then expressed along with single cysteine mutants of PCLep or PCLep 3R substrates, respectively in the YidC-depletion cells (Fig. 5A) or in the SecE depletion cells (Fig. 5B). Both substrates had an additional mutation (H5) that prevents their cleavage by SP1 [33]. The cells expressing substrate and the YidC mutant were grown to 0.5 at OD 600, labeled with [^{35}S]-methionine after IPTG induction and treated with copper phenanthroline (CuP). The sample were then TCA-precipitated, acetone washed, and dissolved in Tris-SDS buffer. The samples were immunoprecipitated

using Anti-YidC (Fig. 5A) or Anti-Lep antibody (Fig. 5B). The samples were then analyzed by SDS-PAGE and phosphorimaging. The results show that PCLep 3R interacts more efficiently with SecY69 compared to PCLep (Fig. 5B) whereas PCLep 3R (Fig. 5A) shows less contact to YidC 430. The crosslinking results are in support of our hypothesis that PCLep inserts at the YidC/Sec interface with both the insertases surveying the inserting protein and acting cooperatively.

Discussion

In this report, we provide evidence for the polarity/charge hypothesis, which states that the membrane insertion pathway selection is determined by the polarity of the translocated periplasmic loop of small membrane proteins. It has been shown earlier using PCLep as a model system that when polarity of the loop is increased either by incorporating negatively or positively charged residues the Sec requirement for insertion can be increased [2] but the translocation of charged residues in periplasmic loops can be complex due to the electrochemical membrane potential (outside positive) promoting translocation of negatively charged residues [30, 34] and hindering translocation of positively charged residues [35]. In contrast, the electrochemical potential does not stimulate or hinder the translocation of a neutral loop of PCLep [35]. We verified that this is also true of a neutral loop with eight asparagines present in hydrophilic loop as PCLep 8N inserted independently of the electrochemical potential (Fig. S2).

We examined the Sec-dependence for loops containing no charged residues to evaluate the contribution of the polar side chains for translocation. As the number of substituted Asn residues in the periplasmic loop was increased from 7 to 10, PCLep insertion changed from YidC only to YidC- and SecYEG-dependent (Fig. 1). Our results showed that charged residues are not needed in the periplasmic loop in order to make insertion YidC/Sec-dependent. Also, we found that translocation of strictly Sec-dependent positively, neutral and negatively charged loops of PCLep

was less Sec-dependent when the overall hydrophilicity was reduced by substituting hydrophobic amino acids into the loop (Fig. 2).

To quantify the hydrophilicity of the loop we used the GES scale. The GES values are based on the water/oil distribution to determine the standard free energy expense of each amino acid [36] and include the contribution of the peptide bond [37].The GES scale was chosen to determine the hydrophilicity of the loop because we have used this scale to previously calculate the standard free energy expense of M13 procoat protein [2], Pf3 coat protein [38] and MscL [39].

As shown in Fig. S4 and Table I, we found a good correlation for the negatively charged and positively charged PCLep series between the YidC/Sec-dependence of membrane insertion and the standard free energy needed for the transfer of the different polar loops of the PCLep proteins across the membrane studied in this paper. A GES value above 282 (KJ/mol) resulted in strict YidC/Sec dependence of insertion for the 3R PCLep while the substitution of three phenylalanines in the loop of 3R PCLep + 3F (GES value of 252 kJ/mol) led to YidC only insertion. Likewise, while -5E (GES value of 317 kJ/mol) inserted in a strict YidC/Sec-dependent manner the substitution of 3 phenylalanines in the loop of -5 PCLep + 3F (GES value of 287 kJ/mol) led to YidC only insertion. With the Asn series, the correlation was not as good (Table 1 and Fig. S3. One factor possibly contributing to the less than perfect correlation of the Asn series is that the 7N PCLep with a higher GES value (251 kJ/mol) still has a glutamic acid residue at position 20, which is easier to insert by

the YidC only pathway and its insertion is facilitated by the membrane potential. When the glutamic residue is substituted with an Asn, which decreases the GES value from 251 to 237 kJ/mol, insertion of 8N PCLep is probably more difficult for the YidC insertase and insertion requires the assistance of the Sec translocase for optimal insertion. Finally, the substitution resulting in two additional Asn residues in the loop (GES value of 291 kJ/mol) led to strict YidC and Sec-dependent insertion of 10N PCLep. Although we have a limited number of data points, we found strict YidC and Sec-dependence of insertion at a GES level of 282, 291 and 317 kJ/mol for the positively, neutral and negatively charged PCLep constructs.

Above a certain hydrophilic threshold of the loop (>296 KJ/mol 4R , > 316 KJ/mol for the 11N PCLep, , and >396 kJ/mol for the -7 PCLep), insertion did not occur even with both the YidC and Sec machineries. The threshold appears higher for the negatively charged mutant and lowest for the positively charged mutant probably because the membrane potential favors the transfer of negatively charged residues over positively charged residues, a fact which is not considered by the GES values.

