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Quantitative Analysis of SecYEG-Mediated Insertion of Transmembrane α-Helices into the Bacterial Inner Membrane

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Abstract

Most integral membrane proteins, both in prokaryotic and eukaryotic cells, are co-translationally inserted into the membrane via Sec-type translocons: the SecYEG complex in prokaryotes and the Sec61 complex in eukaryotes. The contributions of individual amino acids to the overall free energy of membrane insertion of single transmembrane α-helices have been measured for Sec61-mediated insertion into the endoplasmic reticulum (ER) membrane (*Nature* 450:1026–1030) but have not been systematically determined for SecYEG-mediated insertion into the bacterial inner membrane. We now report such measurements, carried out in *Escherichia coli*. Overall, there is a good correlation between the results found for the mammalian ER and the *E. coli* inner membrane, but the hydrophobicity threshold for SecYEG-mediated insertion is distinctly lower than that for Sec61-mediated insertion.

Introduction

Most inner membrane (IM) proteins in the model bacterium *Escherichia coli* require the SecYEG translocon for correct insertion into the membrane.[1] The ribosome–nascent chain complex is first recognized by the signal recognition particle, targeted to the signal recognition particle receptor FtsY, and finally transferred to SecYEG. During further chain elongation, periplasmic parts of the polypeptide are translocated across the IM with the help of the peripherally associated SecA ATPase, while transmembrane α-helices (TMHs) are released laterally into the lipid bilayer.[2]

In previous studies, we have sought to identify the pertinent sequence characteristics that drive the integration of TMHs into the membrane of the endoplasmic reticulum (ER) in mammalian and yeast cells[3–5] and into the mitochondrial IM.[6] These studies have led to a detailed quantitative understanding of how much an individual amino acid X in position *i* in the TMH affects the overall membrane-insertion efficiency, expressed as its individual contribution ($\Delta G_{app}^{X,i}$) to the overall apparent free energy of membrane insertion of a polypeptide segment (ΔG_{app}). To date, only one comparable study has been performed for bacterial IM proteins, using a YidC-dependent, SecYEG-independent model protein.[7] Thus, no quantitative data exists for SecYEG-dependent insertion of TMHs into the IM. Given that the ER membrane and the bacterial IM differ in terms of lipid composition,[8,9] lipid asymmetry between the two leaflets of the bilayer,[10] and the absence/presence of a membrane electrochemical potential,[11] it cannot be taken for granted that the quantitative aspects of TM helix integration are the same in the two systems.

To be able to quantitatively compare ΔG_{app} values between the mammalian ER and the bacterial IM, we have undertaken a detailed analysis of how individual amino acids affect ΔG_{app} for SecYEG-dependent membrane proteins in *E. coli*. Using a new membrane-insertion assay developed specifically for *E. coli* IM proteins, we report ΔG_{app} values for a library of designed and natural hydrophobic polypeptide segments (H segments) inserted into a SecYEG-

dependent host membrane protein. The results show good agreement with the results obtained in the mammalian ER. However, we find that the hydrophobicity threshold for membrane insertion is distinctly lower in *E. coli*, with possible implications for topology prediction schemes and for heterologous expression of mammalian proteins in bacterial hosts.

Results

Model protein and experimental setup

Our membrane-insertion assay is based on a new, engineered version of the *E. coli* IM protein Leader peptidase (Lep)—the same protein used in the ER studies—that allows us to measure the insertion efficiency into the IM of the same H segments that we previously analyzed in the mammalian ER[3,4] and in a YidC-dependent *E. coli* IM protein.[7] Lep has two N-terminal TMHs (TMH1, TMH2) and a large periplasmic domain (the P2 domain). It is co-translationally inserted into the IM, adopting a topology with both the N- and the C-terminus in the periplasm.[12,13] The short N-terminal tail is translocated in a YidC-dependent but SecA/SecY-independent way,[14,15] while the insertion of TMH2 and the translocation of the P2 domain are strictly dependent on SecA and SecY.[15–17] In the new Lep version, which we call Lep^{LacY}, we have introduced two hydrophobic segments in the P2 domain: an H

