Demonstration of a Specific *Escherichia coli* SecY−Signal Peptide Interaction†

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ABSTRACT: Protein translocation in *Escherichia coli* is initiated by the interaction of a preprotein with the membrane translocase composed of a motor protein, SecA ATPase, and a membrane-embedded channel, the SecYEG complex. The extent to which the signal peptide region of the preprotein plays a role in SecYEG interactions is unclear, in part because studies in this area typically employ the entire preprotein. Using a synthetic signal peptide harboring a photoaffinity label in its hydrophobic core, we examined this interaction with SecYEG in a detergent micellar environment. The signal peptide was found to specifically bind SecY in a saturable manner and at levels comparable to those that stimulate SecA ATPase activity. Chemical and proteolytic cleavage of cross-linked SecY and analysis of the signal peptide adducts indicate that the binding was primarily to regions of the protein containing transmembrane domains seven and two. The signal peptide−SecY interaction was affected by the presence of SecA and nucleotides in a manner consistent with the transfer of signal peptide to SecY upon nucleotide hydrolysis at SecA.

Protein transport across, or integration into, biological membranes is a vital cellular process (*1*−*3*). Components of the Sec translocon, the membrane pore through which presecretory proteins (or membrane proteins) achieve membrane translocation (or integration), are the most conserved transport constituents throughout the three kingdoms of life (*4*).

In *Escherichia coli*, the essential components of the translocase (*5*) include the membrane-associated form of SecA (*6*, *7*) and the polytopic membrane proteins SecY, SecE (homologues of the mammalian Sec61α, Sec61γ, and the yeast ER[1] Sec61p, Sss1p, respectively), and SecG (*8*, *9*); the latter three proteins form a stable trimeric SecYEG complex (*10*). SecA is an ATPase that powers the membrane translocation of hydrophilic polypeptides by coupling ATP hydrolysis with protein movement via concomitant SecA membrane insertion and deinsertion cycles (*11*, *12*). SecY protein has 10 transmembrane (TM1−TM10), six cytosolic (C1−C6), and five periplasmic (P1−P5) domains (*13*), and it forms the core of the passageway for the translocating polypeptide chain (*14*). Together with SecE, SecY also provides specific membrane binding sites for SecA. SecE, containing three TMs, stabilizes the SecY protein (*15*), and may serve as a gate for the protein conducting channel (*16*). SecG, with two TMs, is believed to facilitate SecA membrane cycling by switching its own membrane topology (*17*).

The SecYEG complex is believed to constitute the building block for the protein conducting channel (*18*). Electron microscopy of *Bacillus subtilis* SecYE reveals a ringlike structure in both detergent and reconstituted proteoliposomes (*19*). Though it is still unclear if one (*20*), two (*21*), or four (*22*) SecYEG complexes are involved in the formation of an active protein conducting channel, it has been shown that the oligomeric states of these complexes are dynamic. Three-dimensional electron image mapping of *E. coli* SecYEG complexes demonstrated a dimeric packing (*23*), yet the crystal structure of the translocon from *Methanococcus jannaschii* exhibited a monomeric SecY complex (*24*). Previous studies have revealed important contacts among the subunits of the *E. coli* SecYEG complexes; C1, C2, and C6 of one SecY protein are close to a neighboring SecY (*25*), C2 and C3 are close to SecG (*26*), and C4 and C5 are close to SecE (*27*−*29*), while C5 and C6 are important for interacting with SecA (*30*, *31*). The importance of the SecY TMs was also demonstrated recently through substituting the central six residues of individual TMs with those of either TM3 or TM4 from LacY (*32*). It was shown that the function of SecY is tolerant of sequence exchanges involving TM1, TM5, TM6, and TM8, whereas its function is much more sensitive to changes involving TM2, TM3, TM4, TM7, and TM10. While the architecture of subunit packing of SecYEG complexes themselves is emerging from electron image mapping (*23*) and the SecYEG crystal structure at a resolution of 3.2 Å (*24*), the faces within the complexes that embrace the translocating preprotein are still undefined. Through in vitro transcription, translation, and cross-linking of a labeled preprotein, Joly and Wickner (*33*) demonstrated that SecY and SecA are in close contact with the elongating nascent polypeptide. A similar study using yeast ER membranes (*34*) pointed to the close proximity between the signal

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[1] Abbreviations: AMP-PCP, adenylylmethylenediphosphonate; Bpa, benzoyl phenylalanine; BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; BS[3], bis(sulfosuccinimidyl) suberate; BSA, bovine serum albumin; DDM, *n*-dodecyl β-D-maltoside; DSS, disuccinimidyl suberate; DTT, dithiothreitol; ER, endoplasmic reticulum; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; TM, transmembrane segment.