Is the polarity rule applicable to the mitochondrial Oxa1 insertase, which is found in the mitochondrial inner membrane? Unlike the bacterial YidC, the mitochondrial Oxa1 does not cooperate with the Sec translocase since mitochondria lack a Sec translocase. Therefore, one would expect that the polarity of the protein regions in the intermembrane space to be rather low. Interestingly, there are seven mitochondrial-encoded membrane proteins in *S. cerevisiae*, (Cox1, Cox2, Cox3,

ATP6, ATP8, ATP9, and subunit b) that insert into the inner membrane from the matrix and have to translocate a hydrophilic region to the intermembrane space (IMS) Table S1 shows the translocated regions of these mitochondrial proteins typically have a net negative charge like bacterial YidC proteins as pointed out by Herrmann and Bonnefoy [40] and the size of the IMS loop tends to be short with the exception of the C-terminal domain of Cox2. In all cases, the GES values are less than 247 kJ/mol. This is similar to the YidC only membrane proteins in bacteria, where the translocated periplasmic loops are short (28 residues or less) with the highest GES value is 238 kJ/mol for the M13 procoat protein (Table S2).

The recent structure of YidC [15, 16] provided an explanation for why YidC dependent substrates often contain negatively charged residues. The structure revealed that the membrane-embedded core region contains a hydrophilic groove with a positively charged residue (corresponding to arginine 366 in the *E. coli* YidC), which has been proposed to attract the negatively charged residues within the substrate translocated domain [15]. Translocation of positively charged domains may require the Sec machinery because the conserved arginine in the hydrophilic grove of YidC is unable to attract the positively charged loop of the YidC targeted PCLep protein. Rather, it is likely to be repelled by the conserved arginine. As the number of positively charged residues in the substrate is increased, the electrostatic repulsion between the loop of the protein and the positive charge on YidC increases, which possibly explains why the Sec machinery is required to a much greater extent, to insert the protein into the membrane [2]. Our hypothesis is that PCLep with

positively charged residues is still directed to YidC located in close proximity to SecYEG where the translocase can assist in the translocation of the polypeptide chain. Support for insertion occurring at the YidC/SecYEG interfacial region comes from disulfide crosslinking studies where PCLep 3R can be crosslinked to the YidC hydrophobic slide and to the SecY lateral gate region (Fig. 5).

While translocation of a strongly polar loop of PCLep can occur with the help of both YidC and SecYEG, there is a limit to the polarity level that can be translocated. When the 4 arginines, 7 negatively charged residues or 11 asparagine residues are in the loop, then translocation does not occur because the polarity of the loop exceeded the threshold (Fig. 3).

The polarity/charge hypothesis can also explain the length limitation for the translocation of a domain. For example, previously, it was shown that the length of the periplasmic region was a determinant of Sec-independent insertion [41, 42]. However, in these cases, the length was increased by adding a polar segment to the translocated region, which increases the hydrophilicity of the periplasmic loop, in addition to the length. This could be the reason for the Sec-dependence of translocation since the membrane transfer expense exceeded the threshold for the YidC only pathway. Therefore, we increased the length of the periplasmic domain of PCLep by inserting alanine residues, which is predicted to not increase the membrane transfer expense [43]. Interestingly, we found that insertion of PCLep is still Sec-independent when it contained 5 additional alanine residues but the

addition of 10 alanines resulted in translocation events becoming Sec-dependent, suggesting that length of the chain is indeed a translocase determinant. Moreover, we found by increasing the driving force for membrane insertion by incorporating 4 leucine residues into TM2, that translocation of the 10A PC-Lep mutant became almost completely Sec-independent. This is then similar to the size of the periplasmic loop of MscL, which inserts by the YidC only pathway [39]. Most likely, the length limitation for translocation by the YidC insertase has to do with the overall size of the hydrophilic groove that has a limited capacity [21]. It is possible that the limited size of the cavity only allows it to accommodate chains up to 30 residues in length and, loops exceeding this size, would have to be transported across the membrane with the assistance of the SecYEG machinery. This explains why YidC-only substrates have only small protein domains that are translocated. Similar results were found studying the model substrate 2Pf3-Lep that translocates the N-tail region across the membrane [44]. A tail of 41 residues could be translocated across the membrane by the YidC-only pathway, and an increase in the length of the N-tail region or charge density changes the translocase requirements from YidC only pathway to the YidC/Sec pathway.

Notably, there are many membrane proteins, single and multispanning, that have translocated loops greater than 30 residues and are inserted in bacteria by the Sec machinery. We hypothesize that PCLep, on the other hand, is targeted to the YidC insertase and this leads to its limited ability to insert highly polar loops or loops of 30 or 35 residues in size even when it cooperates with SecYEG. Although the reason

for this is not clear it was also seen in a previous study [45]. For example, translocation of PCLep with an OmpA fragment inserted into the periplasmic loop although efficient when short was strongly inhibited or blocked when it was 40, 60, or 80 residues in size. However, when the size of the periplasmic loop was greater than 80 residues in size it was translocated quite well probably because it was then targeted to the Sec machinery in a different fashion and inserted by SecYEG involving the motor SecA ATPase for translocation.

Materials and Methods

Materials

Sodium azide was purchased from Sigma. Isopropyl 1-thio-D-galactopyranoside was from Research Products International Corp. Trans [^{35}S]-label, a mixure of 85% [^{35}S]-methionine and 15% [^{35}S]-cysteine, 1000 Ci/mmol, was from PerkinElmer Life Sciences. Antisera to leader peptidase (anti-Lep) and outer membrane protein A (anti-OmpA) were from our own laboratory collection.

