

# Ligand crowding at a nascent signal sequence

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**W**e have systematically analyzed the molecular environment of the signal sequence of a growing secretory protein from *Escherichia coli* using a stage- and site-specific cross-linking approach. Immediately after emerging from the ribosome, the signal sequence of pOmpA is accessible to Ffh, the protein component of the bacterial signal recognition particle, and to SecA, but it remains attached to the surface of the ribosome via protein L23. These contacts are lost upon further growth of the nascent chain, which brings the signal sequence into sole

proximity to the chaperone Trigger factor (TF). In its absence, nascent pOmpA shows extended contacts with L23, and even long chains interact in these conditions proficiently with Ffh. Our results suggest that upon emergence from the ribosome, the signal sequence of an *E. coli* secretory protein gradually becomes sequestered by TF. Although TF thereby might control the accessibility of pOmpA's signal sequence to Ffh and SecA, it does not influence interaction of pOmpA with SecB.

## Introduction

To export newly synthesized proteins from the cytoplasm to the inner and outer membranes and the intervening periplasmic space, gram-negative bacteria, such as *Escherichia coli*, utilize both a signal recognition particle (SRP)-dependent and a SecA/SecB-dependent pathway. The bacterial SRP consisting of one protein, Ffh (also termed P48), and one 4.5S RNA, cotranslationally recognizes its substrates and in concert with its receptor, FtsY, mediates membrane targeting of nascent polypeptide chains to the SecYE translocon of the inner membrane (for review see Koch et al., 2003). On the contrary, SecA posttranslationally binds to precursor proteins in concert with SecB and, by virtue of being associated with SecY, targets its substrates to the translocon (Müller et al., 2001). Recently, evidence has accumulated indicating that both pathways represent two principally independent routes for two different classes of proteins. Secretory proteins, which harbor cleavable signal sequences and which are destined for the periplasmic space and further to the outer membrane, follow the SecA/SecB pathway. In contrast, for hydrophobic membrane proteins, SRP and FtsY have been demonstrated to be both necessary and sufficient for

integration into the inner membrane (Koch et al., 1999; Koch and Müller, 2000; Park et al., 2002; Beha et al., 2003).

In addition, *E. coli* possesses a group of inner membrane proteins, characterized by large translocated domains, that exhibit a combined dependence on SRP/FtsY and SecA. Detailed studies demonstrated that integration of the transmembrane helices of these proteins requires exclusively SRP (Scotti et al., 1999; Neumann-Haefelin et al., 2000; Lee and Bernstein, 2001), whereas the SecA requirement is confined to the translocation of the extended hydrophilic domains (Neumann-Haefelin et al., 2000) of these membrane proteins.

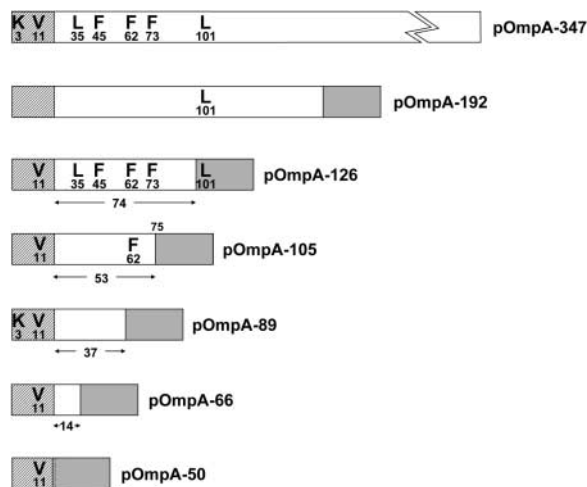
The combined data available therefore suggest that gram-negative bacteria select between two export routes converging at the SecYE translocon and that they must possess discriminatory mechanisms to choose the right cargo. Similar to the situation in the lower eukaryote yeast, in which the SRP-dependent route is reserved to secretory proteins with more hydrophobic signal sequences (Ng et al., 1996), the bacterial SRP selects inner membrane proteins on the basis of their longer and more hydrophobic signal anchor sequences (Valent et al., 1997; de Gier et al., 1998). This implies that signal sequences of secretory *E. coli* proteins do not come into, or at least do not remain in, contact with SRP when they emerge from the ribosome. In fact, when analyzed by cross-linking, ribosome-associated secretory proteins have

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Abbreviations used in this paper: DSS, disuccinimidyl suberate; INV, inside-out, inner membrane vesicle; RNC, ribosome-nascent chain complex; SRP, signal recognition particle; TF, Trigger factor; Tmd-Phe, L-4'-(3-[trifluoromethyl]-3H-diazirin-3-yl) phenylalanine.



**Figure 1. Compilation of pOmpA constructs used.** At the top, the 347-amino acid-long precursor of OmpA is depicted with the hatched box representing the signal sequence. Starting with the first amino acid of the signal sequence, all the positions are indicated at which stop codons were engineered. On top of the numerals, the amino acids of the pOmpA sequence are given that were replaced by the incorporation of Tmd-Phe. Below the precursor of OmpA, nascent chains are listed with the size indicated in number of NH<sub>2</sub>-terminal residues. The gray boxes represent the COOH-terminal 30 amino acids of each nascent chain predicted to be hidden in the ribosomal exit tunnel. For each nascent chain, the positions of cross-linker tested are indicated. Numbers below the bars correspond to the estimated distances in amino acids between the signal sequence cleavage site and the ribosomal exit site. For pOmpA105, the position of the ribosomal exit site relative to the position of the photoprobe is depicted above the bar. For pOmpA-50, the signal sequence cleavage site is represented by a dotted line.

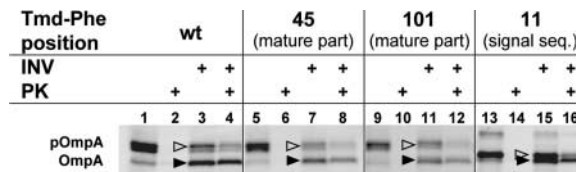
largely been found in contact with Trigger factor (TF) (Valent et al., 1995, 1997; Beck et al., 2000; Beha et al., 2003). TF is a ribosome-associated chaperone and peptidyl-prolyl isomerase involved in folding of both secretory and cytosolic proteins (Hesterkamp et al., 1996). Interestingly, both TF and Ffh have recently been found to use the same ribosomal protein L23 located at the orifice of the exit tunnel (Nissen et al., 2000) as docking site, which might suggest a potential competition for emerging nascent polypeptides (Kramer et al., 2002; Gu et al., 2003; Ullers et al., 2003).

To elucidate the molecular environment and contacts of a signal sequence at the ribosome, we have conducted an in vitro analysis of growing nascent chains of the bacterial secretory protein pOmpA. Using site-specific cross-linking we find that at least TF, Ffh, SecA, and the ribosomal protein L23 itself are crowding around an emerging signal sequence. Our results suggest that TF might fulfil an important regulatory function controlling access of Ffh and SecA, but not SecB, to secretory proteins.