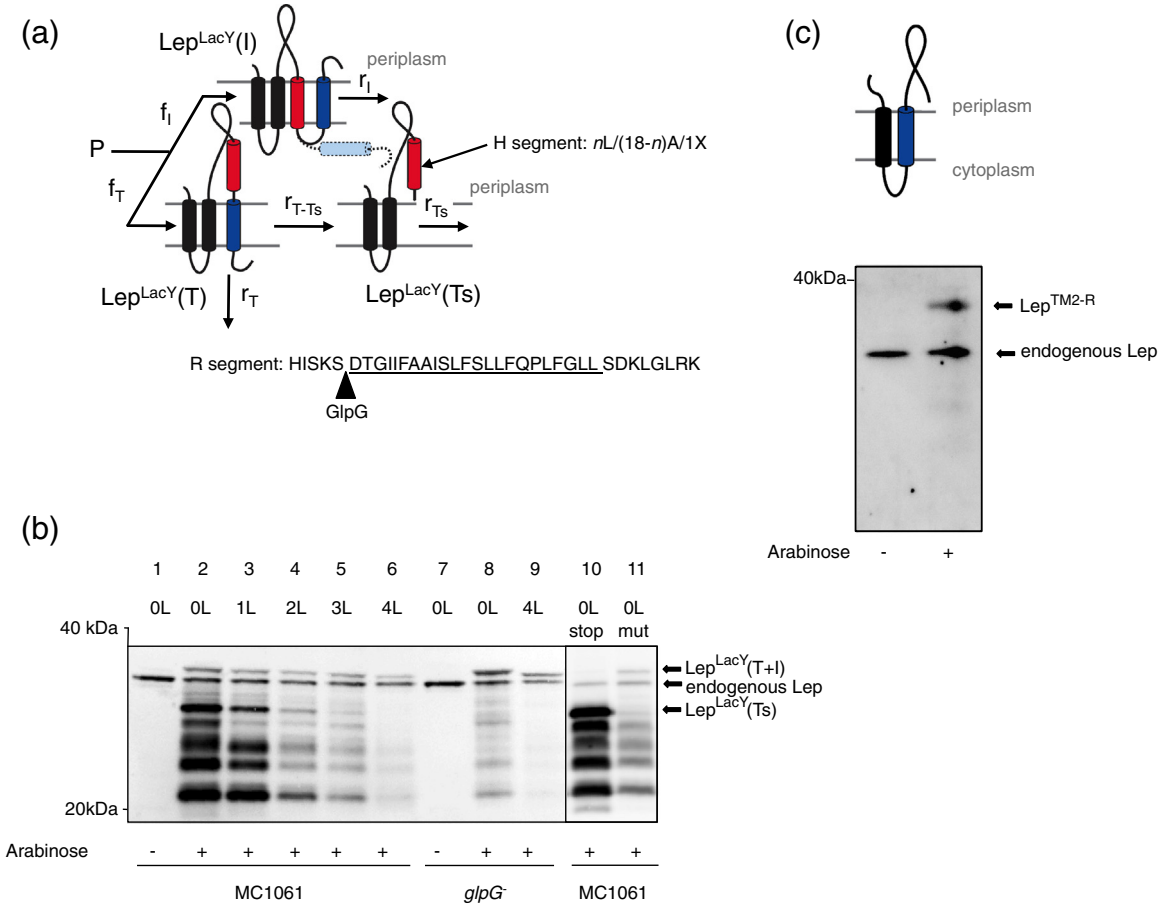

Fig. 1. TM helix insertion assay. (a) Schematic representation of the *in vivo* helix insertion assay, including Lep^{LacY} I, T, and Ts processing and degradation. The Lep^{LacY} model protein contains two natural TM helices (TMH1, TMH2, black), an engineered H segment (red), and a "reporter" R segment corresponding to TMH2 in LacY (blue; the transmembrane part is underlined in the amino acid sequence). The GlpG cleavage site in the R segment is indicated. The loop between TMH2 and the H segment is ~150 residues long, significantly longer than the ~60 residues required for SecA-dependent translocation.[17] (b) Representative Western blots: Lep^{LacY} constructs with *n*Leu/(19 – *n*) Ala H segments (*n* = 0–4; lanes 1–6), with *n* = 0 and a stop codon in the GlpG cleavage site (lane 10) and with *n* = 0 and a mutated GlpG cleavage site in the R segment (lane 11), were expressed in the *E. coli* strain MC1061. Lep^{LacY} constructs in lanes 7–9 were expressed in the Keio strain JW5687-1 (*ΔglpG757*::*kan*). Note that the mobility of full-length Lep^{LacY}(T + I) increases slightly with the hydrophobicity of the H segment. (c) Expression of Lep^{TMH2-R}, in which the second TMH in Lep has been replaced by the R segment, in strain MC1061.

segment (approximately 150 residues downstream of TMH2) and a "reporter" TMH (R segment; approximately 40 residues downstream of the H segment and 45 residues upstream of the C-terminus) (Fig. 1a). The R segment is identical with the second TMH in the *E. coli* IM protein lactose permease (LacY) and has previously been shown to be cleaved by the intramembrane rhomboid protease GlpG if taken out of its natural context and inserted into the IM with an N_{out}–C_{in} orientation.[18] The idea behind the assay is that an efficiently inserting H segment will cause the R segment to be inserted with an inverted N_{in}–C_{out} orientation in the IM (or possibly confine it to the cytoplasm) and hence prevent its cleavage by GlpG, yielding the "inserted" full-length form Lep^{LacY}(I); in contrast, if the H segment is translocated across the IM, the R segment will integrate in the N_{out}–C_{in} orientation [yielding the "translocated" form Lep^{LacY}(T)] and subsequently be cleaved by GlpG [yielding the "small translocated" form Lep^{LacY}(Ts)]. By comparing the relative fractions of Lep^{LacY}(I) and Lep^{LacY}(Ts) molecules, we can determine the degree of membrane insertion of the H segment.

All constructs were expressed in the *E. coli* strain MC1061 for 75 min at 37 °C, after which cells were directly lysed and analyzed by NuPAGE and Western blotting with a Lep antiserum. Cells expressing different Lep^{LacY} forms grow at comparable rates up to at least 90 min (Fig. S1a), and 75 min is an adequate time to ensure that Lep^{LacY} expression, processing, and degradation reach steady state, as evidenced by the constant fraction over time of processed Lep^{LacY}(Ts) for a construct with a 0L/19A H segment (Fig. S1b). Results for a set of H segments of increasing hydrophobicity are shown in Fig. 1b. The R segment is efficiently cleaved (91% cleaved form) in a Lep^{LacY} construct with a weakly hydrophobic H segment of composition 0L/19A (lane 2); despite the 9% uncleaved protein, we assume that this H segment is completely translocated across the membrane because a construct with a strongly polar lysine residue placed in the middle of the poly-Ala H segment (composition 1K/18A; see Supplementary Table S1) gives almost the same amount of uncleaved protein (13%). As the hydrophobicity of the H segment is increased by changing one or more Ala residues to Leu, the degree of cleavage is progressively reduced (lanes 3–5), and there is essentially no Lep^{LacY}(Ts) seen for a 4L/15A H segment (lane 6). As expected, no cleavage is seen for the construct with a 0L/19A H segment in a *glpG*$^-$ strain (lanes 8 and 9) or for a 0L/19A construct with a mutated GlpG cleavage site (SD → LH)[19] in the R segment (lane 11). As a further control, we replaced Lep TMH2 with the R segment such that the latter should insert with an N_{in}–C_{out} orientation; in this case, no cleavage of the R segment is seen (Fig. 1c), showing that GlpG cleaves the R segment only when it spans the IM in an N_{out}–C_{in} orientation.

