Probing the SecYEG translocation pore size with preproteins conjugated with sizable rigid spherical molecules

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Protein translocation in Escherichia coli is mediated by the translocon that in its minimal form consists of the protein-conducting channel SecYEG, and the motor protein, SecA. SecYEG forms a narrow pore in the membrane that allows passage of unfolded proteins only. Molecular dynamics simulations suggest that the maximal width of the central pore of SecYEG is limited to 16 Å. To access the functional size of the SecYEG pore, the precursor of outer membrane protein A was modified with rigid spherical tetraarylmethane derivatives of different diameters at a unique cysteine residue. SecYEG allowed the unrestricted passage of the precursor of outer membrane protein A conjugates carrying tetraarylmethanes with diameters up to 18 Å, whereas a 29 Å sized molecule blocked the translocation pore. Translocation of the protein-organic molecule hybrids was strictly proton motive force-dependent and occurred at a single pore. With an average diameter of an unfolded polypeptide chain of 4–6 Å, the pore accommodates structures of at least 22–24 Å, which is vastly larger than the predicted maximal width of a single pore by molecular dynamics simulations.

In Escherichia coli, about 30% of the proteins synthesized in the cell accomplish their function outside the cytoplasm. Consequently, these proteins need to be translocated across or inserted into the inner membrane. The main system involved in protein translocation and membrane protein insertion is the Sec translocon with, as central component, a membrane-embedded protein-conducting pore, the SecYEG complex (also termed translocon) (1). Most membrane proteins are targeted to SecYEG as ribosome-bound nascent chains involving the signal recognition particle (SRP) and the SRP receptor (FtsY). The ribosome subsequently docks onto the SecYEG complex, and this oligomerization is promoted by SecA and by the ribosome. A cryo-EM structure of the ribosome-bound Escherichia coli SecYEG complex with an inserting membrane protein suggests that SecYEG binds the ribosome as a dimer with only one of the pores accommodating the translocating polypeptide chain (11). A crosslinking analysis of a SecA-associated preprotein translocation intermediate indicates an association with only one of the two SecYEG monomers (12). Thus far, it is unknown if the dimeric represents a functional or structural unit. In this respect, a recent cryo-EM analysis of the homologous mammalian and yeast Sec61p complex indicates the presence of a single pore bound to the ribosome (13).

A central unresolved question concerns the functional width of the translocation pore. Molecular dynamics has been employed to study the plasticity of the pore formed by a SecYEG monomer (14–16). By pushing virtual soft balls through a single SecY pore, a maximal functional size of the pore of 16 Å has been suggested without the need for lateral gate opening (16). On the other hand, experimental studies with microsomes harboring the eukaryotic...
Sec61 complex indicate a pore diameter of 40–60 Å in the active state (17). The SecYEG complex seems rather promiscuous as it can translocate preproteins that are chemically cross-linked to nonpolypeptide constituents (18, 19). Here, we have employed a preprotein conjugated to large rigid spherical molecules with defined molecular dimensions to probe the diameter of the translocation pore in its active state. The data indicate that the active pore diameter by far exceeds the estimate made by the molecular dynamics simulations of the monomeric pore suggesting a more complex pore geometry.

Results

Synthesis of Tetraarylmethanes. To access the size exclusion limit of the protein-conducting pore, organic compounds were synthesized with a precisely defined and systematically increasing size (Fig. 1 and SI Appendix). The nature of these compounds resembles the methane structure in which the carbon atom carries a sp3 hybridization. In this way, the phenyl and biphenyl groups, which are used as substituent, are oriented toward the x, y, and z axes giving the molecule the desired bulkiness and a spherical shape. Steric hindrance associated with the aromatic rings prevents coplanarity of the system and gives the desired rigidity. In addition, each molecule synthesized carries a maleimide group allowing the formation of a covalent protein-organic compound conjugate via a single cysteine present in the precursor of OmpA. The size of the compounds refers to the distance between the apical hydrogen atoms of the phenyl, biphenyl, and substituted biphenyl groups. The sizes are: approximately 8.5 Å for 1-(4-trityl-phenyl)pyrrole-2,5-dione (TAM1), approximately 15 Å for 1-[4-(tris-biphenyl-yl-methyl)phenyl]-pyrrole-2,5-dione (TAM2), approximately 18 Å for 2-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)-N-[4-(tris-(4'-isopropyl-biphenyl-4-yl)methyl]-phenyl]-acetamide (IsoTAM2) and 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-[4-(tris(4'-methoxybiphenyl-4-yl)methyl)phenyl]-acetamide (MeOTAM2), and approximately 29 Å for 5-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetamido)-N,N'-bis(4-(tris(3'-methoxybiphenyl-4-yl)methyl)phenyl)isophthalalimide (MeOTAM3). Due to the presence of the aromatic component in the molecules the basic structure of the molecules has a high hydrophobicity. This hydrophobicity was decreased by modification of the diphenyl groups with methyl-oxy (MeO) groups as shown in MeOTAM2. The rigid conical shaped molecules conjugated to the preprotein proOmpA have a molecular weight of 415.15 (TAM1), 643.25 (TAM2), 849.70 (IsoTAM2), 790.90 (MeOTAM2), and 1608.1 (MeOTAM3), respectively.

