CHAPTER 2

IN VIVO CROSS-LINKING OF THE SEC A AND SEC Y SUBUNITS OF THE ESCHERICHIA COLI PREPROTEIN TRANSLOCASE

Precursor protein translocation across the Escherichia coli inner membrane is mediated by the translocase which is composed of a heterotrimeric integral membrane protein complex with SecY, SecE, and SecG as subunits, and peripherally bound SecA. Cross-linking experiments were conducted to study which proteins are associated with SecA in vivo. Formaldehyde treatment of intact cells results in the specific cross-linking of SecA to SecY. Concurrently with the increased membrane association of SecA, an elevated amount of cross-linked product was obtained in cells harbouring overproduced SecYEG complex. Cross-linked SecA co-purified with hexa-histidine tagged SecY, and not with SecE. The data indicate that SecA and SecY co-exist as a stable complex in the cytoplasmic membrane in vivo.

INTRODUCTION

In Escherichia coli and other bacteria, secretory proteins are translocated across the cytoplasmic membrane as precursors (or preproteins) by a multimeric integral membrane protein complex termed translocase (reviewed in: Wickner et al., 1991; Driessen, 1994). Core components of the translocase are the SecA, SecY, SecE, and SecG polypeptides (Brundage et al., 1990; Hanada et al., 1994; reviewed in: Ito, 1992; Oliver, 1993). SecA is a large homodimeric protein (Driessen, 1993), with preprotein-stimulated ATPase activity (Lill et al., 1989). It is found both free in the cytosol and peripherally associated with the inner membrane (Cunningham et al., 1989; Cabelli et al., 1988, 1991). SecY, SecE, and SecG are integral membrane proteins that can be purified in detergent solution as a heterotrimeric complex (Brundage et al., 1990, 1992). This complex, or a mixture of the individual purified subunits, suffices for reconstitution of the SecA- and energy-(ATP and proton-motive force) dependent preprotein translocation into proteoliposomes (Brundage et al., 1990, 1992; Driessen, 1992; Nishiyama et al., 1993; Hanada et al., 1994).

A variety of biochemical and genetic evidence indicates that the subunits of the translocase are interacting polypeptides. SecY, SecE, and SecG can be isolated and co-immunoprecipitated as a stable complex (Brundage et al., 1990, 1992). SecE stabilizes SecY (Matsuyama et al., 1990), and protects it against proteolysis by FtsH, a membrane-bound ATP-dependent protease (Kihara et al., 1995). Further studies have defined the interacting regions of SecY and SecE (Schatz et al., 1991; Baba et al., 1994; Flower et al., 1995). In wild-type cells, newly synthesized SecY immediately associates with SecE to form a complex that does not dissociate over time or during translocation, whereas SecG appears to be a less stable subunit of the translocase (Joly et al., 1994). In contrast, from genetic data it can be argued that SecE and SecY are dissociable proteins (Bieker and Silhavy, 1990, 1992).

SecA is a dissociable subunit of the translocase, and appears to cycle in an ATP-dependent fashion between cytosol and membrane (Breukink et al., 1992; Van der Wolk et al., 1993; Economou and Wickner, 1994), although it has been argued that this process may not be necessary for the translocation reaction per se (Chen et al., 1996). Membrane binding of SecA occurs with high affinity at the SecYEG complex (Hartl et al., 1990; Douville et al., 1995) and with low affinity to phospholipids (Lill et al., 1990; Hendrick and Wickner, 1991). Using ligand affinity blotting, a direct interaction between SecA and SecY has been demonstrated (Snyders et al., 1997). This interaction appears to involve the amino-terminal halve of SecY and the carboxyl-terminal third of SecA. SecY and SecA can both be photoaffinity cross-linked to the preprotein proOmpA during translocation, suggesting that these two Sec-proteins are in the nearest vicinity of a translocating
polypeptide chain (Joly and Wickner, 1993). The SecYEG-bound SecA is accessible to trypsin
digestion from the periplasmic surface (Kim et al., 1994; Van der Does et al., 1996; Chen et al.,
1996), suggesting that it may traverse the membrane. In the presence of preprotein and ATP, a 30-
kDa carboxyl-terminal domain of SecA has been shown to adopt a membrane-protected, proteinase
inaccessible conformation (Economou and Wickner, 1994; Economou et al., 1995; Price et al.,
1996). This has led to the suggestion that the stepwise translocation of preproteins across the
membrane (Schiebel et al., 1990) is coupled to ATP-dependent cycles of the membrane insertion
and de-insertion of the carboxyl-terminal SecA domain (Economou and Wickner, 1994). Strikingly,
this process seems to be coupled to a reversible membrane topology inversion of the
SecG subunit of the translocase (Nishiyama et al., 1996).

