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The SecA and SecY subunits of translocase are the nearest neighbors of a translocating preprotein, shielding it from phospholipids

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To study the environment of a preprotein as it crosses the plasma membrane of *Escherichia coli*, unique cysteinyl residues were introduced into proOmpA and the genes for these mutant preproteins were fused to the gene of dihydrofolate reductase (Dhfr). A photoactivable, radiolabeled and reducible cross-linker was then attached to the unique cysteinyl residue of each purified protein. Partially translocated polypeptides were generated and arrested in their membrane transit by the folded structure of the dihydrofolate reductase domain. After photolysis to label their nearest neighbors and reduction of the disulfide bond between proOmpA-Dhfr and the cross-linker, radiolabeled cross-linker was selectively recovered with the SecA and SecY subunits of preprotein translocase. Strikingly, neither the SecE nor Band 1 subunits were cross-linked to any of the constructs and the membrane phospholipids were almost entirely shielded from cross-linking. The fact that SecY and SecA are the only membrane proteins cross-linked to the translocating chains suggests that they may form an entirely proteinaceous pathway through which secreted proteins pass during membrane transit.

Key words: cross-linking/preprotein translocase/SecA/SecY/translocation pathway

Introduction

Proteins translocating across membranes have been proposed to pass directly through the membrane phospholipid (von Heijne and Blomberg, 1979; Wickner, 1979; Engelman and Steitz, 1981) or to cross via a protein pore or tunnel (Blobel and Dobberstein, 1975). Examples of phospholipid-mediated translocation include the M13 procoat protein (Ohno-Iwashita and Wickner, 1983; Geller and Wickner 1985), apocytochrome *c* (Rietveld *et al.*, 1985), melittin (Muller and Zimmerman, 1987) and complement C9 (Tschopp *et al.*, 1982). The discovery that two receptor proteins, the signal recognition particle (Walter and Blobel, 1982) and the docking protein (Meyer *et al.*, 1982), were involved in early translocation events in canine pancreas endoplasmic reticulum (ER) suggested that proteins play a pivotal role as receptors. Recently, cross-linking has been used to identify membrane-embedded components responsible for translocation in both mitochondria and the ER. These include Sec61 in yeast ER (Musch *et al.*, 1992; Sanders *et al.*, 1992), translocating chain-associating membrane protein (TRAM) in canine microsomes (Görlich *et al.*, 1992) and the general insertion protein (GIP) (Söllner *et al.*, 1992) and import site protein 42 (ISP42) (Vestweber *et al.*, 1989) in mitochondria. In *Escherichia coli*, translocation across the cytoplasmic membrane has been studied by both genetic and biochemical techniques, identifying many if not all of the principle components. Genetic screens have identified SecA, SecB, SecD, SecE, SecF and SecY, each acting at distinct stages during translocation (Emr *et al.*, 1981; Oliver and Beckwith, 1981; Kumamoto and Beckwith, 1983; Riggs *et al.*, 1988; Schatz *et al.*, 1989; Gardel *et al.*, 1990). Biochemical analyses have led to the purification and functional reconstitution of the SecB chaperone (Weiss *et al.*, 1988), leader peptidase (Watts *et al.*, 1981) and translocase (SecA, SecY, SecE and Band 1) (Brundage *et al.*, 1990). This combined genetic and biochemical approach has made this translocation reaction suitable for mechanistic studies (reviewed in Bieker *et al.*, 1990; Wickner *et al.*, 1991).

Many newly synthesized secretory preproteins bind SecB, which is a cytosolic chaperone (Kumamoto, 1989). SecB maintains precursors in a translocation-competent, loosely folded state (Weiss *et al.*, 1988; Lecker *et al.*, 1989). The SecB-precursor complex binds to the SecA subunit of preprotein translocase (Hartl *et al.*, 1990). Preprotein translocase is a multisubunit enzyme consisting of the membrane-embedded SecY/E domain and the peripheral SecA protein (Brundage *et al.*, 1990). Translocase catalyzes translocation across the cytoplasmic membrane in the presence of ATP and a precursor protein. SecA recognizes the SecB chaperone as well as the leader and mature regions of preproteins (Cunningham and Wickner, 1989). SecA hydrolyzes ATP in the presence of the SecB-precursor complex and membranes. SecY/E protein, the integral membrane domain of preprotein translocase, is comprised of three subunits: SecY, SecE and a protein of an unidentified gene termed Band 1. When a cytoplasmic membrane detergent extract is fractionated to isolate the functional components for translocation, the three subunits copurify. Furthermore, these subunits can be immunoprecipitated as a complex with anti-SecY antisera (Brundage *et al.*, 1990, 1992). After reconstitution of SecY/E into proteoliposomes and addition of SecA and ATP, the purified complex can translocate preproteins. Upon preprotein arrival at the membrane, ATP binding and hydrolysis by SecA produces net movement of the polypeptide during which the leader region is cleaved by leader peptidase (Schiebel *et al.*, 1991). An electrochemical gradient of protons accelerates translocation and drives large segments of protein across the membrane. The precise role in translocation of SecD and SecF, identified by genetic screens (Gardel *et al.*, 1990), has not yet been established. Both are membrane proteins containing large periplasmic domains. This has led to speculation (Gardel *et al.*, 1990) that they play a role late in the translocation process.

While much is known concerning the arrival of preproteins at the membrane and the energetics of translocation, an understanding of the environment of a translocating chain

has been elusive. We now report that intermediates of translocation that contain uniquely positioned photoactivable radiolabeled cross-linkers can specifically label SecA and SecY. Neither SecE nor Band 1 show significant cross-linking after photolysis and the cross-linker is largely shielded from phospholipids. These studies provide strong confirmation of the postulate of a proteinaceous transport mechanism (Blobel and Dobberstein, 1975).