The different Lep^{LacY} forms are rather unstable, and a range of smaller degradation products accumulate for Lep^{LacY}(Ts) (Fig. 1b, lanes 2 and 10) but not for Lep^{LacY}(I) (lane 6). These fragments are not seen in a *glpG*$^-$ background (lane 8) and are largely absent in a *degP*$^-$ background (Fig. S2), suggesting that the cleavage product generated by GlpG is further degraded by the periplasmic protease DegP. To be able to accurately calculate the fractions of Lep^{LacY} with inserted and non-inserted H segment, we measured the degradation rates of the I, T, and Ts forms by pulse-chase experiments with radiolabeled Lep^{LacY} constructs: to generate pure Lep^{LacY}(I), we used a very hydrophobic H segment of the composition 7L/12A; to generate pure Lep^{LacY}(T), we used the mutant version of the R segment that cannot be cleaved by GlpG together with the weakly hydrophobic 0L/19A H segment; and to generate pure Lep^{LacY}(Ts), we introduced a stop codon at the GlpG cleavage site in the R segment and used the weakly hydrophobic 0L/19A H segment. As seen in Fig. S3, the degradation of the I, T, and Ts forms can be well approximated as first-order reactions $A(t) = A(0)\, e^{-rt}$, where $A(t)$ is proportional to the amount of Lep^{LacY} I, T, or Ts relative to the outer membrane protein OmpA (which is stable over the course of the chase) at time t, and r is the degradation rate. We found that the three degradation rates are different ($r_I = 0.013\ s^{-1}$, $r_T = 0.019\ s^{-1}$, $r_{Ts} = 0.007\ s^{-1}$; see Fig. 1a) (Fig. S3) and could further determine $r_{T\text{-}Ts} = 0.059\ s^{-1}$ from the Western blot data for Lep^{LacY} with a 0L/19A H segment (see Materials and Methods). Notably, Lep^{LacY}(Ts) is significantly more stable than the other forms, which may at least in part explain why there is little or no increase in the amount of Lep^{LacY}(T + I) corresponding to the decrease in Lep^{LacY}(Ts) as the hydrophobicity of the H segment is increased (Fig. 1b). From these degradation rates, and assuming steady-state conditions, we corrected the Western blot quantitation as described in Materials and Methods to obtain the fraction of molecules with membrane-inserted H segment, f_I, and with translocated H segment, $f_T = 1 - f_I$. Finally, we calculated the apparent free energy of membrane insertion of the H segment $\Delta G_{app} = -RT \ln(f_I/f_T)$, where R is the gas constant and T is the absolute temperature ($T = 310$ K).

Hydrophobicity threshold and derivation of a biological hydrophobicity scale for SecYEG-mediated insertion of H segments into the IM

Using the Lep^{LacY}-based assay, we measured ΔG_{app} for a set of 19-amino-acid long H segments, separated from the rest of the protein by GGPG… GPGG flanking tetrapeptide sequences intended to break any secondary structure and ensure that all H

segments are retained in a fixed position relative to the IM.[3] ΔG_{app} values have previously been obtained for the same or very similar H segments in the mammalian and yeast ER membrane,[3–5,20] for a YidC-dependent protein in the *E. coli* IM,[7] and in the mitochondrial IM.[6] All H segments analyzed here are listed in Supplementary Table S1.

We first measured ΔG_{app} for a series of H segments of composition *n*L/(19 – *n*)A (Figs. 1b and 2). We found that ΔG_{app} depends linearly on *n* (Fig. 2b), and $\Delta G_{app} = 0$ kcal/mol (i.e., the H segment is inserted into the IM in 50% of the molecules) is obtained for $n_{50\%} = 2.0$. This is significantly lower than what has been measured for Sec61-mediated TM helix insertion into the mammalian ER membrane ($n_{50\%} = 3.5$[3]) and a little higher than the value found for YidC-mediated TMH insertion into the *E. coli* IM ($n_{50\%} = 1.5$[7]).

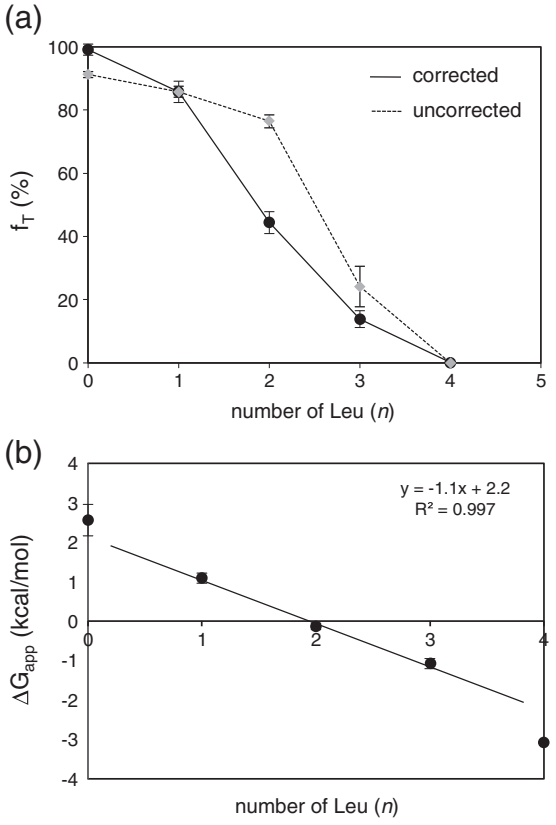

Fig. 2. Determination of the threshold for membrane insertion. (a) Fraction of membrane-translocated Lep^{LacY} (f_T) plotted as a function of the number of Leu residues (*n*) in the H segment. f_T was calculated from Western blot quantitations of Lep^{LacY}(I + T) and Lep^{LacY}(Ts) and corrected as described in Materials and Methods. Uncorrected data, that is, the measured fraction of Lep^{LacY}(Ts), are shown in light gray. (b) Hydrophobicity threshold for insertion of H segments into the IM of *E. coli*. $\Delta G_{app} = -RT \ln(f_I/f_T)$, plotted as a function of *n*. Standard errors are indicated. The linear fit is calculated from the *n* = 1–3 data points, since reliable ΔG_{app} values can only be obtained when $0.15 \leq f_T \leq 0.85$.