## Results

### On the ribosome, the accessibility of the signal sequence changes upon growth of a nascent secretory protein

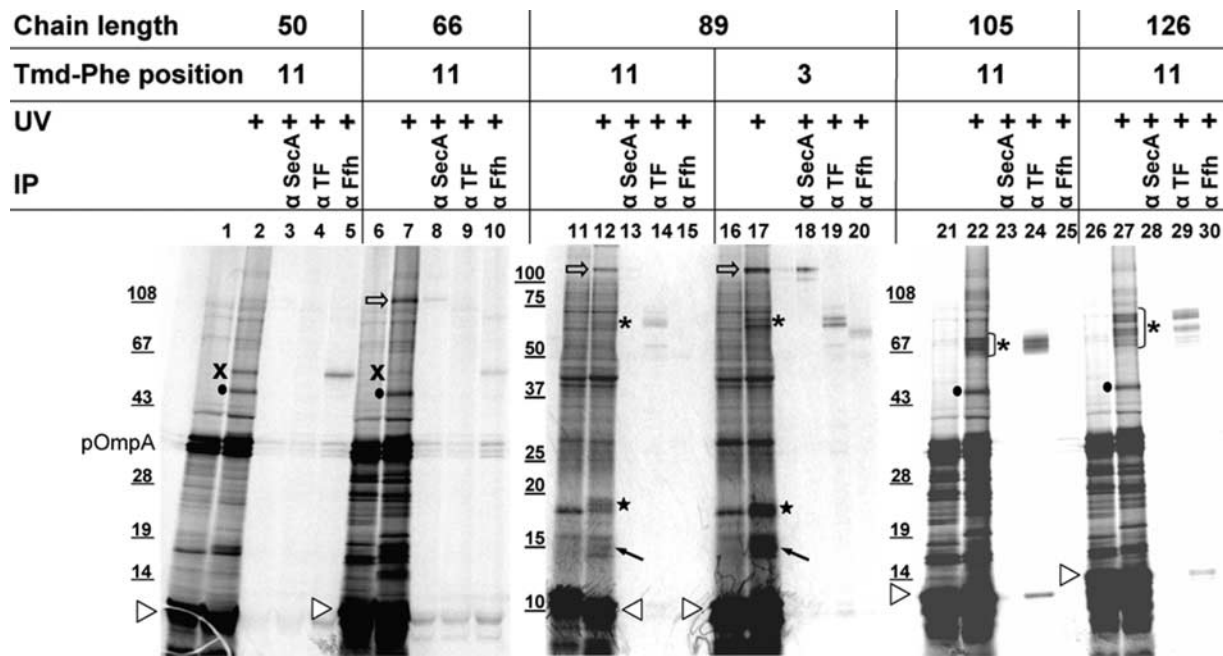
To visualize molecular contacts of a nascent secretory protein of *E. coli*, we site specifically incorporated a cross-linker into the precursor of OmpA (pOmpA) at positions shown



**Figure 2. Site-specific incorporation of Tmd-Phe into pOmpA does not interfere with its translocation into membrane vesicles.** In addition to wild-type pOmpA (wt), the indicated stop codon mutants located in the mature part and the signal sequence of OmpA were expressed in vitro by the help of suppressor tRNA charged with Tmd-Phe. Radiolabeled proteins were separated by SDS-PAGE and visualized by phosphorimaging. Addition of *E. coli* INVs and proteinase K (PK) is indicated. Precursor (pOmpA) and mature (OmpA) forms are labeled with open and closed arrowheads, respectively. Note the aberrant electrophoretic mobility of the mutant precursor carrying Tmd-Phe at position 11 within the signal sequence. The precursor of OmpA often resolves on SDS-PAGE into two closely spaced species, the reason for that being unknown. The background processing of the different pOmpAs obtained in the absence of added INVs (lanes 1, 5, 9, and 13) is most likely due to traces of INVs contaminating the ribosome preparation used.

in Fig. 1. This was achieved by engineering TAG stop codons into the *ompA* DNA and suppressing them in vitro by use of a suppressor tRNA charged with the photoactivatable derivative of phenylalanine, L-4<sup>2</sup>-(3-[trifluoromethyl]-3H-diazirin-3-yl) phenylalanine (Tmd-Phe). To first examine whether or not the incorporation of Tmd-Phe interfered with membrane translocation of pOmpA, the various TAG mutants of pOmpA were synthesized in vitro, and their translocation into inside-out, inner membrane vesicles (INVs) of *E. coli* was tested. This is exemplified for three of the TAG constructs in Fig. 2. Suppression of the stop codons by the tRNA carrying Tmd-Phe is indicated by the synthesis of full-size pOmpA (Fig. 2, compare lanes 1, 5, and 9). When Tmd-Phe had been incorporated into the signal sequence of pOmpA, a slightly aberrant electrophoretic mobility of pOmpA on SDS-PAGE ensued (Fig. 2, lane 13). All derivatives of pOmpA were translocated into INVs to an extent comparable to that of the wild-type pOmpA. This is indicated by the degree to which proteolytic processing of the precursors to the mature forms occurred (Fig. 2, lanes 3, 7, 11, and 15, open and closed arrowheads, respectively) and by the acquirement of resistance toward proteinase K (PK) in the presence of INVs (Fig. 2, compare lanes 2 and 4, 6 and 8, 10 and 12, and 14 and 16). As these derivatives of pOmpA were normally translocated into INVs, they were not likely to display a grossly changed behavior during early steps of biogenesis, including recognition events at the ribosome.

Next, ribosome-associated pOmpA chains between 50 and 126 amino acids in length were synthesized in vitro, each carrying the photoprobe Tmd-Phe at the indicated position within the signal sequence (Fig. 3). Besides full-size pOmpA, these nascent chains of pOmpA were the major translation products (white arrowheads). For each chain, a rather distinct set of cross-linking products (marked by symbols described below) was obtained when samples were irradiated with UV light (Fig. 3, compare lanes 1 and 2, 6 and 7, 11 and 12, etc.).



**Figure 3. Upon growth of the nascent pOmpA chain, the signal sequence changes its molecular environment from SecA and Ffh to TF.** Nascent, ribosome-associated chains of pOmpA of 50, 66, 89, 105, and 126 amino acids length were synthesized *in vitro*. Truncation of the *ompA* mRNA was achieved by the addition of complementary oligodeoxynucleotides and RNaseH cleavage (Behrmann et al., 1998). Stop codons at the indicated positions were suppressed by tRNA<sup>sup</sup> charged with Tmd-Phe. The migration on SDS-PAGE of the nascent chains is indicated by white arrowheads. The sizes of marker proteins are given in kD. The expression of some full-size pOmpA resulting from insufficient truncation of mRNA by RNaseH was usually observed. *In vitro* reactions were supplemented with 270 nM SecA dimer, 234 nM SecB tetramer (468 nM in the case of pOmpA-89), and 80 nM Ffh. UV irradiation (UV) resulted in several adducts, which are marked by various symbols. Identification of cross-links to Ffh (X), SecA (white arrow), and TF (asterisk) by coimmunoprecipitation with specific antibodies is shown. ● marks presumed intramolecular cross-links of full-size pOmpA (compare with legend to Fig. 4). Stars and upwards pointing arrows mark cross-links to ribosomal proteins L23 and L29, as demonstrated in Fig. 6.