Results

Attachment of a sulfhydryl-specific radiolabeled cross-linker

A stable translocation intermediate can provide a 'platform' from which one may study the environment of a translocating polypeptide. Transmembrane-arrested intermediates have been created by the covalent attachment of a large folded structure, such as avidin (Sanders *et al.*, 1992) or bovine pancreatic trypsin inhibitor (BPTI) (Vestweber and Schatz, 1988; Schiebel *et al.*, 1991) near the C-terminus of an authentic preprotein. The use of tightly folded murine dihydrofolate reductase (Dhfr) to block translocation has been used previously to generate intermediates of mitochondrial translocation (Eilers and Schatz, 1986; Rassow *et al.*, 1990). We therefore fused Dhfr in-frame to the C-terminus of the precursor of outer membrane protein A (proOmpA) (Figure 1A) as described in the accompanying paper (Arkowitz *et al.*, 1992). The proOmpA domain of this fusion protein can translocate across bacterial inverted inner membrane vesicles *in vitro* (Arkowitz *et al.*, 1992). In the presence of methotrexate and NADPH, the Dhfr domain folds into a tight, protease-resistant structure. When this hybrid protein is translocated into membrane vesicles in the presence of these Dhfr ligands, the tightly folded structure prevents further translocation. In this intermediate, the Dhfr domain is on the outside of the membrane vesicle and the proOmpA portion is inside the vesicle lumen (see Figure 1B for model). This intermediate is denoted as I_{37} because ~37 kDa have been translocated. If the Dhfr ligands are removed by sedimenting the vesicles with bound intermediate through two successive sucrose cushions, the I_{37} intermediate can undergo a subsequent limited translocation upon warming to 37°C. This small further translocation event results in a loss of the structure of Dhfr and a slight increase in the size of the proteinase K-inaccessible polypeptide (Figure 1C). This intermediate is denoted I_{40} and is a collection of polypeptides with a protease-inaccessible domain slightly larger than that of proOmpA. The I_{40} intermediate does not contain any protease-resistant Dhfr structure and the Dhfr domain cannot bind its ligands. Full translocation occurs upon addition of ATP (Figure 1D).

Site-directed mutagenesis was used to create fusion proteins with unique cysteinyl residues in their proOmpA portion. Wild type proOmpA contains only two cysteines, at residues 290 and 302 (Movva *et al.*, 1980). These cysteines were mutagenized to serines and single cysteine residues were then introduced at unique positions in the C-terminal third of the proOmpA. Dhfr contains a single cysteine at residue six that is conserved across most mammalian species (Blakely, 1984). Since mutagenesis of this cysteine contributes to a destabilization of the Dhfr structure (Eilers *et al.*, 1989), this residue was left intact in all the fusion proteins. A fusion protein of proOmpA–Dhfr without cysteines in the proOmpA region was also constructed, leaving the cysteine in the Dhfr domain as the only cysteine in the fusion protein. The positions of the cysteines in the various constructs are shown in Figure 1A.

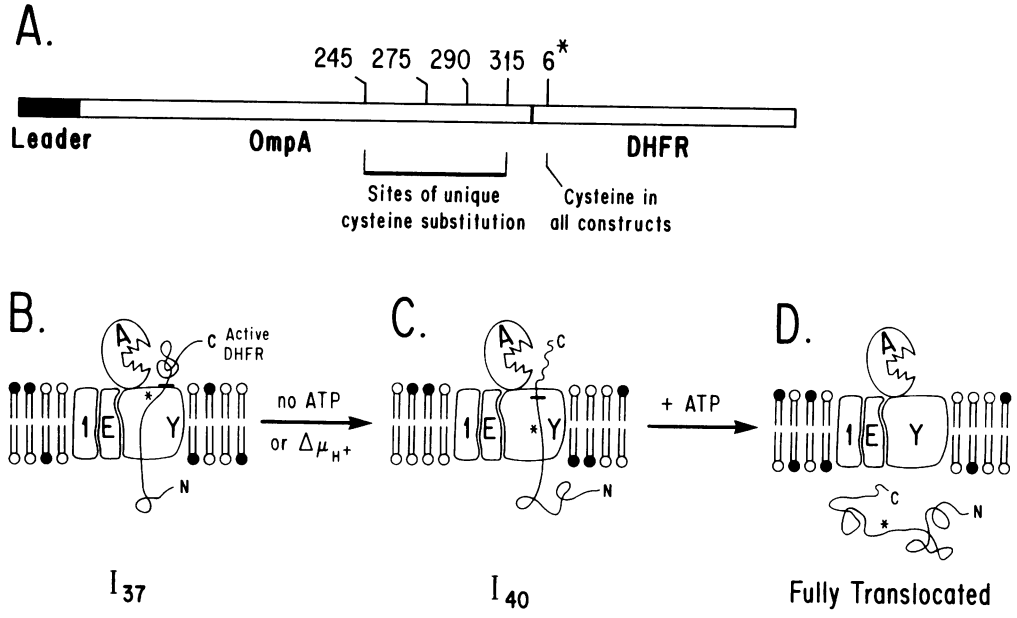
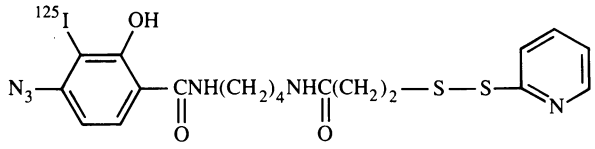


Fig. 1. Schematic representation of the proOmpA–Dhfr fusion protein and its intermediates of translocation. (**A**) Unique cysteines in the OmpA domain are numbered according to their position in the mature domain from the leader peptide cleavage site (Movva *et al.*, 1980). The asterisk represents the cysteine at residue 6 in the Dhfr domain, present in all fusion protein constructs. Sections (**B–D**) represent stages of the translocation of proOmpA–Dhfr into inner membrane vesicles. This model is based on the data from Arkowitz *et al.* (1992). The letters Y, E and A refer to SecY, SecE and SecA respectively and 1 represents Band 1. The asterisk represents the environment of one hypothetical cysteine residue. The slash in the preprotein is the junction between the proOmpA and Dhfr. The model is not intended to address the conformation of the preprotein as it interacts with preprotein translocase.

The cysteines in the fusion proteins were derivatized with a sulfhydryl-specific, radiolabeled and photoactivable cross-linker, *N*-[4-(*p*-azidosalicylamido)butyl]-3′-(2′-pyridyldithio) propionamide (APDP). This heterobifunctional cross-linker has a long spacer arm of ~ 21 Å. It was iodinated at the *meta* position of the phenyl ring between the azido and



hydroxyl groups. This substituted phenyl azide is sulfhydryl-specific by virtue of the pyridyldithio leading group. The derivatization of all proOmpA−Dhfr fusion proteins with [^{125}I]APDP was reversible; upon addition of a reducing agent, the radiolabeled cross-linker was removed (Figure 2A, compare lanes 1−6 with 7−12). ProOmpA