Most of the data on the interaction between Sec-proteins has been gathered from in vitro
studies using isolated membrane vesicles and cytosolic components. We have now employed an in
vitro cross-linking approach to identify SecA-interacting proteins in intact cells. Cross-linking
results in the formation of a 150-kDa protein complex, that in addition to SecA harbours the SecY
protein. These data support recent in vitro suggestions (Van der Does et al., 1996; Chen et al.,
1996) that SecA is an intrinsic subunit of the translocase.

RESULTS

Fig. 1. In vivo formaldehyde cross-linking of SecA to SecY. Anti-SecA (A), -SecY (B), and -SecB (C)
immunoblots of E. coli D10 cells incubated without (lanes 1 and 2) and with (lanes 3 and 4) 0.1 % (w/v)
formaldehyde (H₂CO). Prior to SDS-PAGE, part of the samples was heated at 100 °C (lanes 2 and 4)
to reverse the cross-linking. The 150-kDa complex identified by the cross-linking is indicated by an
open arrow, while the positions of SecA, SecY and SecB proteins are indicated by closed arrows.
Cross-linked SecB di- and trimers are indicated with open arrows. For the detection of SecA and SecY
cross-links, 7.5 % SDS-PAGE was used with an optimal separation of proteins with a Mᵣ between 50
and 300 kDa. For the detection of SecB, 12 % SDS-PAGE was used.
In vivo cross-linking of SecA-associated proteins. Wild-type D10 cells were cross-linked with formaldehyde, and analysed by SDS-PAGE and immunoblotting using an oligoclonal antibody (oAb, mixture of monoclonal antibodies) directed against SecA (Den Blaauwen et al., 1997). In the presence of 0.1 % (w/v) of formaldehyde, a specific cross-linked protein complex was obtained with a relative molecular mass (Mr) of 150-kDa (Fig. 1A, compare lane 1 and 3). In addition, a cross-linked product was observed (lane 3) with a Mr of about 180 kDa (lane 1). The 180 kDa represents dimeric SecA protein as was confirmed by cross-linking of purified SecA (not shown), and was also present in unboiled samples not incubated with formaldehyde (Fig. 1A, lane 1). Most of the cellular SecA is not cross-linked and remained visible as a monomer on SDS-PAGE with a Mr of 100 kDa. Formaldehyde-dependent cross-linking of proteins is reversible, and both complexes were found to disintegrate upon extensive boiling (lane 4). When higher concentrations of formaldehyde were employed, i.e., 0.5 to 1 % (w/v), the specificity of cross-linking was lost, and a smear of protein aggregates with a Mr larger than 250 kDa was identified by the anti-SecA oAb (data not shown). To assess whether the cross-linked complex contains any of the integral membrane subunits of the translocase, blots were developed with polyclonal antibodies (pAb's) that recognize SecY, SecG, or SecE. The anti-SecY pAb detected a protein complex with a Mr of 150-kDa in the cross-linked sample (Fig. 1B, lane 3), while with the anti-SecE and -SecG pAb no specific protein complexes were found in this molecular weight range (not shown).

Cross-linking of the cytosolic chaperone SecB was examined since SecA binds to SecB with high affinity (Hartl et al., 1990), and the molecular weight of a SecB tetramer (64 kDa) could also account for a ~160 kDa SecA-associated complex. With 0.1 % formaldehyde, a minor fraction of the SecB was found to be cross-linked (Fig. 1C). The products were tentatively identified as dimers and trimers, as similar cross-links were found with purified SecB (Fekkes, 1997). No SecB was present in the 100-200 kDa range, and it is therefore unlikely that the 150-kDa complex contains cross-linked SecB. These data suggest that the 150-kDa complex is composed of at least monomeric SecA together with SecY protein.