Assuming that the most COOH-terminal 30 amino acids of each nascent pOmpA chain would be hidden in the exit tunnel of the *E. coli* ribosome (Bernabeu and Lake, 1982), the shortest nascent chain analyzed (pOmpA-50) would not even completely expose the 22-amino acid-long signal sequence (compare with Fig. 1). Unexpectedly, for these short chains, one of the two major cross-linking partners of the signal sequence was identified as Ffh by coimmunoprecipitation (Fig. 3, lanes 2 and 5, X). The nature of the other prominent UV-induced adduct of pOmpA-50 (●) was elucidated by experiments summarized below in Fig. 4. Ribosome-nascent chain complexes (RNCs) of pOmpA-66, in which the entire signal sequence plus 14 additional residues had emerged from the ribosome, yielded a weaker Ffh adduct (Fig. 3, lanes 7 and 10, X) and, different from pOmpA-50, showed significant interaction with SecA (Fig. 3, lanes 7 and 8).

When the nascent pOmpA chains were extended by 23 additional residues (pOmpA-89), cross-linking of Ffh to Tmd-Phe in position 11 (Fig. 3, lane 15) had vanished while the photoprobe placed in position 3 still revealed residual contacts (Fig. 3, lane 20). In contrast, SecA remained a dominating interaction partner also at this length of nascent pOmpA (Fig. 3, lanes 17 and 18). Different from the two shorter chains, both Tmd-Phe constructs of pOmpA-89 were now found also in the vicinity of TF (Fig. 3, lanes 14 and 19) and of additional proteins (Fig. 3, lanes 12 and 17, star and arrow), which will be referred to in the description of Fig. 6. Whereas the TF adducts of the signal sequence of

pOmpA-89 were still rather weak (Fig. 3, lanes 12 and 17, asterisk), they became considerably more pronounced after the length of the nascent pOmpA chains had increased to 105 (Fig. 3, lanes 22 and 24) and 126 (Fig. 3, lanes 27 and 29) amino acids.

Due to the unique locations of the cross-linker, all of the adducts depicted in Fig. 3 reflect binding partners of the signal sequence of pOmpA RNCs. As all these experiments were performed with the same amounts of SecA, Ffh, and TF present, they clearly disclose a length-dependent interaction selectivity of the nascent signal sequence. The combined results then suggest that the signal sequence comes into close proximity to TF only after the nascent chain has grown beyond 70–80 amino acids in length. Below that size, it is freely accessible to Ffh and SecA.

#### Exposure of multiple binding sites for TF within the mature part of a nascent secretory *E. coli* protein

The fact that in order for TF to associate with the signal sequence of pOmpA RNCs, >14 downstream amino acids had to be exposed (as in pOmpA-89, compare with Fig. 1) suggested an involvement of the mature part of pOmpA in binding of TF. Therefore nascent pOmpA chains, 105, 126, and 192 amino acids long, were analyzed, each one carrying Tmd-Phe at a position downstream of the signal sequence as indicated in Fig. 1. Upon UV irradiation, cross-linking products appeared (Fig. 4) whose pattern varied with the position of the cross-linker (Fig. 4, e.g., compare lanes 2, 5, 8,



11, and 14). Most of them were coimmunoprecipitated with anti-TF antibodies (Fig. 4, lanes 3, 6, 9, and 12). One of these TF adducts (asterisk) exhibited almost the same electrophoretic mobility for all Tmd-Phe constructs of a given chain length (Fig. 4, compare lanes 2, 5, 8, and 11). Its size corresponds to the sum of the respective pOmpA nascent chain (e.g., 13 kD for pOmpA-126) and TF, which runs aberrantly on SDS-PAGE at 58 kD. The origin of the more slowly moving TF cross-reactive species remained elusive. Similar patterns of multiple TF cross-links, however, are also obtained when chemical cross-linkers are used (Beck et al., 2000; Kramer et al., 2002; Deuerling et al., 2003). The electrophoretic mobility of cross-linking products that were not recognized by anti-TF antiserum (marked ●) varied with the position of Tmd-Phe within a given chain (Fig. 4, lanes 2, 5, 8, 11, and 14) rather than with its length (Fig. 4, compare lanes 14 and 17). These bands therefore have probably been generated by intramolecular cross-links of the cosynthesized full-size pOmpA.

When Tmd-Phe was in position 101, which in nascent pOmpA-126 was predicted to be inside the ribosomal exit tunnel, no TF cross-links were obtained (Fig. 4, lanes 14 and 15). The same position of the cross-linker, however, yielded a TF adduct, when it had emerged from the ribosome, as in pOmpA-192 (Fig. 4, lanes 17 and 18, asterisk). This confirms that the predominant part of the UV-generated adducts were formed with intact RNCs. Accordingly, dissociation of RNCs by puromycin usually led to a complete disappearance of TF cross-links or at least to a drastic

reduction in intensity, as illustrated for pOmpA-105 (Fig. 4, lanes 19–24, asterisk).

All Tmd-Phe positions shown for pOmpA-126 in Fig. 4 plus additional ones at residues 124 and 145 also resulted in cross-links to TF when RNCs of the longer pOmpA-192 chain were analyzed (unpublished data). These results are consistent with the frequent occurrence of TF-binding sites previously identified on a variety of cytosolic and secretory *E. coli* proteins (Patzelt et al., 2001). If the exposure of such an authentic TF-binding site within the mature sequence of pOmpA was a prerequisite for the interaction of TF with the signal sequence, it would explain why short pOmpA RNCs were not found cross-linked to TF.

### TF prevents cotranslational targeting of pOmpA RNCs

As demonstrated above, in reaching cross-linkable vicinity to TF, the nascent signal sequence of pOmpA lost its accessibility to Ffh and SecA. This finding raised the possibility that TF after binding to the signal sequence of a secretory protein might control its accessibility to proteins specialized in signal sequence recognition, such as Ffh and SecA. We therefore asked for the consequences of a deficiency of TF on pOmpA RNCs. In *E. coli*, one of the hallmarks of RNCs containing SRP substrates is their cotranslational targeting to INVs, which can be visualized by flotation centrifugation (Neumann-Haefelin et al., 2000). In contrast, RNCs of a secretory protein such as pOmpA, which are not recognized by SRP, virtually do not float with INVs. Fig. 5 A (bottom) illustrates that the vast majority of pOmpA-192 RNCs is re-