Since ΔG_{app} depends linearly on *n*, and since there is little positional dependence in the $\Delta G^{X,i}_{app}$ values for non-polar amino acids,[4] we can calculate the contribution of a single alanine and a single leucine to the overall ΔG_{app} of the H segment using the data in Fig. 2b: $\Delta G_{app} = -1.1n + 2.2 = n\ \Delta G^{L}_{app} + (19 - n)\ \Delta G^{A}_{app} = n\ (\Delta G^{L}_{app} - \Delta G^{A}_{app}) + 19\ \Delta G^{A}_{app}$, which gives $\Delta G^{A}_{app} = 0.11$ kcal/mol and $\Delta G^{L}_{app} = -0.99$ kcal/mol.

To establish a full "biological" hydrophobicity scale[3] for the bacterial IM, we further measured the contribution to ΔG_{app} of each of the 20 naturally occurring amino acids (X) when placed in single copy in the middle of an H segment of composition *n*L/(18 – *n*)A/1X. For each of the amino acids, we adjusted *n* such that the measurement was carried out near the point of maximum sensitivity of the assay, that is, at $\Delta G_{app} \approx 0$ kcal/mol. Assuming that the contribution of the GGPG…GPGG flanking regions can be ignored,[3] we calculated the contribution of amino acid X (ΔG^{X}_{app}) to the overall ΔG_{app} from the relation $\Delta G^{X}_{app} = \Delta G_{app} - n\ \Delta G^{L}_{app} - (18 - n)\ \Delta G^{A}_{app}$ (with $\Delta G^{L}_{app} = -0.99$ kcal/mol and $\Delta G^{A}_{app} = 0.11$ kcal/mol).

The results are shown in Fig. 3a. There is a good overall correlation with the ΔG^{X}_{app} values measured in the mammalian ER membrane[3] (slope = 1.0; $R^2 = 0.9$) (Fig. 3b), with non-polar residues having negative ΔG^{X}_{app} values and polar and charged residues having positive values. It is worth pointing out that, as in the ER, Pro strongly reduces membrane insertion ($\Delta G^{P}_{app} = 2.7$ kcal/mol), presumably reflecting the importance of an α-helical structure of the H segment when inserted into the membrane.

The ΔG^{X}_{app} values measured for SecYEG- and YidC-mediated TM segment insertion into the bacterial IM also correlate rather well (slope = 1.7; $R^2 = 0.8$) (Fig. 3c). Of note, however, is that the ΔG^{X}_{app} values are more similar for the non-polar and weakly polar residues (dotted line; slope = 0.8; $R^2 = 0.8$) than for the strongly polar and charged residues (Q, N, H, E, D, R, and K), which have considerably larger ΔG^{X}_{app} values on the YidC scale compared to the SecYEG scale. The simplest interpretation is that there is an energetic difference between insertion of polar/charged residues through the YidC and Sec-type translocases, but this needs to be confirmed by further studies.

Positional dependence of ΔG^{X}_{app}

The contribution of polar and charged amino acids to ΔG_{app} as measured in the ER system depends strongly on the position of the residue in the H segment.[4] To obtain comparable data for the *E. coli* IM, we measured ΔG_{app} for H segments with the composition *n*L/(18 – *n*)A/1X (X = D, E, H, K, N, P,