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sequence of a transported protein and regions of Sec61p containing TM2 and TM7. Hints of the involvement of *E. coli* SecY in signal peptide recognition have been noted since the discovery of the SecY/prlA gene (*35*, *36*). Suppressor analysis of prlA mutations using dysfunctional LamB signal peptides revealed that the suppressor mutations clustered in distinct regions, and TM7 of SecY was proposed to function in signal sequence recognition (*37*). Yet no investigation has focused on the direct interaction between a signal peptide and the *E. coli* translocon. Consequently, it remains unclear whether the nascent polypeptide chain is merely translocating in close proximity to SecY, SecY performs simply a proofreading function (*37*), or SecY is more intimately involved in the specific recognition of the signal peptide.

In this study, we employed a synthetic peptide with the alkaline phosphatase signal sequence harboring a benzoyl phenylalanine (Bpa) residue for UV-induced cross-linking, to probe its interactions during signal peptide−SecYEG association. We find evidence for a direct and specific interaction with the wild-type signal peptide but not with a nonfunctional one. Signal peptide cross-linking with SecY, signal peptide-induced SecA ATPase activity in the presence of SecY, and the nucleotide-dependent preferences of the signal peptide−SecA and −SecY interactions provide insight into the changing environment of the signal peptide during ATP turnover. Moreover, it is found that the signal peptide cross-linked to SecY primarily through TM7 and TM2, while the adjacent periplasmic loop regions are not involved.

EXPERIMENTAL PROCEDURES

Materials. Reactive Blue 4 agarose, BSA (essentially fatty acid free), Folin and Ciocalteu's reagent, *Staphylococcus aureus* V8 protease (EC3.4.21.19), ATP, AMP-PCP, IPTG, PMSF, DTT, DDM, L-(+)-arabinose, and the octapeptide VHLTPVEK were purchased from Sigma Chemical Co. (St. Louis, MO). Preswollen DEAE-52 cellulose was from Fisher Scientific, and acetone-precipitated and ether-extracted *E. coli* phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). All other chemicals and reagents used in this study were either of molecular biology or analytical reagent grade. The *E. coli* wild-type alkaline phosphatase signal peptide (WT), MKQSTIALALLPLLFTPVTKAC-NH_2; 3K4L, MKQKKLALAAAALALSSSASAC-NH_2; and the nonfunctional 1K2L peptide, MKQQQAALAAAALAA-SSSASAC-NH_2, were synthesized using *FastMoc* chemistry and purified by reverse-phase HPLC as described earlier (*38*−*40*). The photoactive amino acid, *ρ*-benzoyl phenylalanine (Bpa), was substituted for phenylalanine in the wild-type sequence, and the peptide was designated WT(Bpa).

Bacterial Strains and Growth. E. coli strain BL21, harboring the plasmid pBAD22 with $His_{(6)}$SecEYG under control of the arabinose promoter, was kindly provided by F. Duong, University of British Columbia, Vancouver, BC. BL21 cells were grown with vigorous aeration at 37 °C in LB broth, containing ampicillin (100 μg/mL). Exponential cultures were induced with L-(+)-arabinose (1−2%) and growth continued for an additional 1−3 h. After the cells were harvested by centrifugation, the pellets were washed once with buffer A (10 mM Tris-HCl, pH 8.0 plus 20% glycerol) and stored at −70 °C. *E. coli* strain BL21.14pCS1, received from D. Oliver, Wesleyan University, and used for the overexpression of SecA, was also cultured in LB medium and induced with IPTG (1 mM) for approximately 3 h. Cells were collected and stored as described above.