Conjugation of proOmpA with Spherical Tetraarylmethanes. The tetraarylmethanes maleimide derivatives were conjugated to a unique cysteine of the precursor protein proOmpA (S245C). A position in the main chain was chosen rather than at the C-terminal end of proOmpA to assure that the organic molecule passes the pore in combination with the polypeptide chain, which substantially adds to size to be translocated. Because the organic compounds are not readily soluble in water, the compounds were dissolved in an appropriate organic solvent and subsequently added to proOmpA that was denatured in urea. After labeling, the derivatization of proOmpA was precipitated with trichloroacetic acid, washed with acetone and dissolved in urea buffer. To determine the extent of labeling, conjugated proteins were reduced with tris-(2-carboxyethyl) phosphine (TCEP) and labeled with fluorescein-5-maleimide (Fmal). The fluorescence intensity of the conjugated and subsequently Fmal labeled proOmpA was compared with that of proOmpA labeled with Fmal only (Fig. S1). The IsoTAM2, MeOTAM2, and MeOTAM3 derivatives were hardly labeled with Fmal indicating that conjugation of proOmpA (S245C) with these compounds was almost 100%. With TAM1 and TAM2 conjugated proOmpA an approximately 10% labeling with Fmal was observed (Fig. S1). However, control experiments with the cysteineless proOmpA indicate a 5% of nonspecific labeling with Fmal. Taking this into account we concluded that for all tetraarylmethane maleimide derivatives the degree of labeling of proOmpA (S245C) is ≥95%. Except for the largest MeOTAM3 (Fig. 2B), labeling of proOmpA (S245C) with the other tetraarylmethane maleimides did not result in a significant change in the mobility of proOmpA on SDS-PAGE. This is likely due to the small molecular size of the conjugates (400–800 Da) and because the derivatization does not affect the overall charge of proOmpA.

Translocation of Tetraarylmethane proOmpA Conjugates by the SecYEG Complex. The proOmpA conjugates labeled with the different tetraarylmethanes were assayed for translocation using inner membrane vesicles (IMVs) of E. coli strain UH203 containing overexpressed levels of SecYEG. Translocation assays were performed in the presence and absence of the ionophores nigericin and valinomycin to assess the role of the proton motive force (PMF). Under the conditions used unlabeled proOmpA translocated efficiently into the IMVs (Fig. 2A, WT) and translocation was two- to 2.5-fold stimulated by the PMF (Fig. 2A, −PMF vs. +PMF, open and filled dots, respectively). In the presence of a PMF the proOmpA tetraarylmethane conjugates translocated into UH203 IMVs as efficiently as unlabeled proOmpA as shown for proOmpA-IsoTAM2 (Fig. 2A, +PMF filled dots), except for the MeOTAM3-labeled proOmpA that was not translocated (Fig. 2B). In contrast to the unlabeled proOmpA, the translocation of the proOmpA tetraarylmethane conjugates was more dependent on the presence of a PMF (Fig. 2A, −PMF vs. +PMF). The translocation of MeOTAM3-labeled proOmpA was not restored by the PMF (Fig. 2B). Fig. 2C summarizes this data showing the translocation rate of the various proOmpA tetraa-
Translocation Arrest by Tetraarylmethane-Conjugated proOmpA. To determine if the tetraarylmethane conjugate arrests translocation because of blocking the translocation pore, we performed a translocation reaction using saturating concentrations of proOmpA, IsoTAM2-proOmpA, MeOTAM3, or proOmpA-dihydrofolate (DHFR). Addition of methotrexate and NADPH to the latter fusion construct leads to tight folding of the DHFR domain and results in an arrest in translocation of proOmpA-DHFR (23). After translocation of the different proteins, IMVs were recovered by centrifugation through a sucrose cushion and used in a second translocation reaction using Fmal-proOmpA as substrate. When the first translocation reaction was performed in the absence of a preprotein, Fmal-proOmpA was readily translocated into the IMVs in the second translocation reaction (Fig. 4A, lane 1). In contrast, IMVs used to translocate proOmpA-DHFR in the first translocation reaction were unable to translocate Fmal-proOmpA (lane 4). IMVs used in a translocation reaction with proOmpA or IsoTAM2-proOmpA showed similar levels of Fmal-proOmpA translocation as IMVs incubated without a preprotein (lanes 2 and 3). In contrast, when first MeOTAM3-proOmpA was translocated into the IMVs, translocation of Fmal-proOmpA in a second round of translocation was completely blocked (Fig. 4B, lane 2). These results indicate that even in the presence of a PMF, the largest molecule tested; i.e., MeOTAM3 causes a block of the translocation pore.

As translocation of the proOmpA tetraarylmethane derivatives is strongly dependent on the PMF we investigated this requirement in further detail. To this end, IsoTAM2-proOmpA was translocated into IMVs both in the presence and in the absence of a PMF. A collapse of the PMF was induced by the addition of the ionophores valinomycin and nigericin. After the translocation reaction, reactions performed in the presence of a PMF were supplemented with valinomycin/nigericin and IMVs were recovered as described above. The reisolated IMVs were used in a second translocation reaction using Fmal-proOmpA as substrate. Both IMVs used to translocate IsoTAM2-proOmpA in the absence and presence of a PMF showed Fmal-proOmpA translocation (Fig. 4C, lane 3 and 4) with an efficiency comparable to IMVs that had not been incubated with proOmpA (lane 2). This result demonstrates that even in the absence of a PMF when translocation is slow (Fig. 2), the smaller conjugates do not block the SecY pore (Fig. 4C).

Translocation by a SecYEG Pore that Is Constrained by a Cross-Linked Lateral Gate. Tetraarylmethanes are relatively hydrophobic molecules. Therefore, the possibility exists that a large part of these molecules cross the membrane by sliding along the interface of the pore and the lipid bilayer, possibly at the lateral gate. To address this possibility, two strategies were adopted: First, we synthesized a tetraarylmethane in which the hydrophobic isopropl groups were replaced by more hydrophilic methoxy groups. In this way, the outer shell of the molecule is more hydrophilic in nature thereby minimizing unwanted interactions with the lipid phase. Like the other conjugates, MeOTAM2-proOmpA was synthesized a tetraarylmethane in which the hydrophobic isopropl groups were replaced by more hydrophilic methoxy groups. In this way, the outer shell of the molecule is more hydrophilic in nature thereby minimizing unwanted interactions with the lipid phase. Like the other conjugates, MeOTAM2-proOmpA was

Fig. 3. The SecY PrlA4 mutation relieves the strong PMF-dependent translocation of IsoTAM2-proOmpA. Translocation reactions were performed in the presence (black dots) and absence (white dots) of a PMF (A) with wild-type IMVs and (B) IMVs derived from the PrlA4 mutant.