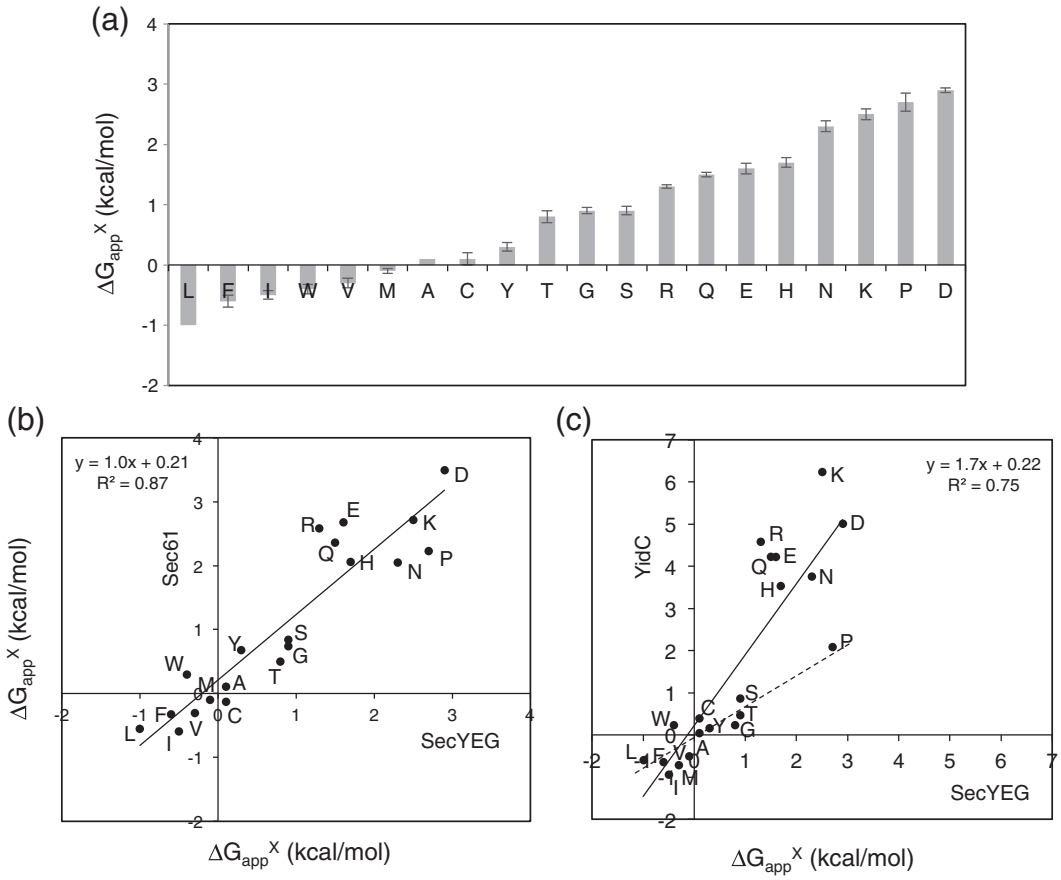

Fig. 3. Biological hydrophobicity scale for SecYEG-mediated TM helix insertion. (a) Lep^{LacY} constructs with H segments of the general composition *n*Leu/(18 − *n*)Ala/1X (X being any of the natural amino acids, with *n* chosen such that −0.5 kcal/mol ≤ ΔG_{app} ≤ 0.5 kcal/mol) were expressed in *E. coli* MC1061. f_T values were calculated from Western blot quantitations with subsequent correction as described in Materials and Methods. ΔG^X_{app} values (averages of ≥5 independent experiments; standard errors are indicated) were calculated as described in the main text. (b) Correlation between ΔG^X_{app} values measured for SecYEG-mediated TMH integration into the IM and for Sec61-mediated TMH integration into the mammalian ER.[3] (c) Correlation between ΔG^X_{app} values measured for SecYEG- and YidC-mediated[7] TM helix integration into the IM of *E. coli*. The value for lysine on the YidC scale given in Fig. 3 of Ref. 7 has been corrected to ΔG^X_{app} = 6.2 kcal/mol (cf., Fig. 2 in the same paper). The dotted line shows the linear correlation including only the non-polar and weakly polar residues (L, I, F, V, M, A, C, W, Y, T, G, S, and P).

Q, R, W, Y), with the X residue placed in positions 1, 3, 6, 10, 14, 17, and 19. ΔG^X_{app} depends strongly on position for polar and charged amino acids, and the aromatic amino acids tryptophan and tyrosine are most favorable for membrane insertion when located near the ends of the H segment (Fig. 4). Overall, these findings agree quite well with the results for the mammalian ER, except for $\Delta G_{app}{}^R$, which appears to have a stronger positional dependence in the ER than in the IM.

ΔG_{app} for marginally hydrophobic TMHs in *E. coli* IM proteins of known three-dimensional structure

Due to the lower hydophobicity threshold for H segment insertion (Fig. 2) and the differences found for both the hydrophobicity scale (Fig. 3) and the single amino acid scans (Fig. 4) when comparing the *E. coli* IM and mammalian ER data, we measured ΔG_{app} for a collection of natural TMHs in the bacterial IM and compared the results with ΔG_{app} values for the same TMHs previously measured in the ER system.

Twelve "marginally hydrophobic" TMHs (i.e., TMHs with a predicted ΔG_{app} ≥ 1.4 kcal/mol according to the "ΔG predictor"[4]) from bacterial membrane proteins of known three-dimensional structure were previously tested for insertion into the ER membrane, and 11 were found to have ΔG_{app} ≥ 0.9 kcal/mol.[21] Interestingly, and in agreement with the lower hydrophobicity threshold for membrane insertion in the IM, all 11 TMHs had an appreciably lower ΔG_{app} (between −0.5 and 0.5 kcal/mol) in the *E. coli* IM (Fig. 5), although only one (CyoC TMH4) inserted