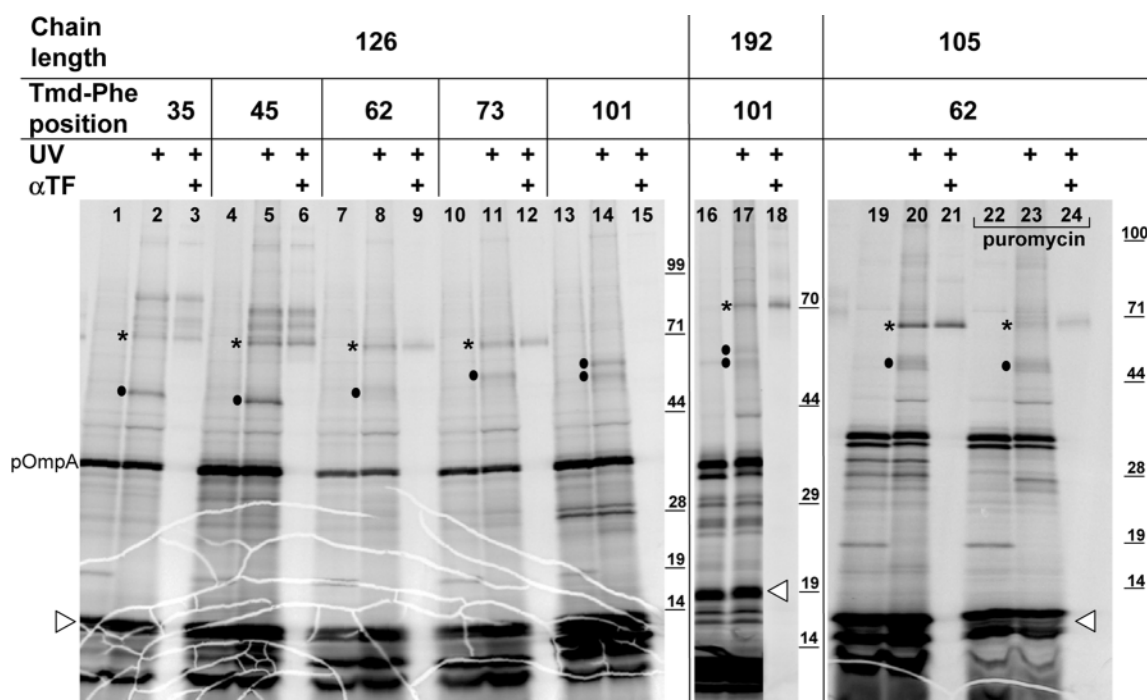


Figure 4. **TF interacts with the mature part of nascent pOmpA chains at multiple sites.** Nascent, ribosome-associated chains of pOmpA of 126, 192, and 105 amino acids length were synthesized *in vitro*, and their position on SDS-PAGE is indicated (white arrowheads). UV irradiation (UV) resulted in the appearance of several radiolabeled bands, most of which could be coimmunoprecipitated with anti-TF antibodies (αTF). The asterisk marks the adducts that exhibit apparent molecular masses corresponding to the arithmetic sum between those of TF and each nascent pOmpA chain. The electrophoretic mobility of adducts in the 50-kD range (marked ●) changed with the position of Tmd-Phe within the nascent chain, suggesting that they might be derived from intramolecular cross-links of full-size pOmpA.

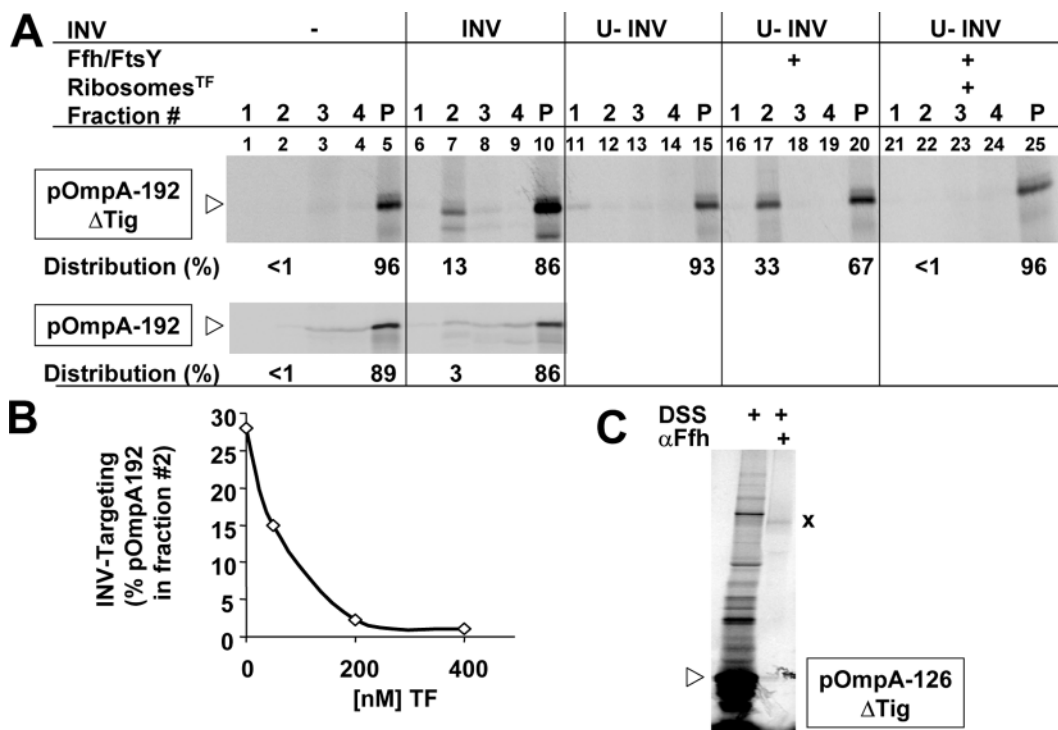
covered from the pellet fraction (P) of the sucrose gradient (Fig. 5 A, lanes 5 and 10), and the addition of INVs does not lead to any significant increase in RNCs floating with the membrane fraction (Fig. 5 A, lanes 2 and 7).

In contrast, when pOmpA-192 RNCs were produced by an in vitro system whose components had been prepared from a TF knockout mutant (Fig. 5 A, top), a minor yet significant fraction of RNCs floated with INVs (pOmpA-192  $\Delta$ Tig, Fig. 5 A, compare lanes 2 and 7). The degree of flotation was clearly less pronounced than previously observed for an authentic SRP substrate (Neumann-Haefelin et al., 2000) but, importantly, was dependent on Ffh/FtsY. After urea treatment of the vesicles to remove membrane-bound Ffh and FtsY, flotation of RNCs was totally abolished (Fig. 5 A, compare lanes 6–10 with 11–15) but was fully restored solely by readdition of purified Ffh and FtsY (Fig. 5 A, lanes 16–20). This Ffh/FtsY-dependent targeting of pOmpA RNCs to INVs was possible only due to the absence of TF because it was reversed in a dose-dependent manner by providing purified TF during synthesis (Fig. 5 B). Saturation of the system with TF was not necessary to see this effect. A complete reversal of targeting was observed already with an amount of TF adhering to  $\Delta$ Tig ribosomes after reconstitution with TF (Fig. 5 A, lanes 21–25). Consistent with the

Ffh-mediated membrane targeting, an interaction of Ffh with pOmpA RNCs when synthesized in the absence of TF was also revealed by cross-linking, as illustrated in Fig. 5 C for pOmpA-126 RNCs, whose signal sequence, like that of pOmpA-192, normally does not bind Ffh (Fig. 3).