Fig. 2. Overproduction of the SecYEG complex results in the increased cross-linking of SecA as 150-kDa complex. Experiments were performed with E. coli NO2947 cells transformed with pET324 (control) or pET340 (SecYEG+). (A) Cells were incubated without (lanes 1 and 3) or with (lanes 2 and 4) 0.1 % (w/v) formaldehyde (H₂CO) and analysed by 7.5 % SDS-PAGE and immunoblotting using anti-SecA oAb. (B) Anti-SecA immunoblots of dilution series of sucrose gradient-purified inner membrane vesicles from control cells and SecYEG+ cells. A ratio of 1:1 indicates 5 µg of total membrane protein.
Overproduction of SecYEG results in an increased cross-linking of the 150-kDa complex. In order to substantiate the polypeptide composition of the formaldehyde cross-linked 150-kDa complex, in vivo cross-linking experiments were performed with cells that overproduce the SecYEG complex. E. coli strain NO2947, which gives the highest levels of SecYEG overproduction (Van der Does, 1997) was transformed with plasmid pET342 carrying the secY, secE, and secG genes in tandem with individual ribosome binding sites, or with plasmid pET324, a control plasmid lacking the sec genes. Formaldehyde cross-linking of SecYEG-overproducing cells (termed SecYEG+ ) again resulted in the formation of the 150-kDa complex, but the amount formed was substantially larger (i.e. 8-fold, based upon densitometric scanning) as observed with the parental strain containing the control plasmid (Fig. 2A). The elevated level of 150-kDa complex formed in the SecYEG+ strain correlated with the approximately 8-fold increased amount of SecA present in sucrose gradient-purified inner membranes (Fig. 2B).

Isolation of the SecA-containing 150-kDa complex. To enable purification and unequivocal identification of the polypeptide composition of the cross-linked 150-kDa complex, a six histidine-tag was introduced at the amino- or carboxy-terminus of SecY, and the N-terminus of SecE. The genes were cloned individually in tandem behind the trc-promoter in the order secY-secE-secG as has been described for the non-tagged genes (Van der Does et al., 1996). Strain NO2947 was transformed with pET324 (control), pET340 (SecYEG+ ), pET349 (SechYnEG+ , designating a His-tag on the SecY protein), pET512 (SechYcEG+ , designating a His-tag on the C-terminus of SecY), and pET320 (SecYhEnG+ , designating a His-tag on the SecE protein). Immunoblot analysis of isolated membranes using SecY, SecE, and SecG-specific pAbs revealed that the His-tagged proteins were overexpressed in similar amounts as the untagged proteins (Fig. 3A). The His-tag at SecY or SecE does not interfere with the activity of the translocase, as urea-treated membranes derived from the various strains exhibited identical SecA translocation ATPase activities that were around 20-fold higher than the activity found for the parental strain (Fig. 3B).

Cells that overproduce the SecYEG complex with His-tagged SecY or SecE proteins were incubated with or without 0.1 % formaldehyde. After disruption by sonication, membranes were isolated, solubilized with octylglucoside and loaded onto Ni2+-NTA columns. After several washing-steps, proteins were eluted with imidazole and analysed by immunoblotting with anti-
SecA oAb. Only in case of the N- or C-terminally His-tagged SecY, the elution fraction from the cross-linked samples showed the presence of the 150-kDa complex (Fig. 4, lanes 3 and 4). The minor amount of non-crosslinked SecA observed in the eluted fractions is most likely due to a dissociation of some of the cross-linked complex at 60 °C. Hardly any SecA or SecA-containing complex was specifically eluted from the column loaded with the sample containing the His-tagged SecE, or with His-tagged SecY when the sample was not cross-linked (Fig. 4, lanes 5 and 2, respectively). Since His-tagged SecY and SecE can be readily purified as individual proteins by the Ni²⁺-NTA affinity chromatography (Van der Does, 1997), the lack of a SecA-containing complex in the imidazole eluted fractions using the His-tagged SecE is not due to the lack of binding of this protein to the column-resin. As a control, elution fractions were stained with anti-SecY or anti-SecE pAb, showing that His-tagged SecY and SecE were purified specifically from the expected samples. These data unequivocally identify SecA and SecY as the cross-linking partners of the 150-kDa complex.