Strains, Plasmids, and Growth Conditions

MC1060 (F-, *Δ(codB-lacI)3*, *galK16*, *galE15*, *relA1*, *rpsL150*, *spoT1*, *hsdR2)*, MK6 (MC1061 *yidC* promoter replaced by the araBAD promoter) and JS7131 (MC1060 *ΔyidC* attlambda::(araC ParaBAD-*yidC*), the *E. coli* YidC depletion strains, are from our collection. CM124 (*secE*Δ19-111, pCM22) and GSO479 (MG1655 secE promoter replaced by the araBAD promoter), the *secE* depletion strains, were obtained from Beth Traxler [27] and from Gisela Storz [46], respectively. In all these strains, the *yidC* and *secE* genes, respectively, are under the control of the *araBAD* promoter.

JS7131 cells were cultured at 37 °C for 3 h in LB media supplemented with 0.2% arabinose (YidC expression conditions) or 0.2% glucose (YidC depletion conditions)[2]. The SecE depletion strain CM124 was cultured in M9 media with

0.2% arabinose plus 0.4% glucose (SecE expression conditions) or 0.4% glucose (SecE depletion conditions) for 8 –9 h to deplete SecE. Prior to induction of the plasmid-encoded proteins in JS7131 and CM124, the cells were exchanged into fresh M9 media [2] and shaken for 30 min at 37 °C. To express the PCLep mutants in CM124, JS7131, and MC1060, the genes were cloned into the pLZ1 vector under the control of the lacUV5 promoter [47].

Protease accessibility assay

Expression of the PCLep proteins encoded on the vector was induced by 1 mM isopropyl 1-thio-β-D-galactopyranoside (final concentration) for 5 min. Cells were labeled with [^{35}S]-methionine for 1 min and converted to spheroplasts. The pulse-labeled cells were collected by centrifugation and resuspended in spheroplast buffer (33 mM Tris-HCl, pH 8.0, 40% (m/v) sucrose). The resuspended cells were treated with 1 mM EDTA and 10 μg/ml lysozyme on ice for 30 min. An aliquot was then digested by the proteinase K (0.75 mg/ml) for 1 h on ice, and the reaction was quenched by the addition of 5 mM phenylmethylsulfonyl fluoride (PMSF) for 5 min. The cells were TCA precipitated and spun down at 14,000 *g* for 10 min. The pellet was washed with 1 ml of ice-cold acetone. The protein pellet was then solubilized with Tris-SDS buffer (10 mM Tris-HCl, pH 8.0, 2% (m/v) SDS). The samples were immunoprecipitated with antiserum to leader peptidase (Lep) to precipitate the PCLep derivatives or with antiserum to OmpA for a control. The samples were analyzed by SDS-PAGE and phosphorimaging. All protease mapping data reported in

the study were performed at least three times and the data shown are representative.

Mutagenesis of PCLep to Create Mutants

All of the mutants studied in this paper were made using PCR mutagenesis. The integrity of the sequence was verified by DNA sequencing.

Disulfide Crosslinking

To detect PCLep interaction with YidC, disulfide crosslinking was used. A cysteine was introduced at the -12 position of the signal peptide of PCLep and a cysteine was introduced at position 430 in the center of TM3 of YidC. *E. coli* MK6 cells expressing YidC 430C and PCLep H5 -12C or YidC 430C and procoat-Lep 3R -12C were induced with 1mM IPTG for 5 min to express the substrate PCLep and labeled with [^{35}S]-methionine (50 μCi/ml) for 1 min. The cultures were treated with 1 mM freshly prepared copper 1,10-phenanthroline for 10 min at 37 °C. The samples were incubated with an equal volume of 20% trichloroacetic acid (TCA) to precipitate total proteins, then acetone washed and solubilized in Tris-SDS (10 mM Tris, 2% SDS), pH 8.0 buffer. Crosslinking between PCLep and YidC was analyzed by immunoprecipitation with YidC C-tail antiserum. To detect crosslinking between PCLep and SecY, a cysteine was introduced at position +33 in the center of coat transmembrane region (TM2) and a cysteine was added to position 69 of SecY. For

expression of these proteins, we used a SecE depletion strain, LR1655-secEY^{-} from our collection, which is a derivative of the *E. coli* strain GSO479 from Gisela Storz [46]. The chloramphenicol resistance gene was removed by the transformation of the suicide plasmid pCP20 [48, 49]. SecY 69C was expressed from pMS119EH bearing the secYEG genes using the IPTG-inducible Tac promoter. PCLep H5 33C or PCLep 3R 33C was expressed with pGZ119. Cells bearing SecY 69C and PCLep H5 33C or SecY 69C and PCLep 3R 33C were treated with 1mM IPTG for 10 min to express the substrate PCLep, labeled with [^{35}S]-methionine (50 μCi/ml) for 2 min, and then treated with 1 mM copper phenanthroline for 10 min, followed by TCA precipitation and immunoprecipitation with Anti-Lep antiserum. Samples were analyzed by SDS-PAGE and phosphorimaging, as described above.

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Figure Legends

Fig. 1: Increased Sec requirement with polar residues in the loop of PCLep. At the top of the figure are the amino acid sequences of PCLep mutants with asparagine residues introduced into the periplasmic region marked in red. The sequences of the two transmembrane segments are marked with green letters. YidC and SecE

requirement for membrane insertion of WT (A), 7N (B), 8N (C), and 10N (D) PCLep were tested. Representative data of OmpA indicate inhibition in OmpA export under SecE depletion conditions (E). *E. coli* JS7131 (YidC depletion strain) expressing the various PCLep proteins were grown for 3 h under YidC expression (0.2% arabinose) or YidC depletion conditions (0.2% glucose), labeled with [^{35}S]methionine for 1 min, and analyzed by the protease-accessibility assay, as described under "Materials and Methods." The Sec dependence of membrane insertion was tested using CM124, the SecE depletion strain. CM124 transformed with the respective pLZ1 plasmid was grown under SecE expression (0.2% arabinose and 0.4% glucose) or SecE depletion conditions (0.4% glucose), labeled with [^{35}S]methionine, and analyzed as described above.