### Upon exit, the signal sequence of pOmpA remains in immediate vicinity to the ribosome via ribosomal protein L23

The moderate extent by which pOmpA RNCs interacted with Ffh in the absence of TF (Fig. 5 C) suggested that the signal sequence might come into contact with additional binding partners when relieved from its association with TF. When pOmpA-126 RNCs were synthesized in the absence of TF and without exogenously added Ffh and SecA, a prominent 24-kD cross-link was formed by disuccinimidyl suberate (DSS) (Fig. 6 A, star). This adduct was coimmunoprecipitated with antibodies raised against ribosomal protein L23 of *E. coli* (Fig. 6 A, lane 5), whereas antisera directed against the ribosomal proteins L19, L22, and L24, which are all exposed on the surface of the large ribosomal subunit (Nissen et al., 2000), did not visibly precipitate any material (Fig. 6 A, lanes 3, 4, and 6). A very faint reaction was observed with anti-L29 antibodies (Fig. 6 A,



**Figure 5. TF interferes with a cotranslational targeting of a bacterial secretory protein.** Nascent chains of pOmpA, 192 and 126 amino acids in length, were synthesized in vitro as before (pOmpA-192) or by a cell-free system whose components had been prepared from a TF-null mutant (pOmpA-192,  $\Delta$ Tig). (A) After synthesis in the presence or absence of the components indicated at the top of the panel, pOmpA-192 chains were subjected to flotation gradient centrifugation. The gradient was fractionated into four equal fractions from the top (fraction nos. 1–4), and proteins were precipitated using TCA and analyzed by SDS-PAGE and phosphorimaging. The pellet fraction (P) was directly dissolved in SDS-PAGE loading buffer. The radioactivity of the pOmpA-192 bands of all fractions was quantitated using Imagequant software. Indicated are the numbers for the relevant fraction 2 (membrane fraction) and P (non-membrane-associated material). Ribosomes<sup>TF</sup>, ribosomes prepared from the  $\Delta$ Tig strain and reconstituted with purified TF. (B) The amount of pOmpA-192 synthesized in the  $\Delta$ Tig system and recovered from fraction 2 after flotation centrifugation of an INV-containing sample (compare with A) is plotted against the concentration of purified TF present during synthesis. (C) pOmpA-126 was synthesized in the  $\Delta$ Tig system in the presence of 160 nM Ffh. After synthesis, samples were treated with the cross-linker DSS.  $\alpha$ Ffh, anti-Ffh antibodies.

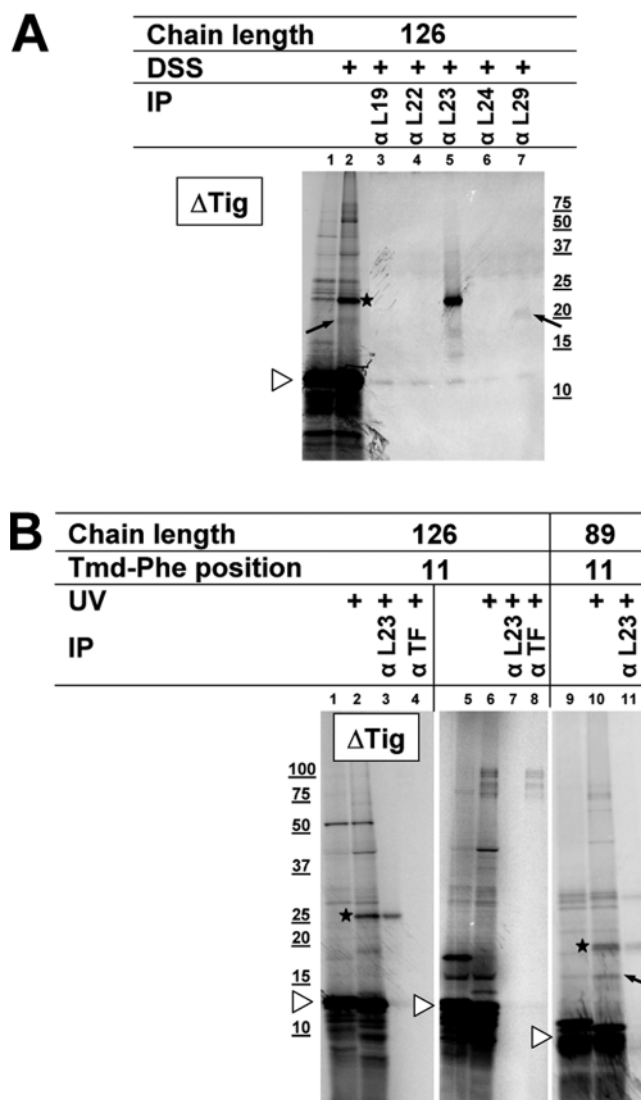
lane 7) precipitating a minor cross-link of 20 kD (Fig. 6 A, lane 2, arrow).

To find out whether this interaction between pOmpA-126 and L23 involved the signal sequence, cross-linking was performed with pOmpA-126 RNCs carrying Tmd-Phe in the signal sequence at residue 11 (Fig. 6 B). As with the chemical cross-linker DSS, L23 was the dominant reaction partner of the signal sequence of pOmpA-126 (Fig. 6 B, lanes 1–4). If synthesis of these RNCs occurred in the presence of TF, L23 adducts were completely eliminated (Fig. 6 B, lanes 6 and 7) in favor of those to TF (Fig. 6 B, lanes 6 and 8). These findings suggest that the signal sequence of a nascent chain of pOmpA remains attached to the ribosome even after the exit of >90 amino acids either via L23 or, normally, via TF. The signal sequence was, however, not the only contact site of pOmpA-126 to L23. Similar L23 cross-links were obtained also for Tmd-Phe positions downstream of the signal sequence cleavage site (unpublished data), which, in the presence of TF, had given rise to cross-links to TF (compare with Fig. 4).

As shown above, the signal sequence of pOmpA-89 RNCs had displayed highest versatility in its molecular contacts, still being accessible to SecA but already found in the vicinity of TF. If L23 were a constitutive part of the molecular environment of a nascent signal sequence in *E. coli*, it was expected to become apparent by a cross-link with the signal sequence of pOmpA-89 despite the presence of TF. This, in fact, is demonstrated in Fig. 6 B (lanes 10 and 11) by coimmunoprecipitation. The L23 adduct of pOmpA-89 had become visible after UV irradiation also in previous experiments (compare with Fig. 3, stars). The additional 15-kD cross-link of pOmpA-89 that usually appeared together with the L23 adduct (arrows in Fig. 3, lanes 12 and 17, and Fig. 6 B, lane 10) could stem from ribosomal protein L29, although a clear identification has not yet been possible. Thus, shortly after exiting the ribosomal tunnel, the signal sequence of pOmpA seems to be apposed to L23, where it remains accessible to proteins such as Ffh and SecA; these contacts are all blocked upon further growth to a total chain length of 70–80 residues, from which point on, the signal sequence remains shielded by TF.

### TF is not involved in controlling access of SecB to pOmpA

A common denominator of the combined results presented thus far is that TF is involved in determining the accessibility of nascent pOmpA, in particular that of its signal sequence, to Ffh and SecA. Hence, we asked if this would also apply to the export-specific chaperone SecB. A controversial issue has remained if SecB was able to associate with the signal sequences of *E. coli* secretory proteins (for review see Muller et al., 2001). We therefore analyzed pOmpA-126 RNCs carrying Tmd-Phe in the signal sequence by UV-induced cross-linking after synthesis in the presence of exogenously added SecB (Fig. 7). While TF (asterisk) yielded the only prominent cross-link of intact RNCs (Fig. 7, top, lane 2), a clear SecB adduct was obtained after releasing pOmpA-126 from the ribosome by puromycin (Fig. 7, lanes 5 and 6, triangle). Virtually the same results were obtained when the cross-linker was placed at position 35 within the mature part