Protein Purification and Proteoliposome Reconstitution. Overexpressed SecA was purified from *E. coli* strain BL21.14pCS1 by affinity chromatography on reactive blue 4 agarose as reported earlier (*39*−*41*). Inverted inner membrane vesicles (IMVs) were prepared from *E. coli* BL21, containing pBAD22 $His_{(6)}$-SecEYG, as described previously (*42*, *43*) with minor modification. The isolation and purification of $His_{(6)}$-SecEYG from IMVs was carried out essentially as described by van der Does et al. (*44*, *45*). SecY was identified after Western blotting using conventional procedures and anti-SecY antibody (1:2000), a generous gift of W. Wickner, Dartmouth Medical School. DEAE-52 purified $His_{(6)}$-SecEYG was reconstituted into proteoliposomes as described (*44*−*46*).

Translocation ATPase Activity. $His_{(6)}$-SecEYG proteoliposomes were evaluated for translocation ATPase activity essentially as described by Lill et al. (*47*) and Douville et al. (*10*) with minor revisions. Reactions (25−50 μL) contained either 50 mM Tris-HCl (pH 8.0) or 50 mM HEPES-KOH (pH 7.5) with 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, BSA (0.5 mg/mL), 0.4 μM SecA, reconstituted $His_{(6)}$-SecEYG proteoliposomes (1−2.5 μg protein/reaction), and, where indicated, 2−180 μM signal peptide. Reactions were started by the addition of 4 mM ATP, and the amount of inorganic phosphate (P_i) released was determined using the malachite green/molybdate colorimetric method (*48*). Calculated values, expressed as pmol of P_i released/min/μg of SecA, were converted to relative percent of SecA ATPase activity.

Peptide Biotinylation. Wild-type signal peptide and wild-type (Bpa) peptide were biotinylated via the cysteine sulfhydryl groups using EZ-Link PEO-maleimide activated biotin as described by the manufacturer (Pierce, Rockford, IL). Approximately 100 μL of PEO-maleimide activated biotin solution (10 mM in PBS) was added to 2.5 mL (1−2 mg) of the reduced peptide, and the mixture was allowed to react at room temperature for at least 2 h. The biotinylated peptides were purified by reverse-phase HPLC, lyophilized, and stored at −70 °C.

Cross-Linking Reactions. UV-photoactivatable cross-linking at 350 nm of WT(Bpa)-biotin to SecA and/or SecYEG was carried out at room temperature for 60 min or as indicated. After the addition of loading cocktail, the samples were incubated at 37 °C for 15−30 min and analyzed by SDS−PAGE, electroblotting, and chemiluminescent detection of the cross-linked biotinylated peptides as described below.

SDS−PAGE and In-Gel Digestion. SecY was separated from the SecYEG complex and signal peptide by 15% SDS−PAGE essentially as described by Laemmli (*49*). In the presence of 0.2 mM sodium thioglycolate, in-gel digestion with *S. aureus* V8 was performed according to Cleveland et al. (*50*), Pedersen et al. (*51*), and White and Cohen (*52*). Briefly, the band containing SecY was visualized by Coomassie Brilliant Blue staining, excised, soaked in 5% sucrose, 125 mM Tris-HCl (pH 6.8), 0.1% SDS plus 1 mM DTT, and then transferred to the bottom of a well in another 5% stacking and 16.5% Tris-tricine (*53*) SDS gel. V8 protease, 1 to 3 μg, was added, and electrophoresis was carried out

until the applied sample reached the bottom of the stacking gel. The power was shut off, and the gel was left overnight at 37 °C, after which electrophoresis was resumed and continued until completion.