Fig. 2: Increase in hydrophobicity of a hydrophilic loop decreases the Sec dependence of translocation. At the top of the figure are the amino acid sequences of PCLep Sec-dependent mutants (substitutions are in red letters) with the polarity of the loop decreased by the introduction of hydrophobic phenylalanines (blue letters). The YidC and SecE requirements for membrane insertion of 11N (A), 11N+2F (B), 11N+3F (C), -5 (D), -5 +3F(E), 3R (F), and 3R+3F PCLep (G) are shown. Membrane insertion, labeling and protease mapping studies were performed as described in Fig. 1, and under "Materials and Methods."

Fig. 3: Increasing the hydrophobicity of transmembrane segment 2 restores translocation of a highly polar periplasmic loop of PCLep. At the top of the

figure are the amino acid sequences of Sec-dependent PCLep mutants (substitutions are in red letters) without and with added four leucine residues (in red letters) introduced into TM2. The YidC and SecE requirements for insertion was investigated for the 4R (A), 4R + 4L (B), -7 (C), -7 + 4L (D) and 11N +4L (E), are shown. Membrane insertion, labeling and protease-accessibility studies were performed as described in Fig. 1, and under "Materials and Methods."

Fig. 4: Increasing the length of the translocated region increases the Sec dependence of PCLep membrane insertion. At the top of the figure are the amino acid sequences of PCLep mutants with extended loops of 5, 10, and 15 alanine residues (in blue letters) at the indicated positions within the periplasmic loop. Where indicated, 4 leucine residues (in red letters) were added to TM2. The YidC and SecE dependence of the 5 Ala (A), 10 Ala (B), and 15 Ala (C) PClep mutants are shown. The PClep mutants with the 4L mutation are shown in the right panel in A-C. The YidC and SecE dependence of membrane insertion was examined as described in Fig. 1, and under "Materials and Methods." NS mean not studied.

Fig. 5: Substrate PCLep 3R interacts with YidC and SecY during translocation. Disulfide crosslinking shows contact between YidC and PCLep. MK6 (the YidC depletion strain) expressing YidC 430C and PCLep H5 -12C or YidC 430C and PCLep 3R -12C was labeled with [^{35}S]methionine for 1 min and then treated with 1 mM freshly prepared CuP for 10 min at 37 ^{o}C. YidC was immunoprecipitated with antibody against C-terminal peptide. The samples were analyzed by SDS-PAGE and

phophorimaging. B. Disulfide crosslinking shows contact between SecY 69C and PCLep 3R. LR1655-secEY^{-} (the SecY depletion strain) expressing SecY 69C and PCLep H5 +33C or SecY 69C and PCLep 3R +33C was pulsed labeled for 2 min and treated with 1 mM CuP for 10 min at 37^{o}C. The samples were immunoprecipitated with anti-Lep antiserum to precipitate PCLep and the samples were analyzed by SDS-PAGE and phophorimaging.

Fig. S1. YidC-dependent membrane insertion of the various 4 Leucine TM PCLep variants with extended loops. The YidC dependence of membrane insertion of 4L PCLep with 5A (A), 10A (B), and 15 A (C) added to the periplasmic loop were tested. *E. coli* JS7131 (YidC depletion strain) expressing the various PCLep proteins were grown for 3 h under YidC expression (0.2% arabinose) or YidC depletion conditions (0.2% glucose), labeled with [^{35}S]methionine for 1 min, and analyzed by the protease-accessibility assay, as described under "Materials and Methods."

Fig. S2. The 8N PCLep inserts into the membrane independent of the electrochemical potential. *E. coli* JS7131 expressing 8N PCLep or -5 PCLep proteins were grown for 3 h under YidC expression (0.2% arabinose). Cells were pretreated with 50 μM CCCP (the protonophore) for 45 s, labeled with [^{35}S]methionine for 1 min. PCLep was detected by immunoprecipitation using Lep antiserum and OmpA, which inserts in an electrochemical potential manner, was detected using OmpA antiserum. The results show that 8N PCLep is inserted and

cleaved to mature CLep when the protonophore CCCP is added to collapse the electrochemical potential. The PC-5Lep is inserted and cleaved to mature C-5Lep in the presence of an electrochemical potential, while it is not inserted across the membrane when CCCP is added and accumulates in the precursor PC-5Lep form. As a control, the membrane electrochemical potential-dependent OmpA was shown to accumulate in a precursor form when the potential is dissipated by the addition of the protonophore CCCP.

Fig. S3. Increased Sec requirement with polar residues in the loop of PCLep. Quantification of the data in Fig. 1.

Fig. S4. Increase in hydrophobicity of a hydrophilic loop decreases the Sec dependence of translocation. Quantification of the data in Fig. 2.