Fig. 2. Translocation of proOmpA tetraarylmethane depends on the PMF. The different proOmpA conjugates were diluted into translocation buffer containing SecA (20 μg/mL), SecB (32 μg/mL), ATP (1 mM), and 10 μg IMVs. At different time intervals the translocation reaction was terminated by protease K treatment on ice. Samples were precipitated with trichloric acid (TCA) and protease protected material was analyzed by SDS-PAGE and immunoblotting using a polyclonal antibody against OmpA that recognizes the C-terminal end of proOmpA. Translocation reactions were performed in the presence and absence of a PMF. To dissipate the PMF nigericin and valinomycin (1 μM final concentration) was added to the reaction mix. (A) Translocation of wild-type proOmpA and the different proOmpA conjugates, in the presence (left) and absence (right) of a PMF. (B) Translocation of MeOTAM3-proOmpA in the presence and absence of a PMF. (C) Plot of the translocation rate versus the molecular size of the tetraarylmethane-conjugated to proOmpAS245C. Closed dots, +PMF; and open dots, −PMF.

ylmethane conjugates in the presence of the PMF plotted against the molecular size of the conjugate.

Previous studies have shown that the PMF-dependent translocation of proOmpA is suppressed in the PrlA4 mutant strain (20). The PrlA4 SecY protein contains two mutations, F286Y and I408N, where the latter is responsible for the suppressor effect (21). To determine if the strong PMF-dependent translocation is slow (Fig. 2), the PrlA4 strain we analyzed the translocation of proOmpA that had not been incubated with proOmpA (lane 2). This result addressed this possibility, two strategies were adopted: First, we synthesized a tetraarylmethane in which the hydrophobic isopropl groups were replaced by more hydrophilic methoxy groups. In this way, the outer shell of the molecule is more hydrophilic in nature thereby minimizing unwanted interactions with the lipid phase. Like the other conjugates, MeOTAM2-proOmpA was
eimidoethane (BMOE) that introduces a spacer of approximately a disulfide bond, and by incubation with the cross-linker bis-malnate (NaTT) to link TM2 and TM7 of the lateral gate by means of SecY(S87C/F286C)EG were treated with the oxidizer tetrathiohydrophilic pore. As described previously (8), IMVs containing of the tetraarylmethane through the membrane via the central motif in the C4 loop. OmpT digestion of SecY that is not treated different spacer lengths (Fig. 5A). Second, a double cysteine SecY mutant (F286C and S87C) was used in which the lateral gate of the translocon at the interface of the hydrophobicity. In this study we investigated the diameter of the active SecYEG pore. For this purpose, different tetraarylmethanes were synthesized and covalently linked, via a maleimide group, to a unique cysteine residue at position 245 of the preprotein proOmpA. They can be used as molecular rulers to access the size of the functional translocation pore. Remarkably, all synthesized tetraaryl-
methanes conjugated to proOmpA were readily translocated into E. coli IMVs except for the largest molecule MeOTAM3 that has a molecular dimension of approximately 29 Å. When the size of the unfolded polypeptide is taken into account, assuming an extended conformation of 4–6 Å, the overall diameter of the translocation pore must be at least approximately 22–24 Å. Surprisingly, this exceeds the expected size for a monomeric pore without a lateral gate opening as determined by molecular dynamics simulations. To test if these molecules indeed pass through a single pore and/or whether lateral gate opening is required, we employed a SecY mutant in which the lateral gate opening was controlled through the use of a site-specific crosslink between TM2 and TM7 that together form the lateral exit site (8). Herein, two unique cysteines were introduced in the lateral gate. These were chemically cross-linked by oxidation or by the use of the chemical cross-linker BMOE that separates the thiolis by approximately 8 Å. When the lateral gate was constrained by oxidation, translocation of both proOmpA and the tetraaryl-methane conjugates was blocked. However, when the lateral gate was cross-linked with BMOE, translocation occurred unrestricted. In addition, the large proOmpA-MeOTAM3 blocked the pore for subsequent rounds of translocation, whereas the smaller IsoTAM2 did not. Therefore, we conclude that the translocation pore can accommodate relatively large structures, which indicates a more complex pore geometry than previously suggested by molecular dynamics simulations (16).

To exclude the possibility that the hydrophobic nature of the tetraaryl methanes influences the translocation of the conjugates we decreased the hydrophobicity of the tetraaryl methane (IsoTAM2) by substituting each aromatic unit with a methyl-oxy group (MeOTAM2). The translocation kinetics of this proOmpA derivative was nearly indistinguishable from that of the other tetraaryl methanes indicating that hydrophobicity is not a major factor (Fig. 2B). As translocation of the proOmpA derivatives was also undisturbed with a SecYEG complex containing a fixed lateral gate, an interface translocation model of the tetraaryl-methane molecules can be ruled out. Rather, the additional space provided by the opened lateral gate may contribute to the size of the active pore. The experimentally determined pore size of approximately 22–24 Å will be closed to the maximal pore diameter, as a further expansion of the tetraaryl methane sphere to 29 Å arrested translocation. This size is substantially smaller than the previously size of approximately 40–60 Å based on fluorescent quenching techniques (17). In this respect, the recent structure of SecYEG from T. maritima with SecA bound in an intermediate state of ATP-hydrolase shows in comparison to the M. jannaschii SecYEG structure, a partial opening of the lateral gate region around TM2 and TM7/8 (7) that points at a more complex pore geometry possibly including an opened lateral gate as an extension of the central pore.

Another characteristic feature of the translocation of proOmpA derivatized with tetraaryl methanes is the much stronger PMF-dependence than of wild-type proOmpA. Our data support the hypothesis that the PMF modulates the opening or even the width of the pore during translocation (20, 24). To further investigate the strong PMF-dependent translocation of proOmpA tetraaryl methane derivatives, translocation of proOmpA-IsoTAM2 was investigated with IMVs containing the PrlA4 mutant of SecY. The SecYEG pore of this mutant is thought to be in a relaxed state, probably because of a destabilization of the closed state (25). In IMVs containing the Prl4 mutant, translocation of proOmpA-IsoTAM2 indeed is independent of the PMF. Also, the translocation kinetics of proOmpA-IsoTAM2 into PrlA4 IMVs is increased as compared to wild-type IMVs as shown previously for wild-type preproteins.