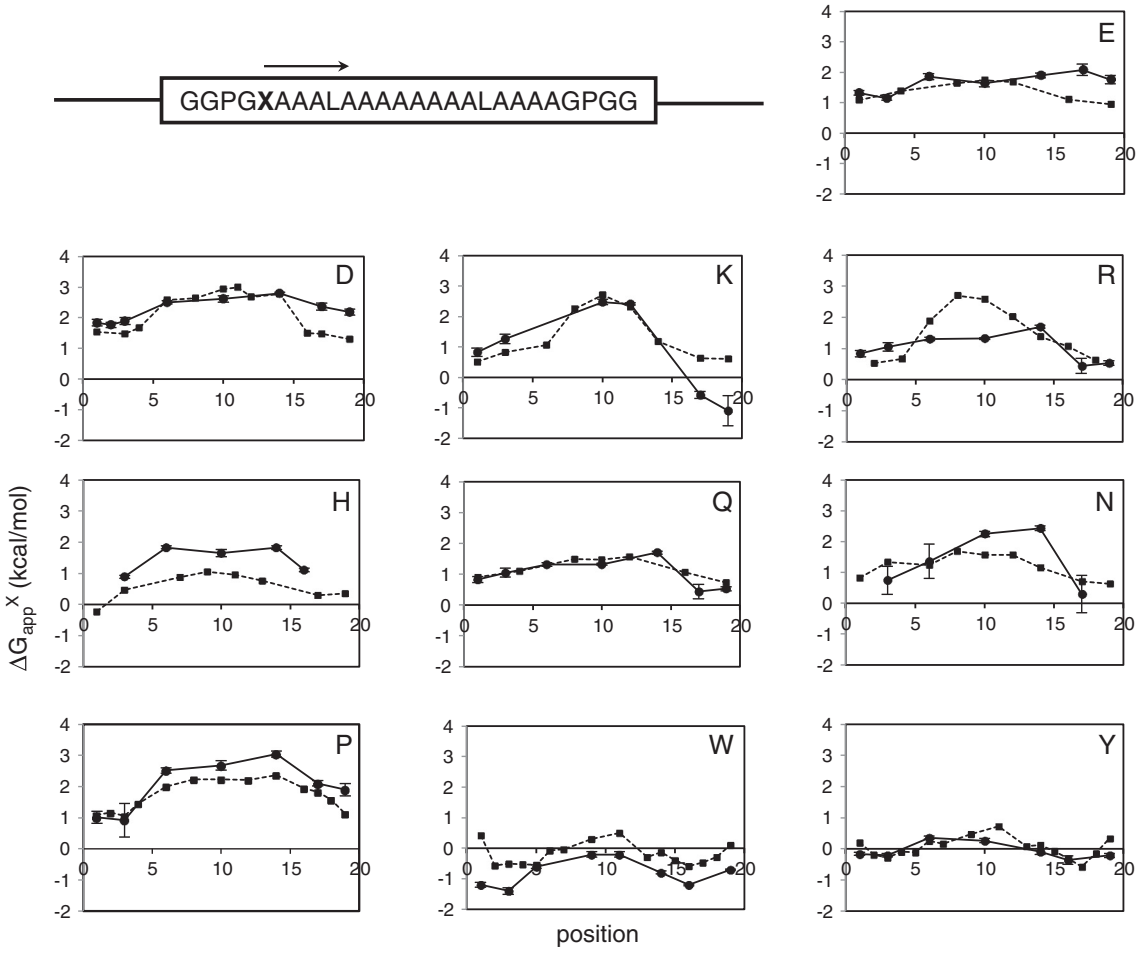

Fig. 4. Positional dependence of ΔG^{X}_{app}. Selected amino acids (X = D, E, H, K, N, P, Q, R, W, and Y) were "scanned" along the H segment. Ala residues in positions 1, 3, 6, 9, 14, 17, and 19 were replaced by an X residue in an H segment with composition *n*Leu/(19 − *n*)Ala where *n* was chosen such that -0.5 kcal/mol $\leq \Delta G_{app} \leq 0.5$ kcal/mol with the given X residue located in position 10. Lep^{LacY} harboring the H segments were expressed and f_T was quantified from Western blots with subsequent correction as described in Materials and Methods. ΔG^{X}_{app} values were calculated as described in the main text (averages of ≥4 independent samples; standard errors are indicated). Broken lines show comparative data obtained for Sec61-mediated integration of H segments into mammalian rough microsomes.[4]

efficiently; this TMH also had the lowest measured ΔG_{app} value in the ER system.

Discussion

The bacterial SecYEG and the eukaryotic Sec61 translocons are closely related in sequence and structure, and both mediate membrane translocation of soluble proteins and membrane insertion of integral membrane proteins.[2] But how closely related are the sequence characteristics that determine the efficiency of membrane integration of a TMH in the two systems? Quantitative data exist for insertion into the ER membrane of mammalian and yeast cells[3–5] and for YidC-mediated insertion into the *E. coli* IM,[7] but not for SecYEG-mediated insertion. We have now established a new *in vivo* assay, based on cleavage of a "reporter" TMH by the rhomboid protease GlpG, to measure the SecYEG-mediated insertion of polypeptide segments (H segments) into the IM of *E. coli*.

Analyzing a large collection of Leu/Ala-based H segments in this new *E. coli* system, we find a good correlation between the contributions of the 20 natural amino acids to the total apparent free energy of membrane insertion, ΔG_{app}, between the SecYEG and Sec61 systems (Fig. 3b). However, we also find some quantitative differences between the two systems. Most importantly, the hydrophobicity threshold for membrane insertion is significantly lower in the bacterial SecYEG system compared to the Sec61 system (Fig. 2). This is also apparent from the fact that certain "marginally hydrophobic" TMHs

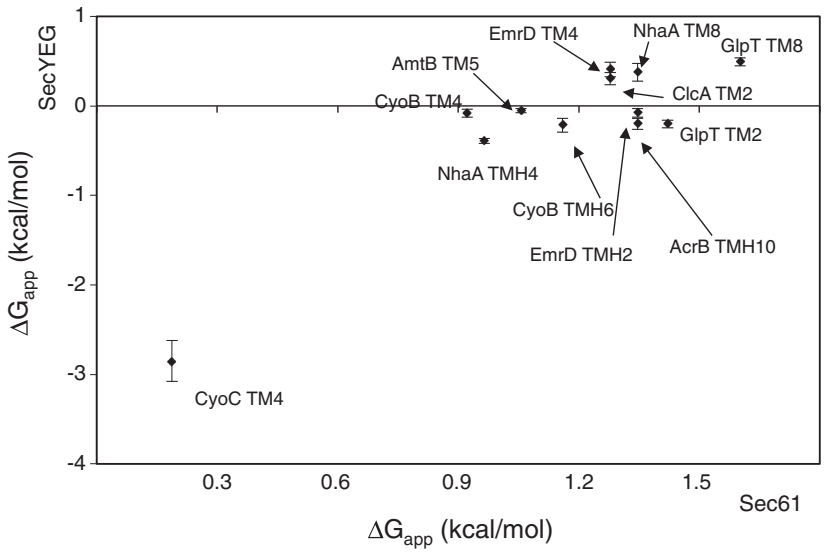

Fig. 5. Membrane insertion of "marginally hydrophobic" TM helices. Twelve TM helices with N_{out}–C_{in} orientation from membrane proteins with solved crystal structures (CyoB, CyoC, AcrB, GlpT, AmtB, NhaA, and EmrD) were cloned as H segments into Lep^{LacY}. Constructs were expressed in *E. coli* MC1061 and analyzed by Western blotting with subsequent correction as described in Materials and Methods (averages of ≥5 independent experiments; standard errors are indicated). The results are compared to previous measurements for Sec61-mediated integration into the ER.[21] The ΔG_{app} value for CyoC TM4 measured in *E. coli* is approximate, as the insertion efficiency is too high for an accurate measurement. Note the different scales on the *x*- and *y*-axes.

found in membrane proteins of known structure insert better in the SecYEG system than in the Sec61 system (Fig. 5). The positional dependence of the contributions of individual polar and charged residues to the overall ΔG_{app} is similar when comparing the SecYEG and Sec61 systems (Fig. 4).