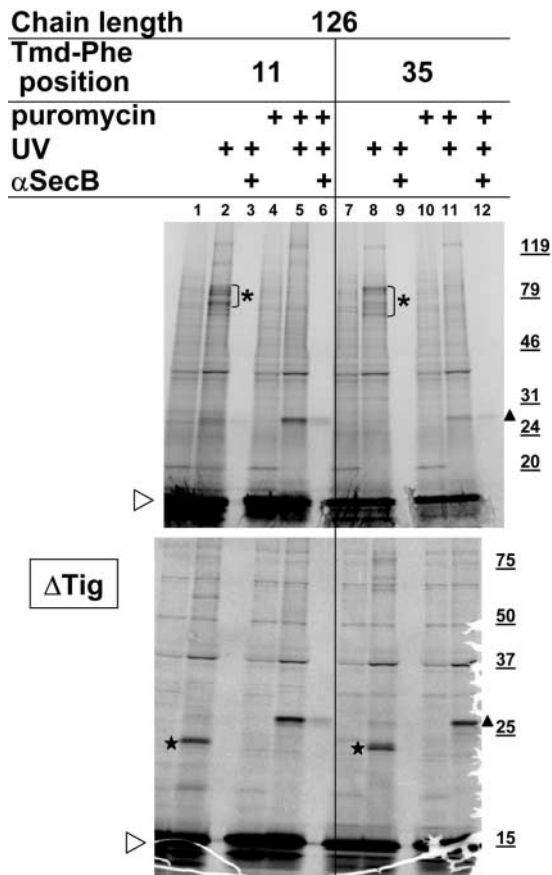


**Figure 6. Transfer of nascent pOmpA from ribosomal protein L23 onto TF.** (A) pOmpA-126 was synthesized in the  $\Delta$ Tig system. After cross-linking with DSS, aliquots were immunoprecipitated with antisera raised against the indicated proteins of the large *E. coli* ribosomal subunit. Adducts to L23 (star) and L29 (upwards pointing arrow) are highlighted. (B) Nascent pOmpA chains of 126 and 89 amino acids in length with the photoprobe incorporated within the signal sequence at position Val-11 were synthesized in the absence or presence of TF as indicated. Cross-links obtained by UV irradiation were identified using antibodies against L23 and TF.

of pOmpA (lanes 7–12). Clearly SecB can associate also with the signal sequence of pOmpA but only after its release from the ribosome.

To find out if TF prevented access of SecB to pOmpA while it was still ribosome associated, the same cross-linking experiments were repeated after synthesis in the absence of TF (Fig. 7, bottom). Instead of TF, both Tmd-Phe mutants were now cross-linked to L23 (star), and SecB adducts again were formed only after addition of puromycin (Fig. 7, lanes 5, 6, and 11, triangle). In obvious contrast to SecA, Ffh, and L23, the binding behavior of SecB to pOmpA is not influenced by TF because it occurs only after dissociation from the ribosome.





**Figure 7. Binding of SecB to newly synthesized pOmpA including its signal sequence is not controlled by TF and occurs after release from the ribosome.** Chains of pOmpA, 126 amino acids in length with the photoprobe incorporated within the signal sequence at Val-11 or beyond at Leu-35, were synthesized in the presence or absence of TF as indicated. In addition, SecA (270 nM of dimer) and SecB (468 nM of tetramer) were also added. UV-induced cross-linking was performed on nascent chains, resulting in adducts to TF (asterisk) or L23 (star). Both cross-links disappeared upon dissociation of RNCs by puromycin in favor of those to SecB, as identified by immunoprecipitation (triangles).

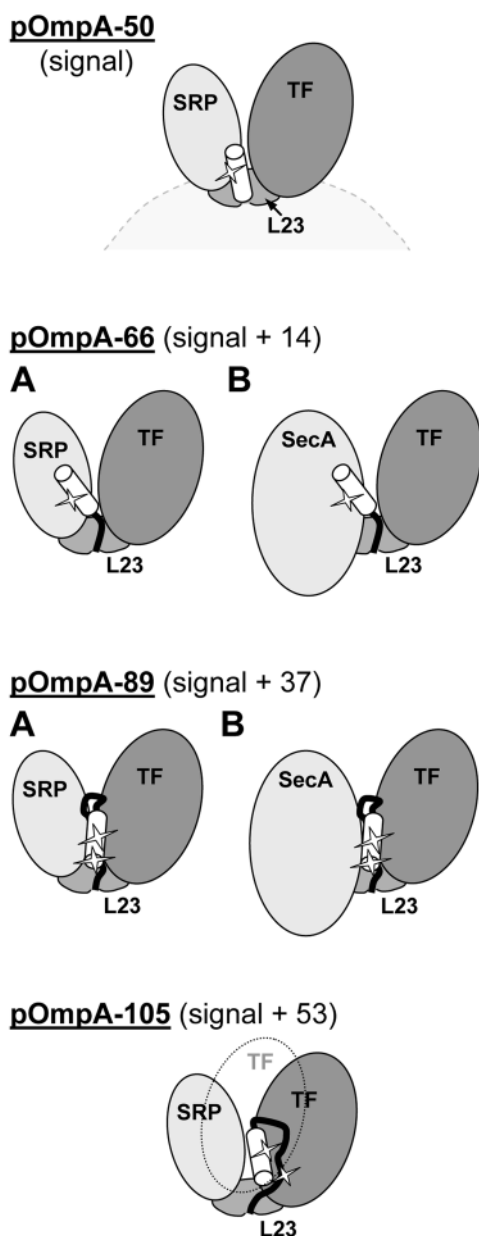
## Discussion

Using site-specific incorporation of a photoactivatable cross-linker, we describe the molecular landscape of a signal sequence after extrusion from the ribosomal exit channel of *E. coli*. The results obtained are summarized in Fig. 8. The scheme depicts the ribosomal protein L23 both as the ribosomal exit site of the nascent chain and the docking site of TF and Ffh (Kramer et al., 2002; Gu et al., 2003; Ullers et al., 2003). With hardly the entire signal sequence exposed (pOmpA-50), the SRP protein Ffh was the only detected cross-linking partner. The same situation was encountered when the signal sequence plus 14 amino acids of the mature part of OmpA had exited the ribosome (pOmpA-66, A). In addition, this nascent chain was also found in contact with SecA (pOmpA-66, B). Although the Ffh cross-link of pOmpA-66 appeared to be weaker than that of pOmpA-50 (Fig. 3), SecA and Ffh are considered as equal binding partners of pOmpA-66 and are therefore represented by two alternative situations in Fig. 8. This is also because by varying

the amounts of Ffh and SecA added to the in vitro reactions, a clear competition between the two proteins for the signal sequence of OmpA-66 could be demonstrated (unpublished data). The signal sequence of OmpA remained accessible to Ffh and SecA up to a distance of 37 amino acids from the predicted exit site (pOmpA-89). At the same time, this nascent chain was the shortest analyzed, whose signal sequence showed contacts with TF and the ribosomal protein L23. Upon further growth (represented by pOmpA-105 in Fig. 8), the only detectable interactions of the signal sequence remained those to TF. If TF was removed from the system, the signal sequence of longer nascent chains of pOmpA (e.g., pOmpA-126) was found attached to the ribosome via L23 and also free to interact with Ffh.