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Mutants	Membrane Insertase YidC	The Sec Translocon SecYEG	GES value (KJ/mol)
Fig. 1			
Wild type	+++	-	238
7N PCLep	+++	+	251
8N PCLep	+++	++	237
10N PCLep	+++	+++	291
Fig. 2			
11N PCLep	NI	NI	316
11N PCLep + 2F	+++	++	288
11N PCLep + 3F	+++	-	286
5E PCLep	+++	+++	317
5E PCLep + 3F	+++	-	287
3R PCLep	+++	+++	282
3R PCLep + 3F	+++	-	252
Fig. 3			
4R PCLep	NI	NI	296
4R PCLep + 4L	+++	+++	296
7E PCLep	NI	NI	396
7E PCLep + 4L	+++	+++	396
11N PCLep +4L	+++	+++	316
Fig. 4			
5A PCLep	+++	-	230
10A PCLep	+++	+	221
15A PCLep	+++	+++	213
5A PCLep +4L	+++	-	230
10A PCLep +4L	+++	-	221
15A PCLep +4L	+++	+++	213

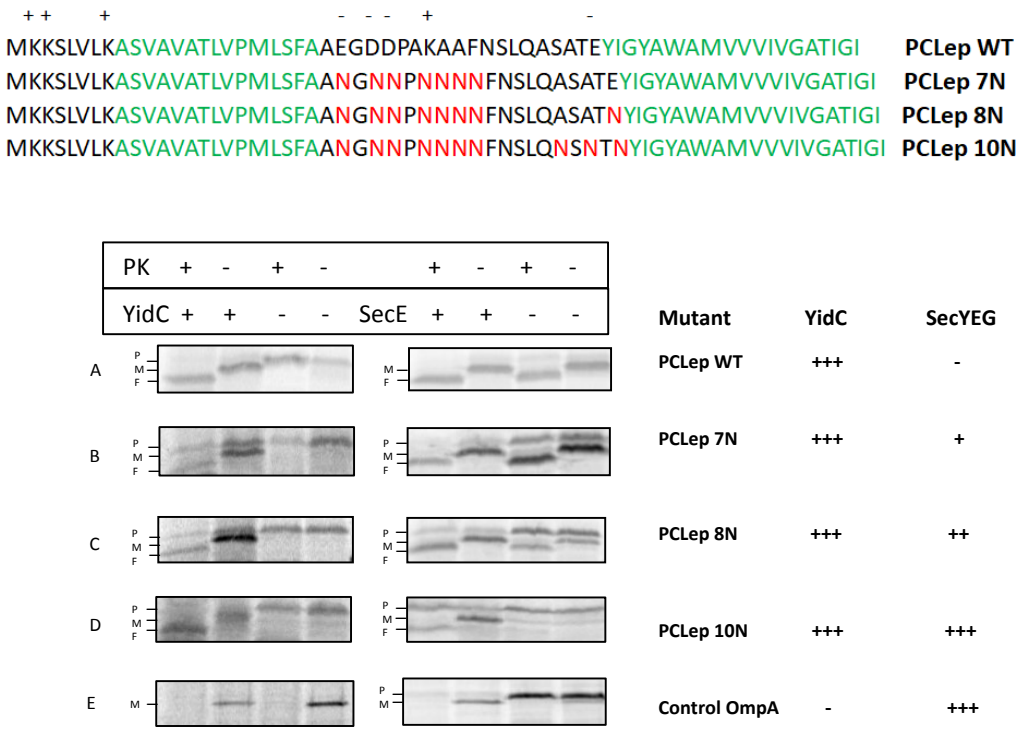
Table 1: GES values of the translocation loops for all the mutants tested in the study along with their dependency on YidC and SecYEG for membrane insertion. The membrane transfer expense for translocation of the periplasmic region of the PCLep constructs calculated using the GES scale. [50] The standard free energy contribution of the membrane potential is not considered here for the transfer of charged residues. '+++' indicates a strict translocase requirement for insertion; '++' indicates a partial translocase requirement; '+' indicates a weak translocase requirement; '-'indicates no translocase requirement. NI indicates not inserted.

Protein	Length Translocated Domain (residues)	Net Charge	GES (kJ/mol)
Cox1	15	0	83.9
	24	-1	84.7
	13	-2	77.9
	3	-1	37.6
	12	-2	104
	18	0	228
Cox2*	15	-2	115
Cox3	7	0	35
	23	-3	123.7
	13	-1	59.9
	4	0	-11.1
Atp6	30	-2	190.7
	13	0	-5.7
	4	0	7.9
Atp8	12	0	29.1
Atp9	13	+1	31.9
	4	0	+22.3
Cob	23	-1	234.4
	36	-1	41.7
	47	-2	246.7
	7	-1	28.2

Table S1. Characteristic of the intramembrane domains of Mitochondrial encoded proteins in S. cerevisiae..* The large C-terminal domain of Cox2 was not included since it requires Oxa2 for translocation.

Protein	Length of Periplasmic Domain (residues)	Net Charge	GES (kJ/mol)
M13 procoat	20	-3	238
Pf3 coat	18	-2	111.4
MscL	28	-1	204.8
TssL	11	0	122.5
Subunit c (F1Fo ATP Synthase	10	-2	102
	6	0	-34.9
CyoA	25 (YidC only domain)	0	200.1

Table S2. Characteristic of the periplasmic domains of YidC only domains of E. coli proteins. * The large C-terminal domain of CyoA was not included since it requires SecYEG for insertion.