Summarizing, our data suggest a high plasticity of the SecYEG translocation pore that can accommodate large nonpolypeptide moieties. Importantly, the data suggest that the lateral gate opening contributes to the functional pore size and that the PMF modulates the width of the translocation pore.

Materials and Methods

Materials. SecA (26) and SecB (27) were purified as described. IMVs with overexpressed levels of SecYEG were obtained from E. coli strain UH203 transformed with pET610 (28). IMVs containing overexpressed levels of SecY (F286C/S87C) were obtained from E. coli strain SF100 transformed with pFE-SecY16 plasmid (8). OmpT was expressed from plasmid pSN9 in strain SF100 and expressed under its own temperature sensitive promoter (29). The proOmpA cysteine mutant S245C was constructed with the QuickChange site-directed mutagenesis kit (Stratagene) using pET2345 containing the cysteineless proOmpA as a template (30). Primers used in addition included a silent MluI cutting site for cloning purposes: S245C forward primer, cccaccgat cgtgtgac gcgtacaacc agggtctg; S245C reverse primer, cagaccctgg ttctgtgac gcgtacaacc agggtctg. The introduced mutations were confirmed by sequencing. ProOmpA(S245C) was purified as described previously (30) and further referred to as proOmpA.

Crosslinking of Lateral Gate. IMVs containing SecY(S87C/F286C)CEG as isolated as previously described (8). IMVs (1 mg of protein/ml) were incubated for 30 min at 37 °C with Na2S2O4 or BMOE at a final concentration of 1 mM and 300 μM respectively. To test the efficiency of the crosslinking IMVs were treated with 1 mg/ml OmpT in 50 mM Tris/HCl pH 7, 0.1% Triton X100 for 30 min at 37 °C. Samples were analyzed by SDS-PAGE gel (12% acrylamide) and Coomassie brilliant blue staining.

Tetraaryl methane Synthesis, proOmpA Labeling, and Translocation Assays. See SI Appendix.

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Supporting information

Materials and Methods

Tetraarylmethane Synthesis

(Z)-3-(4-trityl-phenylcarbamoyl)-acrylic acid (IIIa)

To the free amine IIa (1g, 3 mmol) in THF (6 ml) maleinanhydride (292 mg, 3 mmol) in THF (1 ml) was added and the mixture was stirred for 4 h at room temperature. The solid product was isolated by filtration, washed with THF (2 x 2 ml) and dried in vacuum to give 1.12g (86%) of a white powder.

$^1$H NMR (300 MHz, DMSO-d6) $\delta$ 6.29 (d, $J = 12.1$ Hz, 1H), 6.47 (d, $J = 12.1$ Hz, 1H), 7.07-7.23 (m, 11H), 7.27-7.33 (m, 6H), 7.54 (d, $J = 8.4$ Hz, 2H), 10.43 (s, 1H), 13.11 (bs, 1H)

$^{13}$C NMR (75.4 MHz, DMSO-d6) $\delta$ 64.09 (s), 118.80 (d), 125.97 (d), 127.74 (d), 130.45 (d), 130.87 (d), 131.72 (d), 136.34 (s), 141.83 (s), 146.44 (s), 163.24 (s), 166.82 (s)

MS (EI): 433 [M+]; HRMS calcd. for C$_{29}$H$_{23}$NO$_3$ 433.1678, found 433.1676
1-(4-trityl-phenyl)-pyrrole-2,5-dione (IVa)
Maleamic acid IIIa (1g, 2.3 mmol) and anhydrous sodium acetate (164 mg, 2 mmol) were suspended in acetanhydride (3 ml) and heated at 90°C. After 2 h the reaction mixture was cooled to room temperature, poured into water (15 ml) and extracted with dichloromethane (3 x 20 ml). The combined organic extracts were washed with saturated aq. NaHCO₃ (2 x 20 ml) and water (20 ml), dried over Na₂SO₄ and the solvents were evaporated. Recrystallization from the hot toluene gave 896 mg (94%) of the product as a white solid

^1^H NMR (400 MHz, CDCl₃) δ 6.83 (s, 2H), 7.17-7.28 (m, 17H), 7.31-7.34 (m, 2H)

^1^C NMR (100.6 MHz, CDCl₃) δ 64.90 (s), 124.78 (d), 126.19 (d), 127.72 (d), 129.10 (s), 131.23 (d), 131.95 (d), 134.34 (d), 146.50 (s), 146.60 (s), 169.68 (s)

MS (EI): 415 [M+]; HRMS calcd. for C₂₉H₂₁NO₂ 415.1572, found 415.1568

tris-biphenyl-4yl-methanol (Ib)
The 4-Bromobiphenyl (4.66 g, 20 mmol) was dissolved in diethylether (100 ml) and n-BuLi was slowly added (11.3 ml of 1.6M solution in hexanes, 18 mmol). After stirring for 1 h at room temperature diethylcarbonate (0.727 ml, 6 mmol) was added and stirring continued for 1 hour. The reaction mixture was then poured into water (100 ml), the organic layer separated and the water layer extracted with diethylether (2 x 100 ml). The combined organic extracts were dried over Na₂SO₄ and the solvents evaporated. Purification by chromatography (silicagel, hexane:toluene / 1:1, then pure toluene) yielded 2.79 g (95%) of the product as a white solid.