**Fig. 4.** Purification of 150-kDa complex through His-tagged SecY. Immunoblots of elution fractions after Ni²⁺-purification of the indicated samples. On blots developed with anti-SecA oAb, 150-kDa complex is visible only in fractions from cross-linked samples containing His-tagged SecY (upper panel). His-tagged SecY and SecE are stained in the appropriate samples, both before and after cross-linking (middle and lower panel, respectively). For the anti-SecA blot, samples were run on 7.5 % SDS-PAGE gel, for the anti-SecY and -SecE blots on 15 % gel.

**DISCUSSION**

The general protein secretory pathway in *E. coli* is an extensively studied model system for preprotein transport in bacteria. Various biochemical approaches have shown that SecY, SecE, and SecG interact, forming a heterotrimERIC integral membrane protein complex. This complex functionally interacts with the SecA protein to form a catalytically active assembly termed translocase, which satisfies the minimal requirement to reconstitute efficient preprotein translocation *in vitro*. In this report we have used an *in vivo* cross-linking approach to analyse the SecA interaction with the SecYEG complex. The use of formaldehyde as cross-linker allowed the identification of a specific interaction between SecA and SecY in intact cells. This interaction most likely is a direct one, since it is detected in *in vitro* ligand affinity blotting experiments (Snyders et
It is well established that the SecY, SecE, and SecG subunits interact (Matsuyama et al., 1990; Taura et al., 1993; Hanada et al., 1994; Brundage et al., 1990, 1992; Douville et al., 1994; Joly et al., 1994), and that the entire SecYEG complex is needed for specific SecA binding (Kim and Oliver, 1994; Douville et al., 1995). SecA may also interact with other proteins, such as the integral membrane proteins SecD and SecF (Economou et al., 1995), and the cytosolic chaperone SecB (Hartl et al., 1989). However, the cross-linking approach did not reveal a specific interaction of SecA with any other proteinaceous component of sufficient abundance. Since the detection of complexes by cross-linking is limited by the availability and the proximity of reactive groups, the absence of cross-linked complexes does not imply that there are no other in vivo protein-protein interactions than the ones shown in this paper.

The cross-linking reagent identified a 150-kDa complex that based on the estimated molecular mass in SDS-PAGE contains SecY with monomeric SecA. Cytosolic SecA is normally purified as a stable dimeric protein, and can be cross-linked as such by formaldehyde and succinimidyl esters (Akita et al., 1991; Manting, 1997). Since the SecA dimer does not dissociate at the translocase (Driessen, 1993), it seems that the conformation of the translocase bound SecA differs from that of the cytosolic SecA in a manner that it cannot be efficiently cross-linked as a dimer. Alternatively, a single SecA-SecY cross-linking event may induce a structural rearrangement of the molecules and thereby prevent further cross-linking, or cause an aberrant behaviour of the complex on SDS-PAGE gel. At high formaldehyde concentrations, SecA-containing complexes with a Mr > 250 kDa were detected. However, the poor specificity of cross-linking under those conditions prevented the detection of discrete protein bands. The amount of 150-kDa complex formed was dependent on the strain used for cross-linking, and was for instance higher with strain D10 and SF100 than with NO2947. Nevertheless, with each strain, the same specificity of cross-linking was observed, i.e., formation of the SecA-containing 150-kDa complex only. The difference may be related to the growth physiology and/or due to small variations in the level of functional translocase.