+ + + - - - + -
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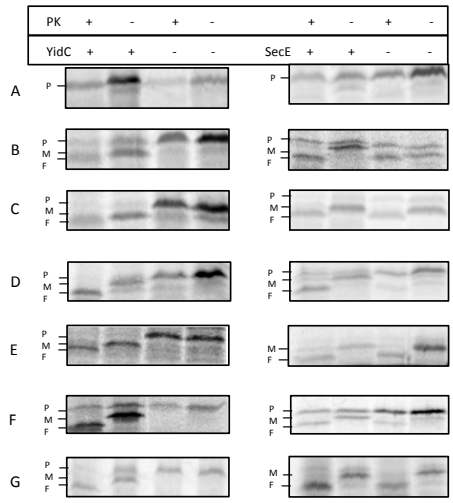
MKKSLVLKASVAVATLVPMLSFAANGNNPNNNNFNSLQASATEYIGYAWAMVVVIVGATIGI PCLep 7N
MKKSLVLKASVAVATLVPMLSFAANGNNPNNNNFNSLQASATNYIGYAWAMVVVIVGATIGI PCLep 8N
MKKSLVLKASVAVATLVPMLSFAANGNNPNNNNFNSLQNSNTNYIGYAWAMVVVIVGATIGI PCLep 10N
PK + - + - + - + -
YidC + + - - SecE + + - -
A
B
C
D
E
P
M
F
Mutant
YidC
SecYEG
PCLep WT
+++
-
PCLep 7N
+++
+
PCLep 8N
+++
++
PCLep 10N
+++
+++
Control OmpA
-
+++

Fig. 1

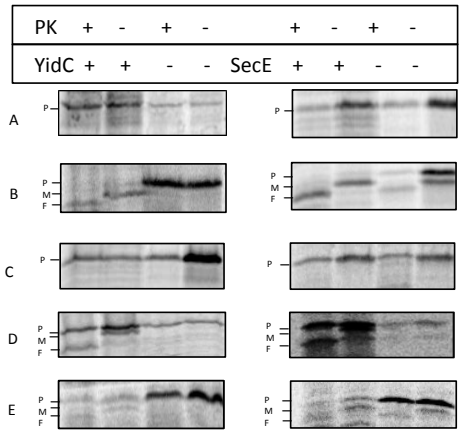
MKKSLVLKASVAVATLVPMLSFAANGNNPNNNNFNSLQNSNNNYIGYAWAMVVVIVGATIGI **PCLep 11N**
MKKSLVLKASVAVATLVPMLSFAANFNNFNNNNFNSLQNSNNNYIGYAWAMVVVIVGATIGI **PCLep 11N+2F**
MKKSLVLKASVAVATLVPMLSFAANGNNPNNNNFNFFQNFNNNYIGYAWAMVVVIVGATIGI **PCLep 11N+3F**
MKKSLVLKASVAVATLVPMLSFAAEEEGDDPAKAAFNSLQASATEYIGYAWAMVVVIVGATIGI **-5 PCLep**
MKKSLVLKASVAVATLVPMLSFAAEEEGDDPAKAAFNFFQAFATEYIGYAWAMVVVIVGATIGI **-5 PCLep+3F**
MKKSLVLKASVAVATLVPMLSFAARGRRPAKAAFNSLQASATEYIGYAWAMVVVIVGATIGI **3R PCLep**
MKKSLVLKASVAVATLVPMLSFAARGRRPAKAAFNFFQAFATEYIGYAWAMVVVIVGATIGI **3R PCLep+3F**



Mutant	YidC	SecYEG
PCLep 11N	N/I	N/I
PCLep 11N + 2F	+++	++
PCLep 11N + 3F	+++	-
-5 PCLep	+++	+++
-5 PCLep+3F	+++	-
3R PCLep	+++	+++
3R PCLep +3F	+++	-

Fig. 2

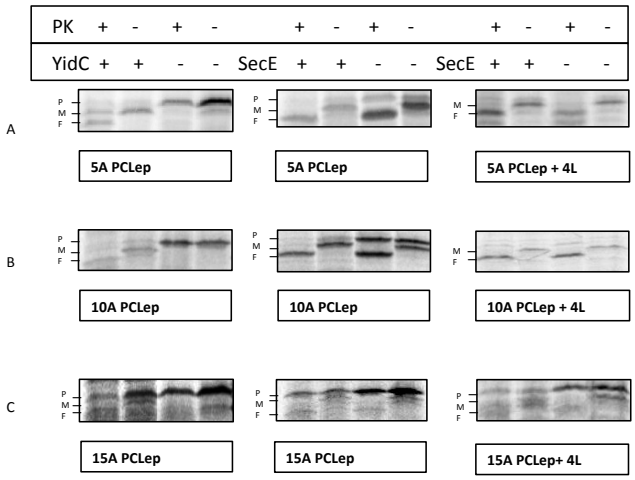
MKKSLVLKASVAVATLVPMLSFAARGRRPARAAFNSLQASATEYIGYAWAMVVVIVGATIGI **4R PCLep**
MKKSLVLKASVAVATLVPMLSFAARGRRPARAAFNSLQASATEYIGLLLLMVVVIVGATIGI **4R PCLep+4L(TM2)**
MKKSLVLKASVAVATLVPMLSFAAEEEEEGDDPAKAAFNSLQASATEYIGYAWAMVVVIVGATIGI **-7 PCLep**
MKKSLVLKASVAVATLVPMLSFAAEEEEEGDDPAKAAFNSLQASATEYIGLLLLMVVVIVGATIGI **-7 PCLep+4L(TM2)**
MKKSLVLKASVAVATLVPMLSFAANGNNPNNNNFNSLQNSNNNYIGLLLLMVVVIVGATIGI **PCLep 11N+4L(TM2)**