In-Gel Chemical Cleavage of SecY. BNPS-skatole, which cleaves protein on the C-terminal side of unoxidized tryptophan residues (*54*), was used essentially as follows. Excised gel pieces, containing SecY, were dried using a speed-vac evaporator, and the vacuum was released under nitrogen. The reaction with BNPS-skatole (1 mg/mL) in 75% acetic acid was conducted at 60 °C for 1 h in the dark to avoid secondary reactions. The supernatant was removed and saved while the gel pieces were hand homogenized in 200 μL of 60% acetonitrile with 0.1% TFA and briefly bath sonicated to assist diffusion of the cleavage products. After centrifugation, the supernatant was saved and the extraction repeated two more times. The pooled supernatants were extracted twice with benzene, to remove residual BNPS-skatole, and dried as above. The cleavage pattern was analyzed after electrophoresis via Tris-tricine SDS−PAGE followed by electroblotting and chemiluminescent detection.

Electroblotting and Chemiluminescent Detection. The V8 digests and chemical cleavage products of SecY, resolved by gel electrophoresis, were transferred onto Immobilon PVDF membranes (Millipore) using a BioRad semidry or tank transfer method. Blocked and washed membranes were incubated for 1 h at room temperature with streptavidin-HRP (1:2500−1:4000). Chemiluminescent detection of the biotinylated complex was accomplished using the SuperSignal West Dura kit (Pierce, Rockford, IL). Band intensities were quantified on an LKB Ultrascan XL laser densitometer.

Additional Methods. Protein concentrations were determined either by the method of Bradford (*55*) or by a modified Lowry procedure (*56*) using BSA as a standard. The latter method was used primarily for IMVs and proteoliposomes.

RESULTS

We have demonstrated that the signal peptide itself, in the absence of mature protein, interacts with SecA, the peripheral ATPase subunit of *E. coli* translocase (*39, 40*). The subsequent step during protein transport involves the passage of the preprotein through the translocon, of which SecY is a predominant part. Consequently, it is tempting to consider whether the signal peptide interacts specifically with SecY as one might expect if it takes an active role in directing the preprotein through the export relay system. To address this issue we used purified $His_{(6)}$-SecEYG (referred to as SecYEG hereafter) and a synthetic peptide corresponding to the wild-type alkaline phosphatase signal sequence which we have characterized extensively in vivo (*38*) and in vitro (*39, 40*). As shown in Figure 1A, in the presence of SecYEG-reconstituted proteoliposomes, SecA ATPase activity increased about 3-fold compared to its endogenous activity. The activity was further enhanced by the presence of a functional signal peptide, WT, but not the nonfunctional one, 1K2L. Maximal peptide dependent activity occurred at about 100 μM WT peptide (Figure 1B). In the detergent micelle environment (0.03% DDM) in which SecYEG was purified, a SecYEG-dependent increase in SecA ATPase activity was also observed (Figure 1C). Sodium azide inhibition of activity was only noted when SecYEG was present (data not shown),