The cross-linking experiments were aimed at the detection of SecA-associated proteins under conditions that no translocation takes place, i.e., the idle state of SecA. Therefore, washed, resting cells were used. A substantial fraction of the cellular SecA is membrane-associated under these conditions, including a tightly bound subfraction (Cabelli et al., 1991). In SecYEG+ cells, an even larger fraction of the cellular SecA is tightly membrane-associated, most likely due to an elevation of the number of high affinity SecA membrane binding sites (Douville et al., 1995). This SecA is not removed upon sucrose-gradient purification of inner membranes, and is resistant to urea-extraction (this study; Van der Does et al., 1996). It exposes its carboxy-terminus to the periplasm, and is active in in vitro preprotein translocation (Van der Does et al., 1996). Since the cross-linking yield correlates with the increase in the SecA-membrane association upon SecYEG overproduction, it is concluded that the elevated cross-linking of SecA to SecY in SecYEG+ cells is due to the enhanced level of functional translocase.

To elucidate the protein composition of the 150-kDa complex, His-tagged SecY and SecE were used. In a similar way as described for the SecY and SecE proteins we constructed a plasmid encoding N-terminally His-tagged SecG, but the protein could not be detected on immunoblots of membranes isolated from the overproducing strain, and the construct was therefore not used in further experiments. The presence of a His-tag at either the amino- or carboxyl-terminus of SecY, or the amino-terminus of SecE, does not interfere with the functionality of the translocase. It is important to stress that the presence of the His tag has no effect on the specificity of the translocase. Unlike the prlA4 suppressor mutant of SecY, the (overproduced) His-tagged SecY was unable to translocate a proOmpA mutant with a defective signal sequence, i.e., with a deletion Ile -8 of the signal sequence (Δ8proOmpA)(Van der Does, 1997). These results imply that the His-tagged proteins catalyse an authentic translocation reaction.

In conclusion, the in vivo cross-linking approach demonstrates a specific interaction between SecA and SecY as a 150-kDa protein complex in the cytoplasmic membrane of intact cells.
cells. It appears that even in resting cells, these proteins stably interact at the membrane translocation sites. Since the SecA cross-linking method discriminates between the SecYEG-bound and non-bound fractions, it provides a convenient tool to probe the SecA membrane-association state in vivo.

**MATERIALS AND METHODS**

**Materials.** Formaldehyde (37 % w/v in H₂O) was from Sigma Chem. Co. (St. Louis, MO), Ni²⁺-NTA agarose was obtained from QIAGEN Inc. (Chatsworth, CA), and Wizard miniprep columns from Promega Co. (Madison, WI). A mixture of mouse monoclonal antibodies (oligoconal) (Den Blauwens et al., 1997) was used to detect SecA on immunoblots. Antibodies against SecE and SecG were obtained by immunizing rabbits with synthetic peptides corresponding to amino acids 64-81 for SecE (GKGATVAFAREAR) and 89-98 for SecG (APAKTEQTQP), coupled to a multi-antigen peptide (MAP) (Research Genetics, Huntsville, AL). Rabbit polyclonal antiseraum against SecB and a synthetic peptide corresponding to the 22 amino-terminal amino acids of SecY were generously provided by W. Wickner (Dartmouth College, Hanover, NH).

**Bacterial strains and plasmids.** E. coli strains D10 (Geller et al., 1986), and NO2947 (Knol et al., 1996) were used. For overproduction of SecYEG in strain NO2947, pET340 was used that harbours the secY, secE and secG genes in tandem under control of the isopropyl-ß-D-thiogalactopyranoside (IPTG)-inducible trc promoter (Van der Does et al., 1996). pET324 was used as control plasmid lacking the sec genes. Plasmids overproducing the SecYEG complex with amino-terminally His-tagged SecY (pET349) and SecE (pET320) were obtained by isolation of a linker region in the NcoI sites in the start codons of the secY and secE genes in pET340. The inserted linking sequence 5'-CCATGCATACATCACCATACTGACGTCGACAAAGCCATGGTGATGGTGATGGTTGTCATCGTCATCTCGCCGCTAGCCTTTCA-3' codes for (H)₆(D)₄KA, i.e., a six His-tag followed by an enterokinase recognition site. A His-tag preceded by an enterokinase site was introduced at the carboxy-terminus of SecY via PCR. The oligonucleotide sequence 5'-CCAGGAATTCTCGTGCCGGGA-3', encoding amino acids 354-358 of SecY, was used as a forward primer. The SalI digestible reverse primer 5'-GAGATCTGACTTTAATGGTATGGTGATGGTTGTTGTCATCGTCATCTCGGCCGCTAGCCTTTCA-3' encodes amino acids 437-442 of SecY, and adds the amino acid sequence (D)₄K(H)₆ to the carboxy-terminus. The PCR-product was cloned into pBluescript II SK⁺ (Stratagene, La Jolla, CA) via EcoRI/SalI digestion, and finally cloned in EcoRI-ClaI digested pET340 via EcoRI/AccI digestion, yielding pET512. All constructs were confirmed by sequence analysis on a Vistra DNA sequencer 725 using the automated Taq sequencing kit (both from Amersham, Buckinghamshire, U.K.). Isolation of plasmid DNA and other DNA techniques followed standard procedures (Sambrook et al., 1989).