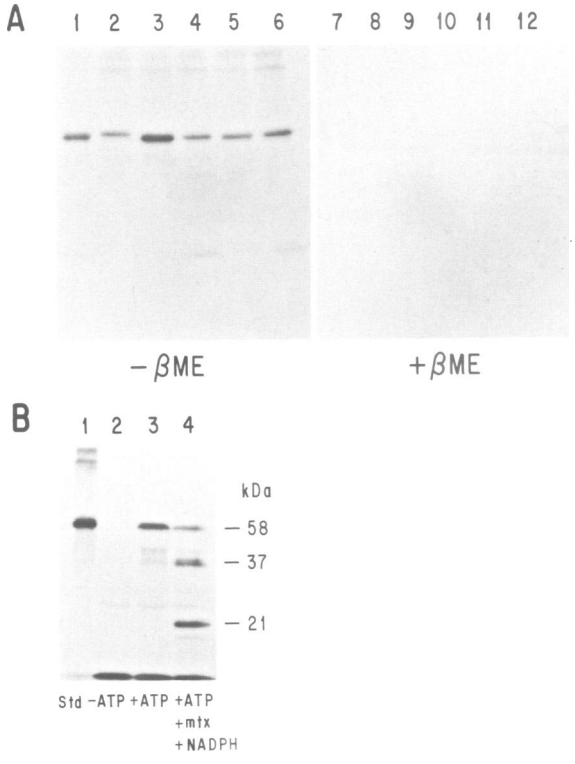


Fig. 2. Reversible conjugation of [^{125}I]APDP and I_{37} formation. (**A**) Autoradiography of unique cysteinyl [^{125}I]APDP−proOmpA−Dhfr constructs that were analyzed by SDS−PAGE in the absence (lanes 1−6) and presence (lanes 7−12) of β-mercaptoethanol. Lanes 1 and 7, no cysteine proOmpA−Dhfr; lanes 2 and 8, C245-proOmpA−Dhfr; lanes 3 and 9, C275-proOmpA−Dhfr; lanes 4 and 10, C290-proOmpA−Dhfr; lanes 5 and 11, C302-proOmpA−Dhfr; lanes 6 and 12, C315-proOmpA−Dhfr. Approximately 50 000 c.p.m. were loaded per lane. (**B**) C245-proOmpA−Dhfr was diluted from urea into buffer A as described in Materials and methods. Lane 1 is a standard representing 10% of the added radioactivity (300 000 c.p.m.); lane 2, translocation reaction without ATP; lane 3, with 1 mM ATP; lane 4, with 1 mM ATP, 1 μM methotrexate (mtx) and 1 mM NADPH. After addition of membrane vesicles and incubation for 30 min at 37°C, the samples (100 μl) were sedimented through 50 μl of sucrose solution (0.2 M sucrose, 50 mM Tris−Cl, pH 8.0 and 50 mM KCl) in a Beckman airfuge for 30 min at 30 p.s.i. at 4°C. The pellets were resuspended in buffer A and digested with 1 mg/ml proteinase K for 20 min on ice. Samples were analyzed without reducing agent by SDS−PAGE and autoradiography.

derivatized at cysteines with fluors, *N*-ethyl maleimide or short peptides can still translocate (Lecker *et al.*, 1990; Tani *et al.*, 1990; S.Mizushima, personal communication). The addition of the cross-linker did not impair the ability of the fusion protein to translocate and form a stable transmembrane intermediate. The fusion protein with a cysteine at residue 245 of proOmpA (C245-proOmpA−Dhfr) is shown as an example. Its translocation required ATP (Figure 2B, lanes 2 and 3). In the presence of the Dhfr ligands methotrexate and NADPH, a translocation intermediate formed. Proteinase K digestion of this intermediate, analyzed by non-reducing SDS−PAGE (lane 4), showed both a Dhfr-sized band (21 kDa) and proOmpA-sized band (37 kDa), indicating that both Cys245 and the cysteinyl residue in the Dhfr domain were derivatized with radiolabeled cross-linker. This digestion of the I_{37} sample is never complete (Arkowitz *et al.*, 1992), indicating that the proOmpA−Dhfr junction is somewhat inaccessible to protease. The covalent modification of the Dhfr cysteine did not impair the ability of this domain to bind methotrexate and NADPH and form a folded and protease-resistant structure.

The environment of a translocating protein

To generate intermediates of translocation, modified preproteins were diluted from urea in the presence of SecB, SecA, methotrexate and NADPH, producing a folded Dhfr domain. Bacterial inverted inner membrane vesicles were added to initiate translocation. The precursor translocated until the tightly folded Dhfr domain reached the membrane, resulting in an arrested translocation intermediate. To assess the environment of the translocating proteins, samples containing either intermediates or fully translocated proOmpA−Dhfr were photolyzed with UV light. The photolysis resulted in a highly reactive nitrene group that inserted non-selectively, producing a covalent bond. The sample was then incubated with SDS and β-mercaptoethanol and analyzed by SDS−PAGE. The reducing agent left the radiolabel attached to the nearest neighbor of the translocation intermediate.

The fusion protein, which did not have a cysteine residue in the proOmpA domain, yielded few translocation-specific cross-linking products (Figure 3A, lanes 5, 6 and 7). Intermediates I_{37} and I_{40} were detected after proteinase K treatment and electrophoresis without reducing agent (Figure 3A, lanes 1−4). No proOmpA-sized material was observed in I_{37} (Figure 3A, lane 2) since there is no cysteine in the proOmpA portion of this protein. The 21 kDa Dhfr protease-resistant band (lane 2) was lost concomitant with translocation to I_{40} (lane 3). The fusion protein containing a cysteine at residue 245 (C245-proOmpA−Dhfr) also yielded few translocation-specific products after UV cross-linking and reduction (Figure 3B, lanes 5−7), though translocation intermediates could be seen after proteinase K digestion (Figure 3B, lanes 1−4). A proOmpA-sized band was present due to the cysteine residue in the OmpA portion of this fusion protein (compare lanes 2 in Figures 3A and B). With cysteine at position 275 or 290 (C275-proOmpA−Dhfr and C290-proOmpA−Dhfr), the I_{37} and I_{40} intermediates (Figures 3C and D, lanes 2 and 3) cross-linked to bands of ~30 (solid oval) and 100 kDa (open oval) (lanes 5 and 6), shown below to be SecY and SecA. The fully translocated proOmpA−Dhfr did not cross-link to these proteins (lane 7). When the sole cysteine was positioned at residue 315 (C315-proOmpA−Dhfr), the cysteine was only