There is also a good correlation between the SecYEG and YidC data, especially for the non-polar and weakly polar residues (Fig. 3c). It appears, however, that strongly polar and charged residues have considerably larger ΔG^{X}_{app} values in the YidC system, possibly pointing to a difference in the way transmembrane segments interact with the two types of translocons. One obvious difference between the SecYEG translocon on the one hand and the Sec61 and YidC translocons on the other is the involvement of SecA in the former. Structural studies suggest that SecA can help induce opening of the lateral gate in SecY,[22] which could conceivably affect the hydrophobicity threshold for membrane insertion.

Nevertheless, the overall similarity between the results obtained with the Sec61, SecYEG, and YidC systems suggests that the underlying physical chemistry is the same, in line with the idea that the primary determinant for membrane insertion can be described in terms of a thermodynamic partitioning of the TMH between the translocon and the surrounding membrane.[23] Given this similarity, it is also not surprising that topology prediction methods perform rather well across the board;[24] however, the small but significant differences between the systems that we have uncovered suggest that some marginal improvement in prediction performance might be gained by training predictors separately on membrane proteins that use one or the other translocon. Finally, it is possible that some of the difficulties experienced when attempting to produce eukaryotic membrane proteins in *E. coli* may reflect these underlying, albeit minor, differences in the workings of the Sec61 and SecYEG translocons.

Materials and Methods

Enzyme and chemicals

All chemicals were from Sigma-Aldrich, except glycerol from Merck and [^{35}S]methionine from PerkinElmer. All restriction enzymes were from Fermentas, the Phusion DNA polymerase for amplification of H segment oligonucleotides was from Finnzyme, and the QuikChange™ Site-Directed Mutagenesis kit and the deoxyribonucleotides were from Stratagene. The anti-rabbit antibody was from GE Healthcare.

Construction of Lep^{LacY}

Lep^{LacY} was generated from a modified version of the *lepB* gene in the pGEM1 vector that contained an SpeI restriction site in Lep codons 226–227 and a KpnI restriction site in codon 253.[3,4] Restriction sites for SacI (amino acid residues 268/269) and ApaI (amino acid residues 279/280) were introduced by site-directed mutagenesis. DNA encoding LacY TMH2 was PCR amplified from the chromosomal *lacY* gene using primers that were complementary to its N- and C-terminal flanks with overhangs containing a SacI restriction site (N-terminal) and an ApaI restriction site (C-terminal) and cloned into Lep^{LacY}. The whole gene was then PCR amplified with primers complementary to the N- and C-termini of Lep^{LacY} with overhangs containing an NcoI restriction site (N-terminal) and a SmaI restriction site (C-terminal) and subsequently cloned into the pING vector,[25] which was previously modified with a new NcoI restriction site.

Double-stranded oligonucleotides encoding H segments either were PCR amplified from previously designed Lep constructs[3,4] with primers complementary to the GGPG/GPGG flanks including a SpeI (N-terminal) or KpnI (C-terminal) restriction site or were generated by annealing two pairs of complementary oligonucleotides (18–48 nucleotides long) with overlapping overhangs followed by annealing of these pairs.[3,26] Double-stranded oligonucleotides encoding TM helices of membrane proteins of known structure (Fig. 5) were PCR amplified with primers complementary to their N- and C-termini with overhangs containing a SpeI restriction site (N-terminal) or a KpnI restriction site (C-terminal) followed by the tetrapeptide sequence of GGPG, GPGG, respectively. All H segment-encoding sequences were ligated into the SpeI- and KpnI-digested pING-Lep^{LacY} vector.

The introduction of a stop codon in the cleavage site of LacY TMH2 and the silencing of the cleavage site in LacY TMH2 were done by site-directed mutagenesis.

Expression and Western blot analysis

Single colonies of freshly transformed *E. coli* MC1061 with a pING-Lep^{LacY} construct (or in a few cases, the *glpG*$^-$ or *degP*$^-$ strains described in the main text) were grown as 3-ml cultures in LB (100 μg ampicillin/ml) overnight at 37 °C and diluted 20–40 times with fresh LB (100 μg ampicillin/ml), and 1-ml cultures were grown for 90 min at 37 °C (to an $OD_{\lambda = 600}$ of 0.35–0.4). Lep^{LacY} expression was then induced by adding 10 μl of a fresh 20% L-arabinose solution. If not otherwise noted, cells were incubated for an additional 75 min at 37 °C, whereafter a 200-μl culture sample was centrifuged at 10,600*g* for 2 min. The pellet was resuspended in 120 μl of sample buffer [140 mM Tris–HCl, pH 8.8, 14% glycerol, 4% SDS, 50 mM DTT, 3.5 mM ethylenediaminetetraacetic acid (EDTA), and 0.02% bromophenol blue], boiled for 10 min at 95 °C, cooled to room temperature, and incubated with 2 μl of DNase1 for 10–15 min at 37 °C. Samples were then directly subjected to NuPAGE (12% Bis/Tris gels) followed by dry protein transfer onto a nitrocellulose membrane using the iBlot system (InVitrogen). Membranes were developed using a standard Western blot protocol [primary antibody: Lep antiserum (rabbit), dilution 7:20,000; secondary antibody: anti-rabbit horseradish peroxidase, dilution 1:16,000]. Membranes were imaged using a Fuji LAS-1000 system and Image reader for LAS-1000 Pro (Fujifilm, Tokyo, Japan) and visualized using the Image Reader V1.8J/Image Gauge V 3.45 software (Fujifilm). A two-dimensional intensity profile of each gel lane was generated using the MultiGauge software, and the multi-Gaussian fit program from the Qtiplot software package‡ was used to calculate the peak areas of the protein bands of interest in the two-dimensional intensity profile.