Mutant	YidC	SecYEG
4R PCLep	N/I	N/I
4R PCLep +4L (TM2)	+++	+
-7 PCLep	N/I	N/I
-7 PCLep +4L (TM2)	+++	+++
11N PCLep +4L (TM2)	+++	++

Fig. 3

MKKSLVLKASVAVATLVPMLSFAAEGDDPAKAAF(5XA)NSLQASATEYIGYAWAMVVVIVGATIGI **5A PCLep**
MKKSLVLKASVAVATLVPMLSFAAEGDDPAKAAF(5XA)NSLQASATEYIGLLLLMVVVIVGATIGI **5A PCLep+4L**
MKKSLVLKASVAVATLVPMLSFAAEGDDPAKAAF(10XA)NSLQASATEYIGYAWAMVVVIVGATIGI **10A PCLep**
MKKSLVLKASVAVATLVPMLSFAAEGDDPAKAAF(10XA)NSLQASATEYIGLLLLMVVVIVGATIGI **10A PCLep+4L**
MKKSLVLKASVAVATLVPMLSFAAEGDDPAKAAF(15XA)NSLQASATEYIGYAWAMVVVIVGATIGI **15A PCLep**
MKKSLVLKASVAVATLVPMLSFAAEGDDPAKAAF(15XA)NSLQASATEYIGLLLLMVVVIVGATIGI **15A PCLep+ 4L**



Mutant	YidC	SecYEG
5A PCLep	+++	+
5A PCLep + 4L	+++ (NS)	-
10A PCLep	+++	++
10A PCLep + 4L	+++ (NS)	-
15A PCLep	+++	+++
15A PCLep + 4L	+++ (NS)	-

Fig. 4

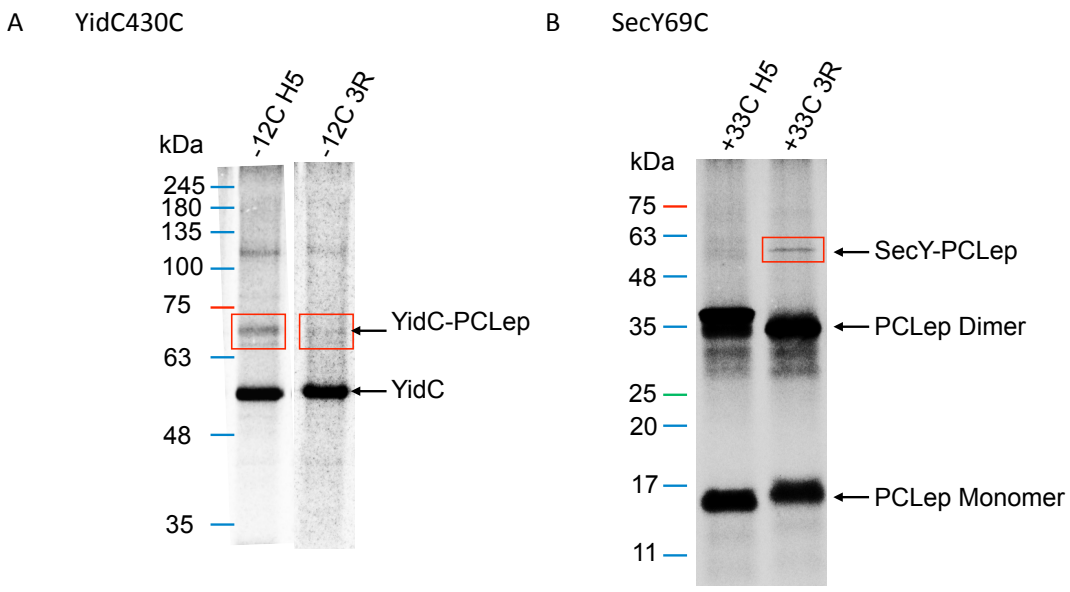

A
YidC430C
-12C H5
-12C 3R
kDa
245
180
135
100
75
63
48
35
YidC-PCLep
YidC
B
SecY69C
+33C H5
+33C 3R
kDa
75
63
48
35
25
20
17
11
SecY-PCLep
PCLep Dimer
PCLep Monomer