^1^H NMR (400 MHz, CDCl₃) δ 2.92 (s, 1H), 7.35 (t, J = 7.3 Hz, 3H), 7.42-7.45 (m, 12H), 7.57-7.62 (m, 12H)
$^{13}$C NMR (100.6 MHz, CDCl$_3$) $\delta$ 81.88 (s), 126.92 (d), 127.26 (d), 127.55 (d), 128.54 (d), 128.97 (d), 140.31 (s), 140.74 (s), 145.94 (s)

MS (EI): 488 [M+]; HRMS calc. for C$_{37}$H$_{28}$O 488.2140, found 488.2129

4-(tris-biphenyl-4yl-methyl)-phenylamine (IIb)
Alcohol Ib(2.76 g, 5.65 mmol) and aniline hydrochloride (1.46 g, 11.3 mmol) were heated at reflux for 3 h in a mixture of acetic acid (10 ml) and toluene (10 ml). Then the reaction mixture was poured into water (100 ml) and extracted with chloroform (3 x 20 ml). The combined organic layers were transferred into a flask containing saturated aq. NaHCO$_3$ (100 ml) and vigorously stirred for 30 min. The organic layer was then separated and the water layer was extracted with chloroform (20 ml). The combined organic extracts were dried over Na$_2$SO$_4$ and the solvents evaporated. Purification by chromatography (silicagel, hexane:ethyl acetate / 5:1, then 3:1) yielded 2.50 g (79%) of the product as a white solid.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.09 (s, 2H), 6.68 (d, $J$ = 8.5 Hz, 2H), 7.12 (d, $J$ = 8.5 Hz, 2H), 7.33 (t, $J$ = 7.7 Hz, 3H), 7.37 (d, $J$ = 8.4 Hz, 6H), 7.44 (t, $J$ = 7.7 Hz, 6H), 7.53 (d, $J$ = 8.4 Hz, 6H), 7.62 (d, $J$ = 7.7 Hz, 6H), $^{13}$C NMR (100.6 MHz, CDCl$_3$) $\delta$ 63.78 (s), 114.82 (d), 126.22 (d), 127.09 (d), 127.30 (d), 128.86 (d), 131.61 (d), 132.21 (d), 137.49 (s), 138.57 (s), 140.76 (s) 143.58 (s), 146.37 (s)

MS (EI): 563 [M+]; HRMS calc. for C$_{43}$H$_{33}$N 563.2613, found 563.2619

(Z)-3-[4-(trisbiphenyl-4yl-methyl)-phenylcarbamoyl]-acrylic acid (IIIb)
To the free amine IIb(1,127g, 2 mmol) in CHCl$_3$ (15 ml) was added maleinanhydride (196 mg, 2 mmol) in THF (1 ml) and the mixture was stirred for 48 h at room temperature. The solid product was isolated by
filtration, washed with THF (2 x 2 ml) and dried in vacuum to give 830 mg (65%) of a white powder.

$^1$H NMR (400 MHz, DMSO-d6) $\delta$ 6.30 (d, $J = 12.1$ Hz, 1H), 6.49 (d, $J = 12.1$ Hz, 1H), 7.24 (d, $J = 8.4$ Hz, 2H), 7.32-7.37 (m, 9H), 7.45 (t, $J = 7.7$ Hz, 6H), 7.61 (d, $J = 8.4$ Hz, 2H), 7.64-7.69 (m, 12H), 10.47 (s, 1H), 13.11 (bs, 1H)

$^{13}$C NMR (75.4 MHz, DMSO-d6) $\delta$ 63.49 (s), 119.03 (d), 126.11 (d), 126.57 (d), 127.47 (d), 128.95 (d), 130.29 (d), 130.86 (d), 130.97 (d), 131.79 (d), 136.50 (s), 137.67 (s), 139.45 (s), 141.65 (s), 145.60 (s), 163.29 (s), 166.82 (s)

MS (EI): 661 [M+]; HRMS calc. for C$_{47}$H$_{35}$NO$_3$ 661.2617 found 661.2632

1-[4-(trisbiphenyl-4yl-methyl)-phenyl]-pyrrole-2,5-dione (IVb)

Maleamic acid IIIb (643 mg, 1 mmol) and anhydrous sodium acetate (40 mg, 0.5 mmol) were suspended in acetanhydride (2 ml) and heated at 90°C. After 4 h the reaction was cooled to room temperature, poured into water (15 ml) and extracted with dichloromethane (3 x 20 ml). The combined organic extracts were washed with saturated aq. NaHCO$_3$ (2 x 20 ml) and water (20 ml), dried over Na$_2$SO$_4$ and the solvents evaporated. Purification by chromatography (silicagel, hexane:chloroform / 1:1) yielded 80 mg (13%) of the product as a white solid.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.86 (s, 2H), 7.30-7.48 (m, 19H), 7.55 (d, $J = 8.4$ Hz, 6H), 7.62 (d, $J = 8.1$ Hz, 6H)

$^{13}$C NMR (100.6 MHz, CDCl$_3$) $\delta$ 64.28 (s), 124.95 (d), 126.44 (d), 127.11 (d), 127.36 (d), 128.86 (d), 129.24 (s), 131.58 (d), 131.91 (d), 134.34 (d), 138.90 (s), 140.61 (s), 145.53 (s), 146.48 (s), 169.68 (s)
tris(4'-methoxybiphenyl-4-yl)methanol (Ic): Dry diethyl ether (10 ml) was added to a solution of 4-bromo-4'-methoxybiphenyl (1g, 3.8 mmol) in dry THF (10 ml), the mixture was cooled to -75 °C, and n-BuLi (2.8 ml, 4.56 mmol, 1.6 M in hexane) was added drop wise over 20 min. The mixture was stirred 30 min. Diethyl carbonate (0.11 ml, 0.95 mmol) in THF (2 ml) was added slowly and the resulting mixture was stirred at -75°C for another hour. The mixture was allowed to warm to 0°C (ice bath) and stirred overnight. The mixture was quenched with methanol (0.6 ml) and the solvent was removed under vacuum. The residue was extracted with ethyl acetate, washed with water, dried over MgSO₄ and concentrated. The product (Ic) (250 mg, 34%) was obtained as a white solid after purification by column chromatography on silica (hexane/EtOAc, 9:1).

$^1$H NMR (CDCl₃) δ 7.55 (d, J= 4.4 Hz, 6H), 7.54 (d, J= 3.6 Hz, 6H), 7.4 (d, J= 10.8 Hz, 6H), 6.98 (d, J= 12Hz, 6H), 3.85 (s, 9H).