The most notable finding seems to be the change in the molecular environment of the signal sequence of pOmpA as the nascent chain becomes longer. Unexpectedly, when just emerged from the ribosome and up to a distance of  $\sim 37$  additional amino acids from the exit site, the signal sequence was found in contact with SecA and Ffh. This situation is not likely to reflect a physiologically relevant stage, as the bacterial SRP in general is not involved in the export of secretory proteins such as OmpA. Furthermore, the bulk of accumulated evidence suggests that SecA interacts with its substrate pOmpA only posttranslationally. It is therefore possible that the binding of Ffh and SecA to the signal sequence of short pOmpA became visible only because of the use of elongation-arrested chains. On the other hand, our data suggest that in the absence of TF, even long nascent chains of pOmpA are amenable to recognition by SRP followed by a cotranslational targeting. Even if this does not seem to be an efficient process, probably due to the low affinity of Ffh for classical signal sequences (Valent et al., 1997; Kim et al., 2001) and to a potential sequestration of the signal sequence by L23, cotranslational targeting of secretory proteins to the SecY translocon bears the risk of interfering with the integration of essential inner membrane proteins. In this sense, TF shielding the signal sequence of secretory proteins against Ffh might play an important function in blocking their entry into the SRP pathway. Similarly, a retarded export of secretory proteins due to titration by TF has recently been described for TF-overproducing *E. coli* cells (Lee and Bernstein, 2002). Hence, TF in addition to, or even more likely in combination with, its well documented chaperone activities, might serve regulatory functions controlling entry of bacterial secretory proteins into their posttranslational export pathway.

Why is the contact between TF and the signal sequence of pOmpA not established from the time of exposure on the ribosome? This is unlikely to be due to a docking site of TF that is further away from the exiting polypeptide chain than that of Ffh. Both proteins dock at L23 (Kramer et al., 2002; Gu et al., 2003; Ullers et al., 2003). Moreover, as illustrated in Fig. 4, pOmpA-105 with Tmd-Phe at position 62 gave rise to TF cross-links, although residue 62 is predicted to be only 13 amino acids distal from the exit site (compare with Fig. 1). On the contrary, in pOmpA-66 carrying Tmd-Phe at position 11, the cross-linker is located 25 residues outside of the ribosome and does not react with TF. This result therefore suggests that it is the signal se-



**Figure 8. At the ribosome, the interaction partners of a signal sequence change with the length of the nascent chains.** Depicted are molecular contacts of the signal sequence of pOmpA at the ribosomal exit site. The large ribosomal subunit is outlined in the top panel. Protein L23 located at the orifice of the ribosomal exit tunnel is indicated. The exiting pOmpA chain is illustrated by a cylinder (signal sequence) followed by a solid line, which represents the part of mature OmpA predicted to have emerged from the ribosome (its size is given in number of amino acids in parentheses). The structure labeled SRP is meant to represent Ffh, the 4.5S RNA is not shown. SecA and TF are indicated. Cross-links are illustrated by stars; for pOmpA-89, cross-links at residues 3 and 11 of the signal sequence are simultaneously depicted. Whereas Ffh and TF have been shown to use L23 as docking site, the presumed ribosomal-binding site of SecA has not been identified. Different from the model showing Ffh and TF simultaneously bound to L23, recent data suggest competitive binding between Ffh and TF to L23 (Ullers et al., 2003). This has also been shown for Ffh and SecA with respect to pOmpA-66 (unpublished data), for which reason binding of the signal sequence to either protein was drawn as two alternative situations (labeled A and B). It should be noted that only single adducts were obtained. Therefore in cases in which the model

sequence, per se, that cannot associate with TF when nascent pOmpA chains are short, and that binding probably can occur only after TF has first associated with the nascent chain at one of the verified recognition sites downstream of the signal sequence. The mechanism by which such a secondary interaction between TF and the signal sequence could be accomplished is unclear. Two speculative scenarios are conceivable. Perhaps TF itself has more than one binding site for nascent chains such that an initially cryptic one for the signal sequence might be exposed only after TF has associated with the substrate via its constitutive binding site. This situation would require some conformational change within the chaperone itself. Alternatively, two TF molecules might bind sequentially first to the mature part of pOmpA and then to the signal sequence, with the previous interaction somehow being required to expose or release the signal sequence as depicted in Fig. 8. Such a subsequent exposure of the signal sequence could be brought about by a TF-assisted conformational change within the nascent chain. Consistent with the idea of two TF monomers binding to one substrate would be the two- to three-fold molar excess of TF over ribosomes (Patzelt et al., 2002) and the formation of TF dimers both in solution (Patzelt et al., 2002) and at the ribosome (Blaha et al., 2003).

Alternatively, a sequential association of the signal sequence of pOmpA first with Ffh and then with TF, as demonstrated here, might be a reflection of a mutually exclusive interaction of both proteins with L23, as recently suggested (Ullers et al., 2003). Several experimental approaches to measure binding of TF to ribosomes suggest that the majority of nontranslating *E. coli* 50S and 70S ribosomes are in complex with TF (Patzelt et al., 2002; Blaha et al., 2003; Maier et al., 2003). As we found the emerging signal sequence of pOmpA accessible to Ffh at a stage of synthesis at which TF has not yet tightly associated with its substrate, this situation might have arisen from a substrate-regulated exchange of TF by Ffh at L23. In theory, this could be achieved by the nascent signal sequence when still traversing the ribosomal peptide tunnel, as evidence has recently accumulated for the ability of the ribosome to sense the nature of a nascent peptide when still embedded in the exit tunnel (Liao et al., 1997; Gong and Yanofsky, 2002; Nakatogawa and Ito, 2002).

Surprisingly, also SecA was found cross-linked to the signal sequence of short nascent chains before they interacted with TF. As mentioned above, this finding is difficult to reconcile with the established function of SecA in the post-translational targeting of secretory proteins to the SecY translocon of *E. coli*. For this to occur, a cotranslational interaction with the signal sequence is hardly required, al-

suggests interaction of the signal sequence with two binding partners (e.g., pOmpA-89), these should be taken as representing two separate populations of adducts. The identified cross-linking partners of the signal sequence are Ffh for pOmpA-50; Ffh and SecA for pOmpA-66; Ffh, SecA, TF, and L23 for pOmpA-89; and TF for pOmpA-105. Upon removal of TF, pOmpA-126 (not depicted) was found cross-linked to L23 and Ffh. The bottom scheme invokes the hypothetical possibility of two TF molecules associating with two different sites of a single nascent chain.



though reports on a cotranslational substrate interaction by SecA exist (Chun and Randall, 1994; Behrmann et al., 1998). A certain relevance of the binding of SecA to pOmpA-RNCs might be deduced from the fact that under our experimental conditions, a similar cotranslational association with pOmpA was not observed for SecB. In accordance with this, only SecA has thus far been detected in association with nontranslating ribosomes (Liebke, 1987; Hoffschulte et al., 1994), with its docking site on the ribosome, however, being totally unknown.

Finally, as recently found for the signal anchor sequence of a membrane protein (Ullers et al., 2003), the ribosomal protein L23 turned out to be yet another protein competing for an interaction with a nascent signal sequence. While this could be shown to occur for an intermediary size of RNCs (pOmpA-89), even in longer nascent chains, in which the signal sequence was found associated with TF, it remained closely spaced to L23, as indicated by the cross-links to L23 in the absence of TF. While this alternative cross-linking to TF and L23 on the one hand reflects the immediate vicinity of both proteins, with TF docking at L23, it simultaneously argues against a free mobility of pOmpA's NH<sub>2</sub> terminus after leaving the ribosomal exit tunnel. The collective data rather suggest that the signal sequence remains fixed to the surface of the ribosome via at least L23 and TF.