Fig. 5

Supplementary Figure

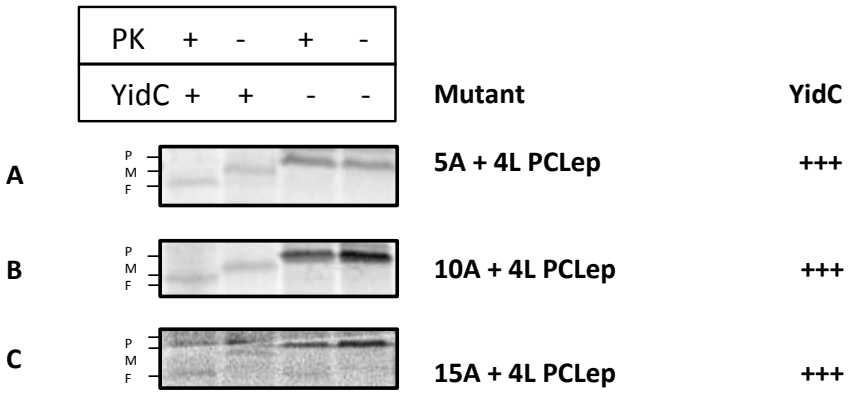


Fig. S1

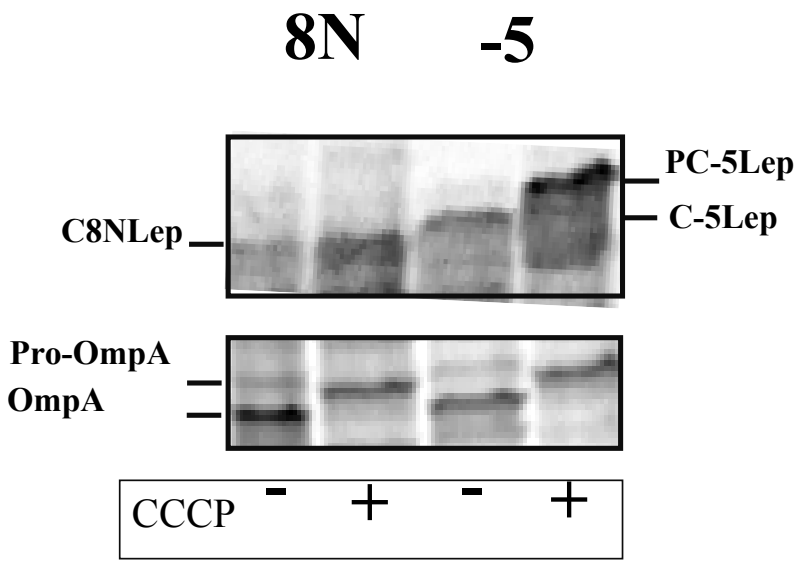
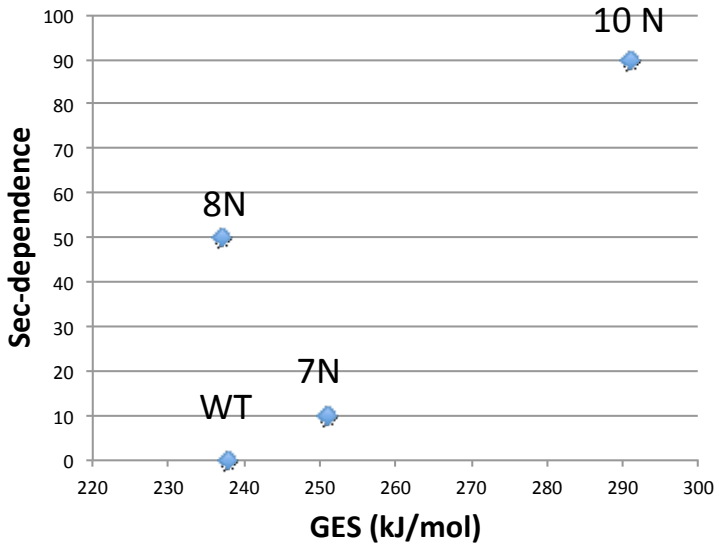


Fig. S2



PClep N mutants

Fig. S3

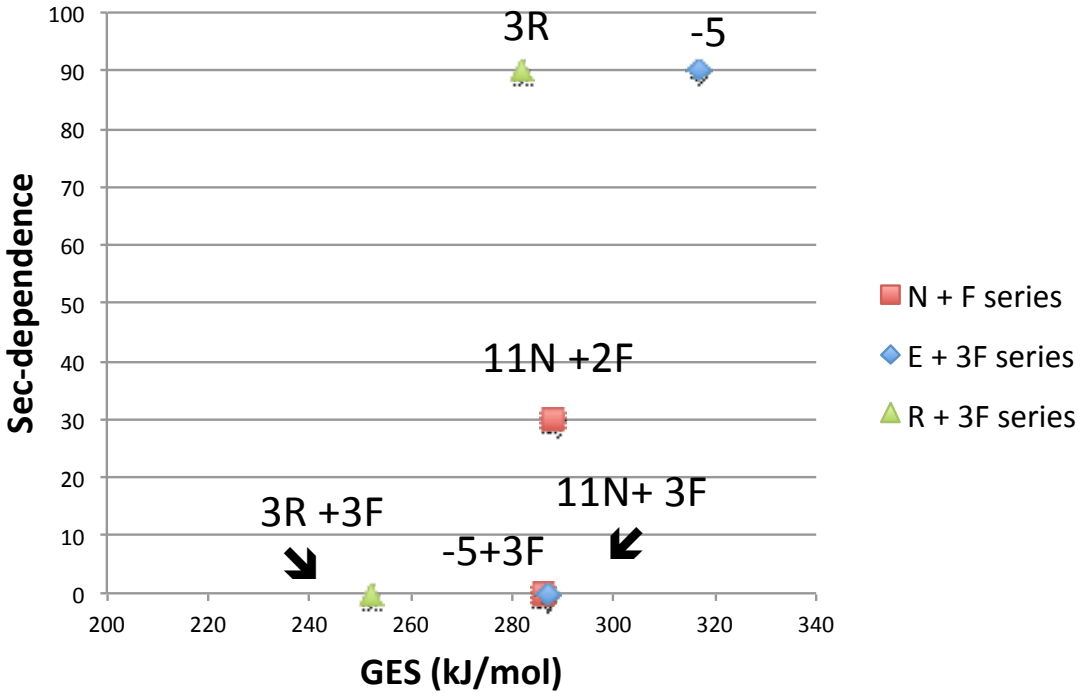

100
90
80
70
60
50
40
30
20
10
0
200
220
240
260
280
300
320
340
Sec-dependence
GES (kJ/mol)
3R
-5
11N +2F
3R +3F
11N+ 3F
-5+3F
N + F series
E + 3F series
R + 3F series

Fig. S4