$^{13}$C NMR (CDCl₃) δ 159.41, 145.49, 139.89, 133.35, 128.58, 128.32, 126.46, 114.45, 94.94, 55.56.

MS (El): 578[M⁺]; HRMS calcd. for C₄₀H₄₃O₄ 578.7023, found 578.6997
4-(tris (4'-methoxybiphenyl-4-yl) methyl) aniline (IIc): Acetic acid (1 ml), Ic (250 mg, 0.43 mmol), and aniline hydrochloride (111 mg, 0.86 mmol) were dissolved in toluene (10 ml), and the mixture was refluxed for 3 hrs. Water was added, the organic layer was separated and the water layer was extracted with CHCl₃ (3x). The organic layers were collected and saturated, aqNaHCO₃ was added and this two-layer system was stirred for 30 min. The organic layer was separated and the water layer was extracted with chloroform (2x). The chloroform solution was dried over MgSO₄ and the solvent evaporated under vacuum. The product (IIc, 50 mg, 20%) was obtained as a white solid after purified by column chromatography on silica (hexane/EtOAc, 9/1).

1H NMR (CDCl₃): 7.54 (d, J = 4.4 Hz, 6H), 7.46 (d, J = 3.6 Hz, 6H), 7.33 (d, J = 10.8 Hz, 6H), 7.08 (d, J = 8Hz, 2H), 6.96 (d, J = 12Hz, 6H), 6.62 (d, J = 8Hz, 2H), 3.85 (s, 9H), 3.62 (s, 2H, NH₂).

13C NMR (CDCl₃): 159.0, 146.0, 142.1, 138.0, 137.3, 133.4, 132.1, 131.7, 128.1, 125.8, 120.0, 114.2, 63.5, 54.8.

MS (EI): 652.9 [M+]; HRMS calcd. for C₄₆H₃₉NO₃ 653.8133 found 653.8141

2-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl)-N-(4-(tris (4'-methoxybiphenyl-4-yl)methyl) phenyl) acetamide (IIIc): A mixture of (2,5-dioxo-2, 5-dihydro-pyrrol-1-yl)-acetic acid (12 mg, 0.076 mmol) and SOCl₂ (0.2 ml) were refluxed for 0.5 hour. The excess of SOCl₂ was removed by evaporation under vacuum. The residue was dissolved in toluene (2 ml) and removed under vacuum. The final product was dissolved in CH₂Cl₂ (1 ml), IIc (50 mg, 0.076 mmol) and Et₃N (12 µl, 0.084 mmol) dissolved in CH₂Cl₂ (1 ml). The reaction mixture was stirred overnight,
diluted with ethyl acetate and washed with aq. HCl, aq. NaHCO₃ and water. The organic layer was dried (MgSO₄) and the solvent was removed under vacuum. The residue was purified by column chromatography on silica (hexane/ethyl acetate, 7/3) to give (IIIc, 30 mg, 50 %) as a white solid.

¹H NMR (CDCl₃): δ 7.58 (d, J = 8Hz, 2H), 7.53 (d, J = 4.4Hz, 6H), 7.46 (d, J = 3.6Hz, 6H), 7.39 (d, J = 8Hz, 2H), 7.31 (d, J = 10.8Hz, 6H), 6.96 (d, J = 12Hz, 6H), 6.77 (s, 2H), 4.31 (s, 2H), 3.82 (s, 9H), 1.60 (s, 1H, NH₂)

¹³C NMR (CDCl₃): δ 172.3, 170.3, 159.30, 145.31, 138.41, 135.11, 133.29, 131.93, 131.59, 129.22, 128.18, 125.95, 120.11, 119.31, 114.41, 110.13, 63.36, 41.52.

HRMS calcd. for C₅₂H₄₂N₂O₆ 790.9045, found 790.9089

tris-(4'-bromo-biphenyl-4-yl)-methanol (Id). Diethyl ether (24 ml) was added to a solution of 4.4'-dibromo-biphenyl (3.12g, 10 mmol) in THF (24 ml), the mixture was cooled to -75 °C, and n-BuLi (7.5 ml, 12 mmol, 1.6M in hexane) was added drop wise over 20 min. The mixture was stirred for 30 min. and after 1 hour, diethyl carbonate (0.3 ml, 2.5 mmol) in THF (2 ml)
was added slowly. The mixture was allowed to warm to 0 °C (ice bath) and was stirred for 3 hrs. The mixture was quenched with methanol (2 ml) and the solvent was removed under vacuum. The residue was extracted with ethyl acetate, and the extract was washed with water, dried (MgSO₄) and concentrated. The residue was purified by column chromatography on silica (CHCl₃/hexane, 9:1). The product \textbf{Id} (991 mg, 47 %) was isolated as a white solid.

\begin{align*}
\text{\textbf{H NMR (CDCl₃)}} & \delta 7.59 (d, J= 8.5 \text{ Hz}, 6\text{H}), 7.56 (d, J= 8.5 \text{ Hz}, 6\text{H}), 7.48 (d, J= 8.5 \text{ Hz}, 6\text{H}), 7.46 (d, J= 8.5 \text{ Hz}, 6\text{H}), 2.88 (s, 1\text{H}). \\
\text{\textbf{C NMR (CDCl₃)}} & \delta 146.19, 139.63, 139.29, 132.88, 128.87, 128.65, 126.83, 121.96, 84.59
\end{align*}

\textbf{tris-(4'-isopropyl-biphenyl-4-yl)-methanol (IId)}. The Grignard reagent was prepared from 2-bromopropane (0.96 ml) and Mg (313 mg) in Et₂O (50 ml) at reflux under N₂. Alcohol \textbf{Id} (750 mg, 1.03 mmol) and PdCl₂ (dppf) (34 mg, 0.0414 mmol) were placed into dry flask under N₂ and Et₂O (50 ml) and THF (20 ml) were added and the reaction mixture was cooled at -78 °C. The Grignard reagent was added slowly during 15 min. The reaction mixture was stirred another 10 min. at -78 °C and subsequently at room temperature overnight. 5% aq. HCl (50 ml) was added and the reaction mixture was extracted with Et₂O (30 ml). The combined organic layers were dried (MgSO₄) and the solvent was evaporated under vacuum. The residue was purified by column chromatography on silica (hexane/ethyl acetate, 9:1) to give product \textbf{IId} (305 mg, 90.5 %) as white solid.
\(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.59 (d, J= 8.5 Hz, 6H), 7.56 (d, J= 8.5 Hz, 6H), 7.48 (d, J= 8.5 Hz, 6H), 7.46 (d, J= 8.5 Hz, 6H), 3.0 (m, 3H), 2.01 (s, 1H), 1.36 (d, J= 6.8 Hz, 18H).