**Growth of bacteria and isolation of cytoplasmic membranes.** NO2947 cells transformed with the various plasmids were grown aerobically at 37 °C on LB-broth containing 50 µg/ml of ampicillin to an OD₆₆₀ of 0.7. Exponentially growing cultures were then supplemented with 0.5 mM IPTG and growth was continued to an OD₆₆₀ of 1.0. Membrane vesicles were prepared by French pressure treatment (Chang et al., 1979). SecA translocation ATPase activity in the presence of urea-extracted membranes (Cunningham et al., 1989) was measured as described (Lill et al., 1989), using 100 µg/ml membrane proteins, 20 µg/ml SecA, 2 mM ATP, and in the absence or presence of 30 µg/ml proOmpA and 50 µg/ml SecB.

**Cross-linking and protein analysis.** In vivo cross-linking of intact cells was performed as described (Prossnitz et al., 1988). Protein samples were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5-15 % acrylamide gels (Laemmli, 1970), followed by western blotting onto immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). The Protein II system from Bio-Rad (Hercules, CA) was used for electrophoresis to obtain maximal separation of the cross-linked complexes. Immunoblots were developed using CSPD chemoluminescent substrate, according to the manufacturers recommendations (Tropix, Bedford, MA). Protein concentrations were determined using Lowry assays (Lowry et al., 1951). For quantitative analysis of immunoblots developed with the chemiluminescence kit, films of various exposures were densitometrically scanned using a Dextra DF-2400T scanner (Dextra Technology Corp., Taipei, Taiwan) and analysed using SigmaScan/Image (Jandel Corp., San Rafael, CA).

**Purification of His-tagged proteins.** After cross-linking, cells (10 ml, O.D₆₆₀ of 1.0) were washed three times with 50 mM TrisCl, pH 8.0, containing 2 mM of PMSF and 5 mM p-aminobenzamidine, resuspended in 1 ml of the same buffer, and disrupted through sonication. Remaining cells were spun down (10 000 g), and membranes were collected from the supernatant by centrifugation (120 000 x g, 20 min). The
pellet was solubilized in 250 µl buffer A (1.25 % [w/v] n-octyl-β-D-glucopyranoside, 20 % [w/v] glycerol, 300 mM NaCl, and 50 mM TrisCl, pH 8.0) for 1 h on ice, after which nonsolubilized material was removed by centrifugation (120 000 x g, 20 min). To assure the presence of equal quantities of protein during the purification procedure, 20 µl of the supernatant was analysed by SDS-PAGE followed by Coomassie brilliant blue staining and immunoblotting using anti-SecA antibody. Solubilized proteins were incubated for 2 h at 4 °C by constant shaking with Ni²⁺-NTA agarose beads that were pre-washed with buffer A. The solution was transferred into Wizard miniprep columns, and flow-through fractions were collected by centrifugation (Eppendorf centrifuge, 2 000 rpm, 1 min). The column material was washed five times with 200 µl of 30 mM imidazole in buffer A for 15 min, followed by centrifugation. Proteins that were specifically bound were finally eluted with 100 µl of 250 mM imidazole in buffer A. Samples were analysed by SDS-PAGE and immunoblotting.

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