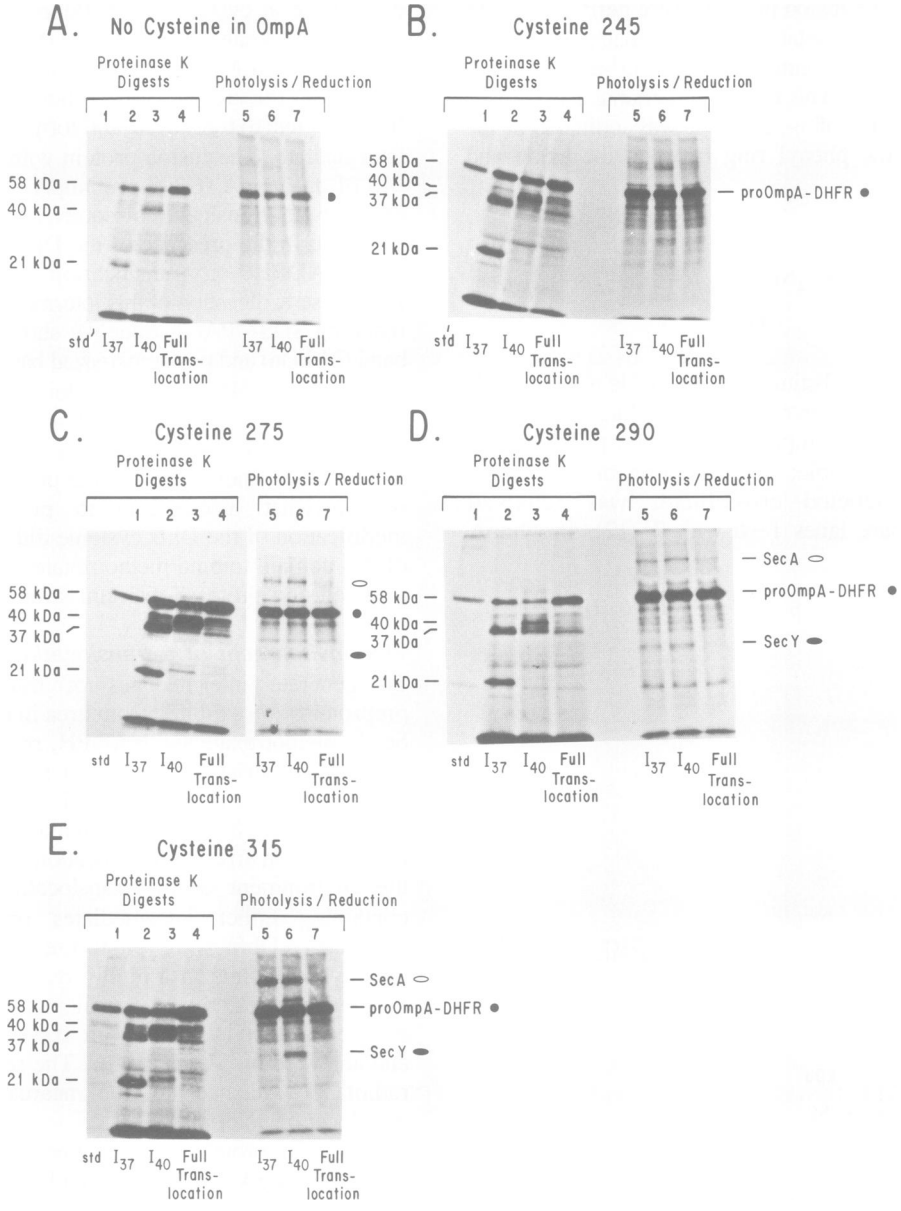


Fig. 3. Translocation and nearest neighbor analysis. (**A**) The no cysteine proOmpA−Dhfr (1.5×10^6 c.p.m.) was diluted from urea into buffer A containing 1 μM methotrexate, 1 mM NADPH and 1 mM ATP as described under Materials and methods. After the 30 min incubation with membrane vesicles, the samples were centrifuged as described in Figure 2B. The pellets were resuspended in buffer A with 0.5 mg/ml BSA and again centrifuged. The second pellets were resuspended in buffer A with 40 μg/ml SecA, 100 μM DTNB and pooled. Samples were either left on ice (lanes 2 and 5, I_{37}), warmed to 37°C (lanes 3 and 6, I_{40}), or warmed to 37°C with 1 mM ATP, 10 μg/ml creatine kinase and 5 mM creatine phosphate (full translocation, lanes 4 and 7). After 20 min, each sample was divided in half. The first half was digested with 1 mg/ml proteinase K on ice for 20 min (lanes 2−4). The second half was irradiated with an inverted UV lightbox at 302 nm. Samples were irradiated from a distance of 4 cm for 5 min at room temperature (Traut *et al.*, 1989). The irradiated samples (lanes 5−7) were analyzed by SDS−PAGE with β-mercaptoethanol and the proteinase K-digested samples were analyzed by SDS−PAGE without reducing agent. Lane 1 represents 10% of the sample used in lanes 2−7. The gel was then analyzed by autoradiography. (**B**) Same conditions as (A) except C245-proOmpA−Dhfr (3×106 c.p.m.) was used. The gel in (A) was exposed to X-ray film twice as long as (B). (**C**) Same as (B) except C275-proOmpA−Dhfr was used. (**D**) Same as (B) except C290-proOmpA−Dhfr was used. (**E**) Same as (B) except C315-proOmpA−Dhfr was used.

10 amino acids from the proOmpA−Dhfr junction. I_{37} with cysteine in this position yielded radiolabeled SecA but not SecY upon photolysis (Figure 3E, lane 5). When I_{40} was generated, the cross-linker could react with SecY as well as SecA (lane 6). The fully translocated protein reacted with neither SecA nor SecY (lane 7).

In all cross-linking experiments with the proOmpA−Dhfr mutants, the most radioactive protein was the proOmpA−Dhfr itself (solid circle), presumably because of the efficiency of the intramolecular reaction. The weak labeling of SecA seen in Figures 3A and B may be due to [^{125}I]APDP attached to the cysteinyl residue in the Dhfr domain. In some cases, a band migrating just above proOmpA−Dhfr was labeled. With C315-proOmpA−Dhfr, this band was labeled at the I_{40} stage but not after completion of translocation. This protein is probably not SecD, as the mobility of SecD is greater than this cross-linked protein (data not shown).