## Materials and methods

### Construction of stop codon mutants of *ompA*

Plasmid p717OmpA (Beck et al., 2000) served as template for the introduction of TAG stop codons. This was achieved for positions Val-11 and Phe-45 by a two-step PCR method involving three primers each (Dilsiz and Crabbe, 1994). These mutant plasmids were transformed into strain DH5 $\alpha$  (*supE44*  $\Delta$ *lacU169* [ $\Phi$ 80*lacZ* $\Delta$ M15] *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*). For the positions Lys-3, Leu-35, Phe-62, Phe-73, Leu-101, Trp-124, and Phe-145, the whole plasmid was amplified using *Pfu* Turbo DNA Polymerase (Stratagene) and two complementary mutant primers each containing the amber codon. In these cases, plasmids were transformed into XL1 Blue (*recA1* *endA1* *gyrA96* *thi-1* *hsdR17* *supE44* *relA1* *lac* [*F'* *proAB* *lacI'* $\Delta$ M15 Tn10 (*tet*)]). All constructions were verified by nucleotide sequence analysis. Note that due to the insertion of a Val after the NH<sub>2</sub>-terminal Met in the signal sequence of pOmpA encoded by p717OmpA, the numbering of individual amino acids differs by one residue from the authentic pOmpA sequence (Beck and Bremer, 1980).

### In vitro synthesis

The various pOmpA derivatives were synthesized by the reconstituted transcription/translation system previously described (Koch et al., 1999; Beck et al., 2000). Salt-washed ribosomes and the fraction of cytosolic translation factors were prepared from *E. coli* strains MRE 600 (Cammack and Wade, 1965), SL119 (Lesley et al., 1991), and C600  $\Delta$ *tig* (Deuerling et al., 1999). For use in the  $\Delta$ Tig system, a TF-free preparation of T7 RNA polymerase was obtained from Promega. Readthrough of stop codons by the use of suppressor tRNA charged with Tmd-Phe has been detailed elsewhere (Beck et al., 2000). When appropriate, INVs were added 10–15 min after starting in vitro synthesis reactions.

Synthesis of pOmpA RNCs was achieved by addition of the following oligodeoxynucleotides: pOmpA-50, 5'-CCAGTTGGTTTTTCATGGGTC-3' (2.5  $\mu$ g/25  $\mu$ l reaction); pOmpA-66, 5'-AAGCCAACATACGGGTTAAC-3' (3  $\mu$ g); pOmpA-89, 5'-AGCTTTGTATGCACCGTTTTTC-3' (2.5  $\mu$ g); pOmpA-105, 5'-GGTCGTCAGTGATGGGTAACC-3' (3  $\mu$ g); pOmpA-126, 5'-TAAACGTTGGATTTAGTGTGTC-3' (3  $\mu$ g); and pOmpA-192, 5'-GGAGTCCTCGCCCTGACC-3' (3  $\mu$ g). RNaseH (Biozym) and a 10Sa RNA antisense oligodeoxynucleotide were added routinely (Beck et al., 2000). To release the ribosome from RNCs, puromycin was added to the reaction mixture at a final concentration of 0.8 mM with a further incubation for 10 min at 37°C.

### Cross-linking

For chemical cross-linking using DSS (Pierce Chemical Co.), triethanolamine acetate was replaced by Hepes-NaOH during the previous translation reaction (Beck et al., 2000). UV irradiation ( $\lambda = 365$  nm) of Tmd-Phe-containing samples was done for 10 min, placing the UV lamp directly over closed reaction tubes in an ice bucket.

### Purification of *E. coli* Ffh

The chromosomal *ffh* gene of *E. coli* strain MZ9 ( $\Delta$ *lac*,  $\Delta$ *phoA*, *phoB*<sup>+</sup>, *phoR*<sup>+</sup>) was amplified by PCR using the P48BamHI (5'-ACAAGCGGAT-CCCCGATTAGCGACACAGGGA-3') and P48NdeI (5'-CGAGAGCATAT-GTTTGATAATTTAACCGAT-3') primers. The PCR product was digested by BamHI and NdeI and cloned under T7 promoter control downstream of a 10His tag in pET19b (Novagen) or pET10N (Truscott et al., 2001), resulting in plasmids pET19b-P48 and pET10N-P48.

For the purification of 10His-Ffh, *E. coli* strain BL21 (DE3) pLysS (Studier et al., 1990) containing pET19b-P48 or pET10N-P48 was grown at 37°C in LB medium containing ampicillin and chloramphenicol and 0.4% glucose. 10 ml of an overnight culture was used to inoculate 1 liter of fresh glucose-free medium. When cells had grown at 30°C to an OD<sub>580</sub> (optical density at 580 nm) of 0.45, 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside was added and growth continued to an OD<sub>580</sub> of 1.35. Cells were then harvested and resuspended in buffer A (50 mM potassium phosphate, pH 7.2, 300 mM NaCl) containing the protease inhibitors aprotinin (2  $\mu$ g/ml), leupeptin (0.5  $\mu$ g/ml), E64 (10  $\mu$ g/ml), and PMSF (1 mM). After breaking cells by three passages through a French pressure cell at 8,000 psi, an S-30 was prepared, supplemented again with protease inhibitors, and applied in two batches to a 5-ml HiTrap chelating column (Amersham Biosciences) loaded with NiCl<sub>2</sub> and equilibrated with buffer A. The gel matrix was washed first with buffer A and subsequently with buffer A containing 150 mM imidazol until no more material absorbing at 280 nm was eluted. 10His-Ffh was eluted with buffer A containing 300 mM imidazol, and 1.5 ml eluate was immediately applied to a 5-ml HiTrap desalting column (Amersham Biosciences), previously equilibrated with 50 mM triethanolamine acetate, pH 7.5, 50 mM KCH<sub>3</sub>COO, 5 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>. 10His-Ffh was eluted with the same buffer adjusted to 50% glycerol and stored at -20°C.

### Miscellaneous

Published protocols were used for the preparation of native and urea-extracted INVs and the protease protection assay (Koch et al., 1999), flotation centrifugation (Neumann-Haefelin et al., 2000), immunoprecipitation on fourfold scaled-up reactions using polyclonal antibodies (Schafer et al., 1999), and purification of SecA (Helde et al., 1997), FtsY (Koch et al., 1999), and T7 RNA polymerase (Beck et al., 2000). Antibodies were raised in rabbits against Ffh (Koch et al., 1999) and SecA. Antibodies against *E. coli* TF and purified TF were a gift from E. Deuerling (University of Heidelberg, Heidelberg, Germany). To reconstitute  $\Delta$ TF ribosomes with TF, 0.5 nmol of  $\Delta$ TF ribosomes was incubated with 1.2 nmol purified TF for 30 min at 30°C. After incubation, ribosomes were centrifuged for 1 h at 90,000 rpm in a Beckman Coulter TLA-100 ultracentrifuge, and the ribosome pellet was resuspended in 50 mM triethanolamine acetate, pH 7.5, 50 mM KCH<sub>3</sub>COO, 5 mM Mg(CH<sub>3</sub>COO)<sub>2</sub> to give a final concentration of 10  $\mu$ M. As a control,  $\Delta$ TF ribosomes were incubated with buffer (20 mM triethanolamine acetate, pH 7.5, 100 mM NaCl, 10 mM EDTA) instead of TF and treated as described above. TF binding to  $\Delta$ TF ribosomes was verified by Western blotting with  $\alpha$ -TF antibodies.

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