\(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 148.32 145.75, 140.31, 138.37, 128.53, 127.23, 127.10, 126.95, 126.79, 82.11, 33.86, 24.24

MS (EI): 614.1 [M+]; HRMS calcd. for C\(_{46}\)H\(_{46}\)O 614.8656 found 614.8649

4-[tris-(4'-isopropyl-biphenyl-4-yl)-methyl]-phenylamine (IIId). A solution of acetic acid (6 ml), IIId (300 mg, 0.488 mmol), and aniline hydrochloride (126 mg, 0.98 mmol) in toluene (3 ml) was stirred at 100 °C for 24 hrs. The solvent was evaporated under vacuum, and then methanol (6 ml) and aq. HCl (2 M, 2 ml) were added. The resulting slurry was refluxed for 24 hrs and the solvent was evaporated under vacuum. The residue was dissolved in chloroform and washed with aq. NaHCO\(_3\). The chloroform solution was dried (MgSO\(_4\)) and concentrated under vacuum. The residue was purified by column chromatography on silica (CHCl\(_3\)/hexane, 9:1) to give product IIIId (305 mg, 90.5 %) as a pale solid. \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.57 (d, J= 8.6 Hz, 6H), 7.5 (m, 12H), 7.37 (d, J= 8.6 Hz, 6H), 7.10 (d, J= 8.5 Hz, 2H), 6.67 (d, J= 8.6 Hz, 2H), 3.52 (s, 2H), 3.0 (m, 3H), 1.36 (d, J= 6.8 Hz, 18H).

\(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 148.77, 148.07, 142.79, 139.43, 138.59, 138.43, 132.31, 131.69, 129.03, 127.11, 127.04, 114.58, 87.61, 33.86, 24.26.

HRMS calcd. for C\(_{52}\)H\(_{51}\)N 689.9731 found 689.9775

2-(2, 5-Dioxo-2, 5-dihydro-pyrrol-1-yl)-N-{4-[tris-(4'-isopropyl-biphenyl-4-yl)methyl]-phenyl}-acetamide (IVd). A mixture of (2,5-dioxo-2, 5-dihydro-pyrrol-1-yl)-acetic acid (61.7 mg, 0.39 mmol) and SOCl\(_2\) (1.2
ml) was refluxed for 0.5 h. The excess of SOCl$_2$ was removed by evaporation under vacuum. The residue was then dissolved in toluene (2 ml) and evaporated again. Finally the product was dissolved in CH$_2$Cl$_2$ and IIId (250 mg, 0.36 mmol) and Et$_3$N (0.055 ml, 0.39 mmol in CH$_2$Cl$_2$ 2 ml) were added. The reaction mixture was stirred overnight, diluted with ethyl acetate and washed with aq. HCl, aq. NaHCO$_3$ and water. The organic layer was dried (MgSO$_4$) and the solvent was removed under vacuum. The residue was purified by column chromatography on silica (hexane/ethyl acetate, 1:1) to give IVd (126 mg, 42.2 %) as a white solid.

$^1$H NMR (CDCl$_3$) δ 7.8 (s, 1H, NH), 7.5 (m, 12H), 7.4 (d, $J$= 4.2 Hz, 2H), 7.37 (m, 8H), 7.24 (d, $J$= 8.5 Hz, 2H), 6.69 (s, 2H), 4.3 (s, 2H), 3.0 (m, 3H), 1.36 (d, $J$= 6.8 Hz, 18H)

$^{13}$C NMR (CDCl$_3$) δ 160.3, 164.1, 148.16, 145.59, 138.83, 138.27, 134.77, 132.0, 131.6, 129.04, 127.11, 127.05, 126.27, 119.3, 64.1, 41.53, 34.02, 24.22

MS (Maldi-Tof): 849.7057 [M+Na]
5-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetamido)isophthalic acid (Ie). A mixture of (2,5-dioxo-2, 5-dihydro-pyrrol-1-yl)-acetic acid (200 mg, 1.29 mmol) and SOCl₂ (4 ml) was refluxed for 1 hr. The excess of SOCl₂ was removed by evaporation under vacuum. The residue was then dissolved in toluene (2 ml) and the solvent was evaporated. Final product was dissolved in dioxane and added prop wise at 0 °C to a solution of 5-aminoisophthalic (233 mg, 1.29 mmol) acid in dioxane. The reaction
mixture was stirred for 2 hrs, poured into the water, extracted with EtOAc (3 x 50 ml), combined organic layers were dried (MgSO₄) and solvent was evaporated under reduce pressure. Product Ie (350 mg, 99 %) was obtained as a yellow powder and was used without further purification in next reaction.