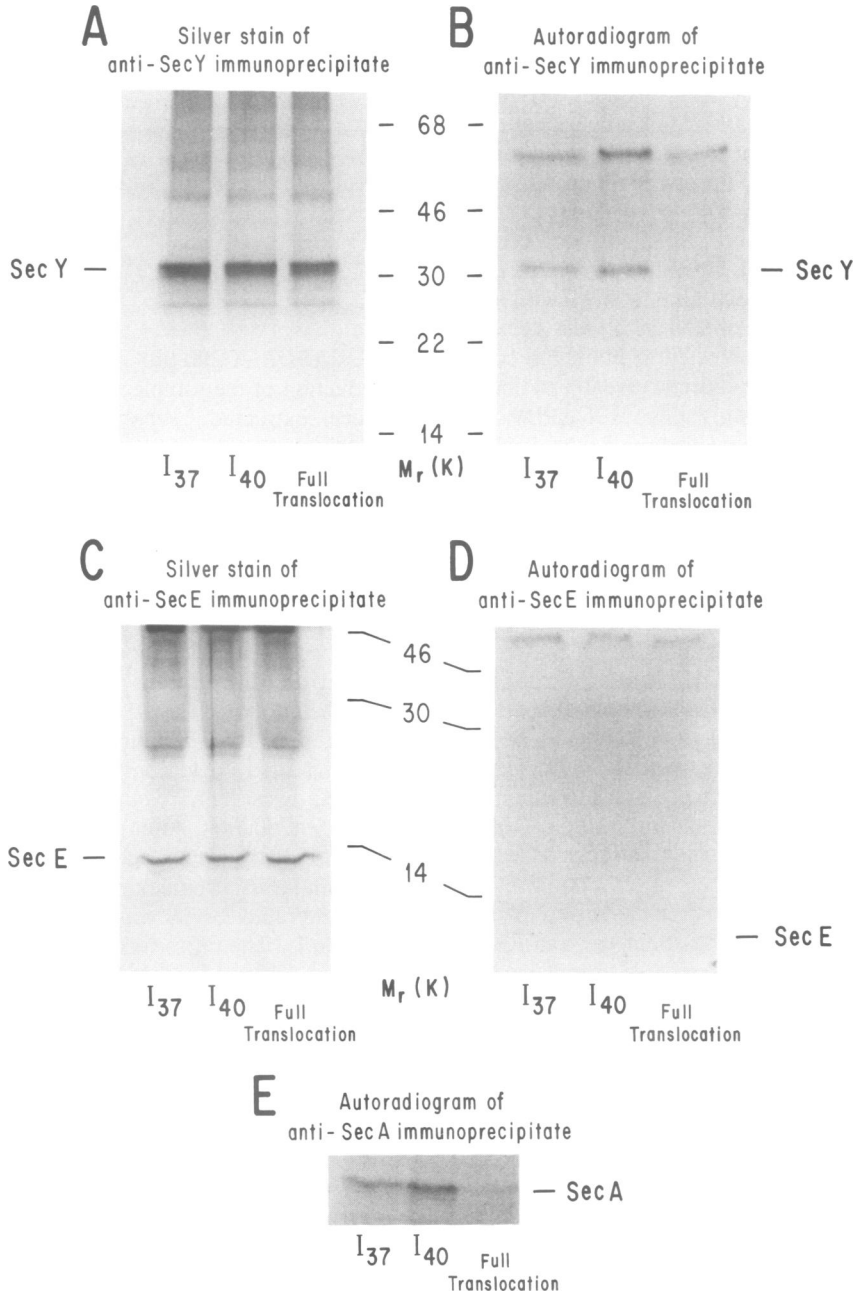


Fig. 4. Identification of cross-linked products. C290-proOmpA−Dhfr was prepared on a large scale at the I_{37} stage. The translocation reaction from Figure 3D was scaled up 6-fold except that 12×10^6 c.p.m. of preprotein was used. The vesicles were sedimented through the same sucrose solution but at 215 000 *g* for 35 min at 4°C. The pellets were resuspended in buffer A plus 0.5 mg/ml BSA and centrifuged again as above. The second pellets were resuspended in 100 mM Tris−Cl, pH 8.0, 5 mM $MgCl_2$, 100 μM DTNB and 40 μg/ml SecA. The sample was then divided into three aliquots: the first aliquot was kept on ice, the second aliquot was warmed to 37°C and the third aliquot was warmed to 37°C with 1 mM ATP, 10 μg/ml creatine kinase and 5 mM creatine phosphate. After 20 min the samples were irradiated as in Figure 3. Samples were processed for immunoprecipitations as described in Materials and methods. (**A**) is a silver stained 15% acrylamide gel of the SecY immunoprecipitation and (**B**) is the corresponding autoradiograph. (**C**) is the silver stained gel of the SecE immunoprecipitation and (**D**) is the corresponding autoradiograph. The SecE immunoprecipitations were electrophoresed on 'high Tris' polyacrylamide gels as described in Brundage *et al.* (1990). (**E**) is the autoradiograph of the SecA immunoprecipitation electrophoresed on a 10% acrylamide gel. The silver stain for the SecA immunoprecipitation is not shown since the IgG obscured the area of the gel where SecA migrates.

Identification of cross-linked products

To identify the proteins labeled after photolysis and reduction, immunoprecipitation was performed in the presence of SDS and Triton X-100. The 30 kDa band seen in Figure 3 was specifically precipitated by antisera directed against the N-terminus of SecY (Figure 4B). ^{125}I-labeled SecY was recovered by immunoprecipitation after cross-linking and reduction of the I_{37} and I_{40} intermediates (lanes 1 and 2) of C290-proOmpA−Dhfr. ~0.3% of the radioactivity from I_{37} was transferred to SecY. The band migrating above SecY in all the immunoprecipitates was proOmpA−Dhfr, which non-specifically adsorbs to the protein-A−Sepharose resin. Label was not transferred to SecY from [^{125}I]APDP−proOmpA−Dhfr, which had completed translocation (lane 3). SecY was the only integral membrane protein specifically cross-linked (Figure 3). Since

SecE is part of the translocation complex (Brundage *et al.*, 1990, 1992), we assayed label transfer from [^{125}I]APDP–proOmpA–Dhfr to SecE using antisera directed against a peptide comprising residues 67–90 of a cytoplasmic loop of SecE. The silver stained gel (Figure 4C) showed that SecE was immunoprecipitated. Quantification of our SecE and SecY immunoprecipitations by [^{125}I]protein A showed that >50% of the SecE and SecY in the samples were immunoprecipitated. However, no labeled SecE was found with the C290-proOmpA–Dhfr intermediates (Figure 4D, lanes 1 and 2). Even with longer exposure, no detectable radiolabeled SecE was seen (data not shown). In samples not subjected to immunoprecipitation, a 5-fold longer exposure did not reveal a radioactive band in the area where SecE migrates. [^{125}I]APDP itself, when not joined to I_{37}, can derivatize the SecY, SecE and Band 1 subunits of translocase in comparable yield (data not shown). This shows that the selective labeling of the SecY subunit seen when the cross-linker is bound to I_{37} and I_{40} is due to the polypeptide topographies.

Antisera specific for SecA was used to identify the 100 kDa band. I_{37} and I_{40} intermediates of ^{125}I-labeled C275-, C290- and C315-proOmpA–Dhfr transferred label to SecA (C290-proOmpA–Dhfr; Figure 4E, lanes 1 and 2). No SecA was labeled when the [^{125}I]APDP–proOmpA–Dhfr had completed translocation prior to photolysis (lane 3). If urea-washed (SecA-stripped) inner membrane vesicles were used, no label was transferred by cross-linking to either SecA or SecY. Both SecA and SecY cross-linking were restored in translocation reactions to which SecA had been added (data not shown).