Pulse-chase assay

E. coli strain MC1061, transformed with a pING-Lep^{LacY} construct, was grown overnight at 37 °C in M9 minimal media supplemented with 0.4% D-fructose, 0.1 mM $CaCl_2$, 100 μg/ml thiamine, 2 mM $MgSO_4$, amino acid mix minus methionine, and 100 μg/ml ampicillin and then back-diluted to an $OD_{\lambda = 600}$ of 0.1 with fresh M9 minimal media and grown as a 4.5-ml culture for 4.5 h at 37 °C. Lep^{LacY} expression was then induced by adding 45 μl of a fresh 20% L-arabinose solution. Proteins were labeled for exactly 1 min with [^{35}S]methionine (10 μCi/ml culture) 5 min after induction and the labeling was then quenched with a vast excess of cold methionine (40 μl of 200 mM methionine per milliliter of culture). Samples of 500 μl of culture were taken at the time points indicated in Fig. S3, and proteins directly precipitated in 10% trichloroacetic acid, first on ice and then at 7 °C overnight. The sample for time point 0 was taken separately at the same time by labeling for exactly 1 min with [^{35}S]methionine (10 μCi/ml culture) followed directly by protein precipitation in 10% trichloroacetic acid on ice.

Precipitated proteins were pelleted for 5 min at 20,000***g*** at 4 °C, washed with 800 μl of acetone, and pelleted again for 5 min at 20,000***g*** at 4 °C, and the pellet was dried for 10 min at 95 °C. After resuspension in Tris–SDS (10 mM Tris–HCl, pH 7.5, and 2% SDS), samples were boiled for 10 min at 95 °C and subsequently centrifuged for 5 min at 20,000***g***. The supernatant was transferred into 650 μl of TSET (50 mM Tris–HCl, pH 8, 150 mM NaCl, 0.1 mM EDTA, and 2% Triton TX-100) containing 5 μl of Pansorbin solution and incubated for 30 min on ice, and Pansorbin was then removed by a 1-min centrifugation at 8600***g***. Supernatant (330 μl) was incubated with OmpA antiserum (0.5 μl) and 330 μl with Lep antiserum (1.5 μl) for 40 min on ice, supplemented with 15 μl of Pansorbin, and rotated overnight at 7 °C. Pansorbin was then washed three times (1: 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, and 0.2% Triton TX-100; 2: 10 mM Tris–HCl, pH 7.5, 500 mM NaCl, 2 mM EDTA, and 0.2% Triton TX-100; 3: 10 mM Tris–HCl, pH 7.5), resuspended in 20 μl of sample buffer, boiled for 5 min at 95 °C, and finally subjected to SDS-PAGE. Gels were dried and visualized in a Fuji FLA-3000 phosphorimager using the Image Reader V1.8J/Image Gauge V 3.45 software and quantified as described under Expression and Western blot analysis.

Calculation of f_T values corrected for degradation rates

Consider the reaction scheme in Fig. 1a. The total production rate of Lep^{LacY} is P, of which a fraction f_I goes into Lep^{LacY}(I) and a fraction f_T goes into Lep^{LacY}(T). At steady state, we have:

$$\frac{d[I]}{dt} = f_I \cdot P - r_I \cdot [I] = 0 \quad (1)$$

$$\frac{d[T]}{dt} = f_T \cdot P - r_T \cdot [T] - r_{T-Ts} \cdot [T] = 0 \quad (2)$$

$$\frac{d[Ts]}{dt} = r_{T-Ts} \cdot [T] - r_{Ts} \cdot [Ts] = 0 \quad (3)$$

Solving for f_I/f_T in terms of the rate constants and [T + I]/[Ts] (which can be measured from the Western blots) gives:

$$\frac{f_I}{f_T} = \frac{r_I \cdot r_{T-Ts}}{r_{Ts} \cdot (r_T + r_{T-Ts})} \cdot \frac{[T+I]}{[Ts]} - \frac{r_I}{(r_T + r_{T-Ts})} \quad (4)$$

$$f_I = 1 - f_T \Rightarrow f_T = \frac{1}{\frac{f_I}{f_T} + 1} \left(\text{with } \frac{f_I}{f_T} \text{ given in Eq. (4)} \right)$$

All rate constants except r_{T-Ts} were measured by pulse-chase analysis (Fig. S3). r_{T-Ts} was obtained from Eq. (3) and the Western blot data for Lep^{LacY} with a 0L/19A H segment, which we assume is fully translocated (i.e., [T + I] = [T]):

$$r_{T-Ts} = r_{Ts} \cdot \frac{[Ts]}{[T]} = r_{Ts} \cdot \frac{[Ts]}{[T+I]}$$

Taking [Ts]/[T + I] from Fig. 1b (lane 2) and with r_{Ts} = 0.007 s^{-1} (Fig. S3c), we obtain r_{T-Ts} = 0.059 s^{-1}.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2013.04.025

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Abbreviations used:
IM, inner membrane; TMH, transmembrane helix; ER, endoplasmic reticulum; EDTA, ethylenediaminetetraacetic acid.

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