1H NMR (DMSO)
160.13, 155.64, 146.66, 144.50, 142.41, 138.53, 136.96, 132.26, 131.66, 129.94, 126.36, 124.80, 119.73, 114.51, 112.92, 112.81, 79.17, 55.51
MS (EI): 653 [M⁺], C₄₆H₃₉NO₃ (653.81)

5-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetamido)-N¹,N³-bis(4-(tris(3'-methoxybiphenyl-4-yl)methyl)phenyl)isophthalamide (Ve): Crude diacyl dichloride Ile from previous reaction was dissolved in dry CH₂Cl₂ (5 ml) and cooled at 0 ºC. Amine IVe (100 mg, 0.153 mmol) and Et₃N (47 µl, 0.168 mmol) were dissolved in dry CH₂Cl₂ and were added dropwise into the solution of diacyl dichloride Ile in dry CH₂Cl₂ at 0 ºC. Reaction mixture was allowed to room temperature overnight, diluted with EtOAc and washed with diluted HCl, sat. NaHCO₃ and water. Organic layer was dried over Na₂SO₄ and solvents were evaporated under reduce pressure. Pure product Ve (71 mg, 60 %) was obtained as a brownish powder after purification by column chromatography on silica (hexane/EtOAc, 5:5).

1H NMR (400 MHz, CDCl₃) δ 7.41 (m, 24H), 7.26 (m, 18H), 7.06 (m, 10H), 6.81 (m, 7H), 6.77 (s, 2H), 4.31 (s, 2H), 4.75 (s, 18H)
13C NMR (100 MHz, CDCl₃) δ 170.61, 166.5, 165.3, 160.1, 146.05, 142.17, 138.68, 135.8, 131.49, 129.97, 126.57, 125.5, 119.7, 113.03, 112.74, 64.2, 55.44, 34.21
MS (EI): 1608.1 [M+18]; C₁₀₆H₈₄N₄O₁₁, 1589.82
**ProOmpA Labeling.** ProOmpA (3 mg/ml) in 8 M Urea and 50 mM Tris/HCl, pH 7 was treated with 1 mM TCEP for 30 mins at room temperature. For the labeling, the tetraarylmethane derivatives were dissolved in different organic solvents (TAM1: methanol; TAM2: chloroform/methanol (1:3, v/v); IsoTAM2: ethylacetate; MeOTAM2 and MeOTAM3: dimethylformamide) and then added to purified proOmpA (2 mg/ml in 8 M Urea, 50 mM Tris/HCl, pH 7) at a final concentration of: a) 4 mM TAM1; b) 4 mM TAM2; c) 4.5 mM IsoTAM2; and d) 8.3 mM MeOTAM2 or MeOTAM3. The suspensions were incubated for 2 hrs (a and b) or overnight (c and d) at room temperature under constant stirring. To increase the efficiency of the reaction, after the first hour of incubation, an aliquot of the fresh solutions of the compounds was added to the suspensions. ProOmpA was recovered from the suspension by 10 % TCA precipitation for 30 mins at 4 °C, followed by several washes of the protein pellet with ice-cold acetone. This effectively removed non-reacted compounds. Samples were dried for 10 mins at 37 °C and resuspended in 8 M Urea, 50 mM Tris/HCl, pH 7.

To verify the labeling efficiency, a second labeling step was performed with fluorescein-5-maleimide (Fmal). Isolated proOmpA conjugates were treated with 1 mM TCEP for 30 minutes at room
temperature and subsequently incubated with 2 mM Fmal for 1 hr at room temperature (1). Samples were analyzed on a 12% SDS-PAGE gel and fluorescence was detected at 520 nm and compared with a 10% standard of Fmal-labeled proOmpA using the Lumi-Imager F1™ Workstation (Roche Molecular Biochemicals).

**Translocation assays.** *In vitro* translocation reactions (50 µl) were performed at 37 °C as previously described (2) using 20 µg/ml of SecA, 32 µg/ml of SecB, 1 µg of urea-denaturated proOmpA with or without the various labeled organic compounds or when indicated proOmpA-DHFR (3), 10 mM phosphocreatine and 50 mM creatine kinase in a buffer consisting of 50 mM Tris/HCl, pH 7, 30 mM KCl, 0.5 mM bovine serum albumin (BSA), 10 mM DTT and 5 mM MgCl₂. *In vitro* translocation reactions (50 µl) were performed and analysed at 37 °C as previously described. *E. coli* UH203 (ompA⁻) or SE6004 (prlA4) IMVs were added to a final concentration of 0.2 mg/ml. Reactions were started by the addition of 2 mM ATP and terminated at various time intervals by chilling on ice. Samples were treated with proteinase K (1 mg/ml) for 30 min on ice, precipitated with 10 % (w/v) TCA, washed with ice cold acetone and analyzed by 12 % SDS-PAGE and immunoblotting using a polyclonal antibody against OmpA. Immunoblots
were developed using the chemiluminescent substrate disodium 4-chloro-3-(methoxyspiro {1,2-dioxetane-3,2’-(5’-chloor)tricyclo [3.3.1.1^3,7]decan}-1-4-yl)phenyl phosphate (CDP star, Roche Molecular Biochemicals). When indicated, the PMF was collapsed by the addition of nigericin and valinomycin at a final concentration of 2 µM.

When indicated, IMVs (from a 50 µl reaction) were recollected by centrifugation through a 0.8 M sucrose solution (15 mins; 75,000 rpm TLA110 rotor, 4 °C). The pelleted IMVs were resuspended in 50 µl of translocation buffer, and used in a second translocation reaction with Fmal-labeled proOmpA as substrate. Translocation reactions were analyzed by 12% SDS-PAGE gels and in gel fluorescence (excitation 520 nm) using the Lumi-Imager F1™ Workstation (Roche Molecular Biochemicals).

References

Supplementary figures

Supplemental Fig. 1. Labeling of proOmpAS245C with tetraarylmethanes derivatives. ProOmpAS245C was incubated with the indicated tetraarylmethanes derivatives. After 2 hrs at room temperature non-reacted tetraarylmethane was removed by TCA precipitation and several washes of the protein pellet with acetone where after the proOmpA tetraarylmethane conjugate was dissolved in 50 mM Tris/HCl pH 8.0 and 8 M urea. To determine the labeling efficiency a small fraction of the proOmpA tetraarylmethane conjugate was incubated with Fmal for 2 hrs at room temperature thereafter the sample was analyzed by SDS-PAGE and in gel UV fluorescence. To determine the amount of nonspecific labeling of proOmpA with Fmal, a cysteine-less variant of proOmpA was used.