Studies of these five mutants, each with a different unique cysteine, have yielded information about the translocation process at the membrane. With the most N-terminal cysteine construct, C245-proOmpA–Dhfr, the cross-linker was presumably in the lumen of the membrane vesicle at both the I_{37} and I_{40} stages of translocation and thus did not transfer label to SecA or SecY. The next two cysteine mutants, C275-proOmpA–Dhfr and C290-proOmpA–Dhfr, transferred label to both SecA and SecY when at I_{37} and I_{40} and thus were probably in or near the membrane. The most C-terminal cysteine construct, C315-proOmpA–Dhfr, could be cross-linked to SecA initially at the I_{37} stage and then to both SecA and SecY at the I_{40} stage. This cross-linking pattern suggests that the cysteine of I_{37} is near SecA, while the cysteine of I_{40} is in a pocket near SecY and SecA.

Phospholipid analysis

We examined the lipids from cross-linking reactions to determine whether translocating chains of entirely exported proteins such as proOmpA are exposed to the lipid bilayer *per se*. The bacterial inner membrane lipids are ~75% phosphatidyl ethanolamine (PE) and 20% phosphatidyl glycerol (PG) (Raetz, 1986). The acidic lipids, PG and cardiolipin, are required for translocation *in vivo* (de Vrije *et al.*, 1988). *In vitro*, negatively charged phospholipids stimulate SecA binding, the translocation ATPase and overall translocation (Lill *et al.*, 1990; Hendrick and Wickner, 1991). We therefore assayed the membrane phospholipids for label transfer after irradiation in the presence of [^{125}I]APDP–proOmpA–Dhfr translocation intermediates. A large scale preparation of the I_{37} intermediate of [^{125}I]APDP–C290-proOmpA–Dhfr was photolyzed. After reduction of the sample with 2-mercaptoethanol, the lipids were extracted, separated by silica gel thin layer chromatography and assayed by autoradiography. A small amount of radioactivity was found in the lipid extract, indicating that a modest amount of cross-linking was occurring to the bilayer lipids. To examine whether cross-linking to lipids of [^{125}I]APDP can occur at all within the bilayer, we used as a model a mutant bacteriorhodopsin in which a unique cysteinyl residue has been shown to be exposed to lipid (Altenbach *et al.*, 1990). [^{125}I]APDP bacteriorhodopsin was prepared with the cross-linker attached to this unique cysteine. After derivatization in denaturants, the protein was refolded in the presence of all *trans* retinal, detergents and *E.coli* phospholipids. The protein was then reconstituted into proteoliposomes by detergent dilution. After photolysis, reduction and extraction with organic solvents, radiolabeled phospholipid was asayed by thin layer chromatography. Approximately 2.2% of the starting radioactivity was recovered in PE and 1.2% in PG (Table I). The cross-linking to PE was only reduced 30% by the addition of reduced glutathione, an aqueous scavenger of activated nitrenes (Bayley and Knowles, 1978) (7890 c.p.m. versus 11 306 c.p.m. without glutathione). These data confirm that the reaction had indeed occurred within the membrane bilayer. This demonstrates that this aryl azide, anchored in the membrane, can react with phospholipids and be detected. If the I_{37} [^{125}I]APDP–proOmpA–Dhfr translocation intermediate were next to the lipid, then the lipid extract should contain significant radioactivity. When an equal amount of [^{125}I]APDP bound to I_{37} and to bacteriorhodopsin was used to assay photocross-linking of lipids, the ratio of PE and PG labeled by bacteriorhodopsin to I_{37} was 7.1 and 6 respectively (Table I). Furthermore, control experiments showed that even this very low level of lipid labeling observed in the I_{37} sample was not ATP-dependent as 1397 c.p.m. were detected in the sample with ATP compared with 1318 c.p.m. without ATP for PE and 702 c.p.m. with ATP compared with 737 c.p.m. without ATP for PG. This indicated that the cross-linking to lipid arose from the low background of adsorbed proOmpA–Dhfr rather than from authentic translocation intermediate. These results indicate that the I_{37} translocation intermediate was largely shielded from cross-linking to the lipid.

Table I. Analysis of lipid cross-linking after TLC

	Bacteriorhodopsin (lipid c.p.m./10^6 c.p.m. total)	I_{37}
Phosphatidylethanolamine	22 327	3143
Phosphatidylglycerol	11 989	2004

Approximately 40 μg of phospholipid was present in the bacteriorhodopsin sample compared with 500 μg phospholipid in the I_{37} sample. The phospholipids were extracted and chromatographed as described under Materials and methods.

Discussion

We have determined that SecA and SecY are the nearest neighbors of proOmpA as it crosses the *E.coli* plasma membrane. Of all the Sec proteins, SecY, by virtue of its multiple membrane-spanning helices, was the most likely

candidate to provide the actual path for translocation. Though SecA and SecY are minor proteins of the plasma membrane, it is striking that [^{125}I]APDP–proOmpA–Dhfr can transfer label to these two proteins so selectively that they can be directly visualized on autoradiograms without immunoprecipitation (Figure 3). The order of interaction of the translocating chain with translocase subunits is initially SecA (Hartl *et al.*, 1990; Lill *et al.*, 1990) followed by SecY. Our studies now provide a direct confirmation of this order of interaction. The membrane phospholipid is largely shielded from the transiting polypeptide chain. In addition, neither SecE nor Band 1 was cross-linked to the different cysteine mutants. These translocase subunits may be structurally necessary for SecY and SecA function and proper association. SecE mutations can suppress leader peptide mutations, suggesting that SecE interacts with the leader domain (Stader *et al.*, 1989). However, it is unknown if the mature domain of preproteins interacts with SecE at all. Genetic and biochemical studies show that only one of the three membrane-spanning segments of SecE is required for association with SecY and for *in vitro* translocation of a precursor protein (Schatz *et al.*, 1991; Nishiyama *et al.*, 1992). Band 1, which is also not cross-linked, copurifies with preprotein translocase and is immunoprecipitated with SecY and SecE when SecY antibodies are used (Brundage *et al.*, 1990, 1992; Arkowitz *et al.*, 1992). Isolation of the Band 1 gene is essential to assess its role in translocation.

Certain *prl* alleles of both *secA* and *secY* can suppress leader peptide mutations, suggesting that they interact with these sequences (Emr *et al.*, 1981; Fikes and Bassford, 1989; Stader *et al.*, 1989). Our work demonstrates that mature regions of preproteins can also interact with SecA and SecY. After I_{37} is formed, the interaction with SecA is not dependent on, or affected by, nucleotides or non-hydrolyzable ATP analogs (data not shown). Only when ATP is added and translocation completed is the interaction disrupted. While SecA is not an integral membrane protein, it is tightly associated with the cytoplasmic membrane such that high concentrations of urea are required to remove it *in vitro* (Cunningham *et al.*, 1989). SecA interacts with SecY; *in vitro*, it can shield SecY from proteolysis and SecA binding to membranes is inhibited by antibodies to SecY (Hartl *et al.*, 1990). SecA and SecY may form a protein groove or path through which translocating chains pass. Since the *E.coli* plasma membrane supports oxidative phosphorylation, such a path in SecY must be 'gated', in that it only opens as it is occupied by a preprotein loop transferred from SecA. Electrophysiological studies have shown ion-conducting channels in *E.coli* membranes (Simon *et al.*, 1989; Simon and Blobel, 1992); it is not yet known whether these channels require SecA and ATP and are related to SecY/E. SecY spans the membrane 10 times. These transmembrane segments may be intimately involved in the passage of secreted proteins. The ER functional analog of SecY may be TRAM, which spans the membrane eight times and is postulated based on cross-linking studies to be involved with translocating chains (Görlich *et al.*, 1992).

The finding that C275-proOmpA–Dhfr and C290-proOmpA–Dhfr give similar cross-linking patterns suggests that they are in similar environments, yet these cysteines are separated by 15 amino acids. Furthermore, the cross-linking patterns of these two constructs do not change upon the I_{37} to I_{40} transition. How can such a long region of the polypeptide chain be engaged with translocase? One possibility is that these translocating chains have loops or folds that travel through the translocation complex. Further resolution of this process will require mapping those residues of SecY to which each construct transfers label at each stage of translocation.

The results with the mature domain of proOmpA fusion proteins may not reflect the paths taken by leader peptides and stop-transfer sequences as they cross membranes. Both types of sequences are rich in hydrophobic residues and may interact with lipid rather than protein. Model leader peptides can partition into bilayers and alter phospholipid structure (Killian *et al.*, 1990; McKnight *et al.*, 1991). Whether this is indicative of any physiological role of lipids in stop-transfer or leader mechanisms is unclear.

The findings presented here have implications for the mechanism of translocation. The fact that SecA and SecY can be cross-linked to a preprotein at various positions during the process demonstrates that these proteins are intimately involved in the reaction. Other cross-linking studies in ER and mitochondrial systems have identified protein components near translocating chains, yet in these organelles there has not been either a complete enumeration of all translocation components or an evaluation of whether cross-linking to lipid also occurred. The combination of biochemical fractionation, reconstitution studies and genetic screens probably have identified most, if not all, the components necessary for translocation in *E.coli*.

Other techniques of monitoring the translocation process may also contribute to our understanding the environment of the translocating polypeptide chain. Spectroscopic techniques, such as electron spin resonance and fluorescence spectroscopy with cysteine-specific reagents, can be used to probe the environment of a translocating chain. Additional cysteine mutants may also allow a higher resolution map of the environment of this translocating polypeptide.

Materials and methods

Materials

Inverted *E.coli* inner membrane vesicles were isolated from *E.coli* KM9 (*unc*$^-$:: Tn*10*, *rel*A1, *spo*T1, *met*B1; Klionsky *et al.*, 1984) as described by Chang *et al.* (1978). SecA was purified according to the method of Cunningham *et al.* (1989) and SecB was purified by the method of Weiss *et al.* (1988) as modified by Lecker *et al.* (1989). All proOmpA–Dhfr proteins were purified by the method of Crooke *et al.* (1988), as for proOmpA. ^{125}I (350–500 Ci/mmol) and [^{125}I]protein A (30 mCi/mg) were purchased from Amersham. Iodogen, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and APDP were purchased from Pierce. Silica gel TLC plates (5×20 cm, 250 μm layer) with a preadsorbent strip were purchased from Whatman. Irradiation of samples was performed with a UV lightbox from Spectroline (Transilluminator Model TR-302). The plasmid pUC19, proteinase K, creatine phosphate and creatine kinase were purchased from Boehringer Mannheim. Restriction enzymes were from New England Biolabs Inc. Sephadex G-25 gel filtration media was from Pharmacia and dithiothreitol, NADPH, ATP, BSA and methotrexate were from Sigma.

Construction and expression of proOmpA–Dhfr proteins

The construction of proOmpA–Dhfr was described in Arkowitz *et al.* (1993). Oligonucleotide directed mutagenesis was performed according to the method of Higuchi *et al.* (1988). Oligonucleotides were synthesized at the UCLA Molecular Biology Institute by T.Sutherland. The following nucleotides were used: *Eco*RI site, N-terminus TCACACAGGAAACAGAATTC; *Sal*I site, C-terminus AATATGTCGACAGCCTGCGGCTGAGTTACAAC; Ser290, sense GGCAACACCTCTGACAACGTGAAA; Ser290, antisense TTTCACGTTGTCAGAGGTGTTGCC; Ser302, sense ACTGATCGACTCCCTGGCTCCGGAT; Ser302, antisense ATCCGGAGCCAGGGAGTCGATCAGT; Cys245, sense GCATCGGTTGTGAC-

GCTTACAAC; Cys245, antisense GTTGTAAGCGTCACAACCGATGC; Cys275, sense GACAAGATCTGCGCACGTGGTATG; Cys275, antisense CATACCACGTGCGCAGATCTTGTC; Cys315, sense GAAGTTAAA-TGTATCAAAGACGTT; Cys315, antisense AACGTCTTTGATACAT-TTAACTTC.

The sense strand oligonucleotides were used with the *SalI* oligonucleotide and the antisense strand oligonucleotides were used with the *Eco*RI-oligonucleotide. After amplification, the resulting fragments were gel purified and reamplified together with the *Eco*RI- and *SalI*-containing oligonucleotides. The fragment was gel purified, digested with *Eco*RI and *SalI* and ligated into the pUC-Dhfr plasmid digested with *SalI* and *Eco*RI (Arkowitz *et al.*, 1993). The region around the mutation was sequenced. The proOmpA−Dhfr gene was then digested with *Eco*RI and *Hin*dIII. The resulting fragment was ligated into the *Eco*RI and *Hin*dIII sites of pTrcOmp9 (Crooke *et al.*, 1988) downstream from the trc promoter. The plasmids were transformed into strain JM109 (Yanisch-Perron, 1985). The entire proOmpA part of the gene was then sequenced to confirm the mutation. A second site mutation occurred while constructing the proOmpA−Dhfr fusion with no cysteine in the proOmpA region altering Lys267 to Ile. Consequently, the constructs derived from it, which included C245-proOmpA−Dhfr, C275-proOmpA−Dhfr and C315-proOmpA−Dhfr, also contained this change. Additionally C275-proOmpA−Dhfr contains an Ala to Val change at residue 271.

Iodination and conjugation of APDP

APDP was dissolved in DMSO (3 mg in 50 μl) and diluted 20-fold into 100 mM potassium phosphate, pH 7.5. 10 μl of the diluted APDP was then added to 90 μl of potassium phosphate buffer in an Eppendorf tube that was coated with 40 μg iodogen (Markwell and Fox, 1978). Two millicuries of carrier free ^{125}I in 0.1 N NaOH were added and after 30 s, the reaction was terminated by pipetting the mixture to a fresh tube. 100 μg of reduced proOmpA−Dhfr in 6 M urea and 0.1 M potassium phosphate, pH 7.5 were then added. The final urea concentration was 2.5 M. Methotrexate and NADPH were added to 100 μM and 1 mM respectively. In the case of the no cysteine proOmpA−Dhfr, methotrexate and NADPH were omitted in the conjugation of APDP to the protein. After 1 h at room temperature, the reaction was filtered over a 2 ml Sephadex G-25 column equilibrated with 6 M urea, 0.1 M potassium phosphate, pH 7.5. Fractions containing protein were stored at −70°C and were stable for several months.

Reduction of proOmpA−Dhfr

400 μg of protein were reduced with 1 mM DTT for 30 min at 37°C in 6 M urea and 0.1 M potassium phosphate, pH 7.5. The mixture was then filtered over a 2 ml Sephadex G-25 column equilibrated in the same buffer. Elution of protein was assayed with Bradford reagent (Bio-Rad). Reduced protein was stored on ice while the cross-linker was iodinated.

In vitro translocations

Translocation into membrane vesicles was performed in buffer A (50 mM HEPES−KOH, pH 8.0, 50 mM KCl and 5 mM $MgCl_2$) containing 100 μM DTNB to prevent reduction of the cross-linker. Radiolabeled preproteins were diluted from urea into buffer A containing 0.5 mg/ml BSA, 40 μg/ml SecA, 20 μg/ml SecB, 10 μg/ml creatine kinase, 5 mM creatine phosphate and 2 mM succinate. Where indicated, 1 μM methotrexate, 1 mM NADPH and 1 mM ATP were added. The mixture was incubated for 5 min on ice followed by 5 min at 37°C. Translocation was then initiated by the addition of 0.25 mg/ml inner membrane vesicles and reactions were incubated for 30 min at 37°C.

Immunoprecipitation of proteins

UV cross-linked samples were dissolved in 1% SDS for 5 min at room temperature. The samples were centrifuged for 5 min in a microcentrifuge at 16 000 *g* and diluted 10-fold with 0.1% Triton X-100 in Tris buffered saline (20 mM Tris−Cl, pH 7.6 and 150 mM NaCl). SecY and SecA antisera were cross-linked to protein A−Sepharose (Pharmacia) by the method of Schneider *et al.* (1982) except that dimethyl adipimidate (Pierce) was used. Aliquots equivalent to 150 μl of resin were used for each immunoprecipitation. Incubations were performed at room temperature for 4 h. SecE was immunoprecipitated with 200 μl antisera for 2 h at room temperature followed by incubation with 100 μl of protein A−Sepharose for 2 h. Samples were centrifuged for 2 min and resuspended in Tris-buffered saline containing 0.1% SDS and 0.1% Triton X-100. After 2 min this step was repeated. Samples were then eluted from the beads by incubating in 80 μl of 160 mM Tris−Cl, pH 6.8, 4% SDS, 20% glycerol, 10 mM β-mercaptoethanol and 0.01% (w/v) bromophenol blue, and incubating for 10 min at 37°C. The samples were centrifuged for 5 min and the supernatant divided in two. Half the sample was analyzed by SDS−PAGE and silver stained. The other half was analyzed by SDS−PAGE and autoradiography. Quantification of immunoprecipitations was performed according to the method of Brundage *et al.* (1992) except that [^{125}I]protein A was used.

Bacterio-opsin derivatization

A mutant bacterio-opsin, which contained a single cysteine at residue position 139, was modified by the method of Flitsch and Khorana (1989) as follows. The protein was dissolved in 50 mM Tris−Cl, pH 8.0, 6 M urea and 2.5% SDS. 100 μl of a 5 mg/ml solution was reduced with 1 mM DTT for 2 h at 37°C. The protein was filtered over a 2 ml Sephadex G-25 column in 100 mM sodium phosphate, pH 7.5, 6 M urea, 2% SDS and 10 mM EDTA at room temperature. Protein elution was monitored by the A_{280} profile (ϵ = 65 000 $M^{-1}cm^{-1}$; Huang *et al.*, 1981). Reduced protein was stored on ice while the cross-linker was iodinated. APDP was iodinated as for proOmpA−Dhfr except that sodium phosphate was used instead of potassium phosphate. The reduced protein (0.1 mg) was incubated with [^{125}I]APDP in 3.6 M urea, 1.2% SDS, 6 mM EDTA and 100 mM sodium phosphate, pH 7.5 for 1 h at room temperature. Free cross-linker and iodine were removed by gel filtration in 100 mM sodium phosphate, pH 7.5, 6 M urea, 2% SDS and 10 mM EDTA. The peak protein fraction was analyzed by SDS−PAGE in the presence and absence of β-mercaptoethanol to check for the reversible attachment of cross-linker. The protein was folded by a procedure similar to that of Huang *et al.* (1981). The derivatized protein (3 nmol) was incubated in the presence of all *trans* retinal (3.5 nmol), 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 1% (w/v) *E.coli* phospholipids at room temperature. The final SDS and urea concentrations were 0.4% and 1.2 M respectively. The folding and chromophore regeneration of denatured bacteriorhodopsin proteins usually reached 60−80% (Braiman *et al.*, 1987). After 2 h, the protein was dialyzed versus 30 mM sodium phosphate, pH 6.2, 150 mM NaCl (4×1 l) for 2 days. The proteoliposomes were stored at −80°C and sonicated briefly before use.

Phospholipid extraction and TLC

UV irradiated samples were reduced with 15 mM β-mercaptoethanol for 15 min at 37°C and precipitated with trichloroacetic acid. The lipids were extracted according to the procedure of Bligh and Dyer (1959) as modified by Rothman and Kennedy (1977). The aqueous phase was reextracted two times with chloroform and the organic phase reextracted two additional times with 0.1 M KCl and 0.1 N HCl. The pooled organic phase was then dried under a stream of N_2. The lipid was solubilized in 50 μl chloroform−methanol (2:1) and spotted on a silica gel TLC plate. The plate was developed in choroform−methanol−water (65:24:4). Standards (PE and PG) were stained by spraying the plate with 10% H_2SO_4 in ethanol and baked at 130°C (Skipski and Barclay, 1969). The PG and PE areas of the TLC plate were scraped and counted in a gamma counter.

Other methods

Protein concentrations were determined by Bradford reagent with BSA as the standard. Sample preparation for SDS−PAGE and transfer to nitrocellulose were according to Ito *et al.* (1980) and Towbin *et al.* (1979). Silver staining was performed according to the method of Wray *et al.* (1981). All autoradiography was performed at −70°C with intensifying screens.

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Note added in proof

The ER homologue of SecY has recently been reported and is Sec61 [Görlich *et al.* (1992) *Cell*, **71**, 489–503].