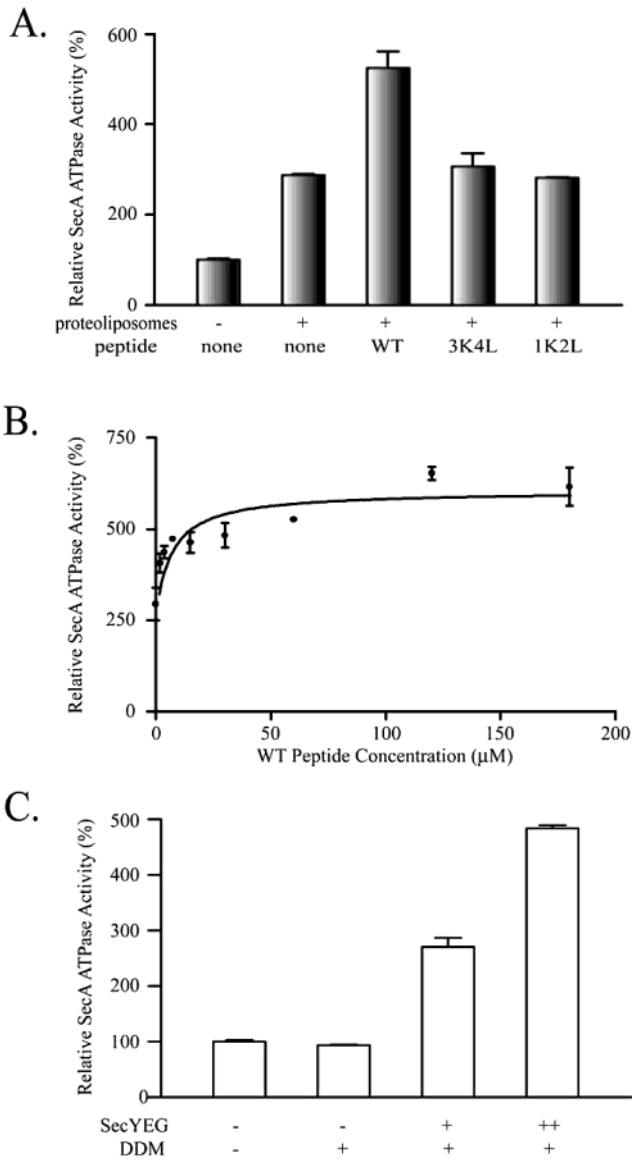


FIGURE 1: SecA ATPase activity in the presence of SecYEG. All reactions in A and B were performed in either 50 mM Tris-HCl (pH 8.0) or 50 mM HEPES-KOH (pH 7.5), containing 50 mM KCl, 5 mM $Mg(OAc)_2$, 1 mM DTT, 4 mM ATP, BSA (0.5 mg/mL), SecA (40 μg/mL), and reconstituted SecYEG proteoliposomes (1.0−2.5 μg of protein/reaction). The concentration of peptide used in panel A was 120 μM, and increasing concentrations of WT peptide (0−180 μM) were used in panel B. (C) The SecA ATPase activity in the presence of purified SecYEG (0.5 and 1 μg/reaction, + and ++, respectively) was determined in the above buffer but with a reduced magnesium concentration (0.5 mM). The activity of SecA alone in aqueous solution in the absence of SecYEG was set at 100%. Each data point represents an average of triplicate assays ± SE.

which is consistent with the notion that such inhibition is due to the stabilized SecA-inserted state at SecYEG (*44*). The data show that the SecYEG in detergent micelles or proteoliposomes forms an active complex with SecA and that the fidelity of the signal peptide−SecA interaction is maintained in the presence of SecY.

In this study, we take advantage of the photoaffinity label, benzoyl phenylalanine (Bpa), incorporated at the C-terminal end of the signal peptide hydrophobic core to explore the signal peptide−SecY interaction through cross-linking. This signal peptide was also labeled with biotin, WT(Bpa)-biotin, through the C-terminal cysteine residue to provide a means

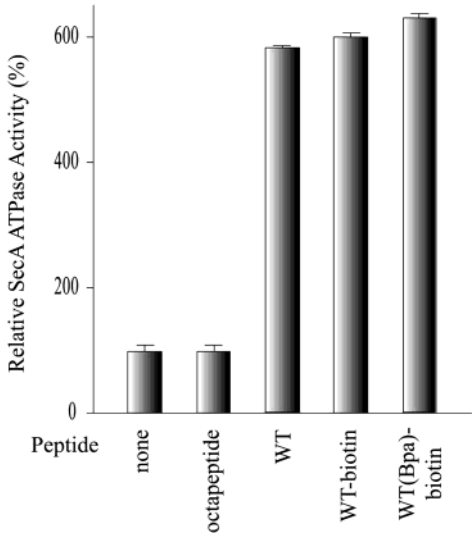


FIGURE 2: Effect of signal peptide derivatives on SecA/lipid ATPase activity. All assays were performed in 50 mM HEPES-KOH (pH 7.0), containing 30 mM KCl, 30 mM NH_4Cl, 0.5 mM $Mg(OAc)_2$, 1 mM DTT, 4 mM ATP, BSA (0.5 mg/mL), SecA (40 μg/mL), *E. coli* phospholipids (SUV, 300 μg/mL), and 20 μM peptide where indicated. The activity of SecA in the absence of peptide was set at 100%. WT-biotin refers to the peptide with the wild-type alkaline phosphatase signal peptide linked to biotin; WT-(Bpa)-biotin is the corresponding peptide which also includes the Bpa affinity label in place of phenylalanine. The sequence of the unrelated octapeptide is given in Experimental Procedures. Each data point represents an average of triplicate assays ± SE.

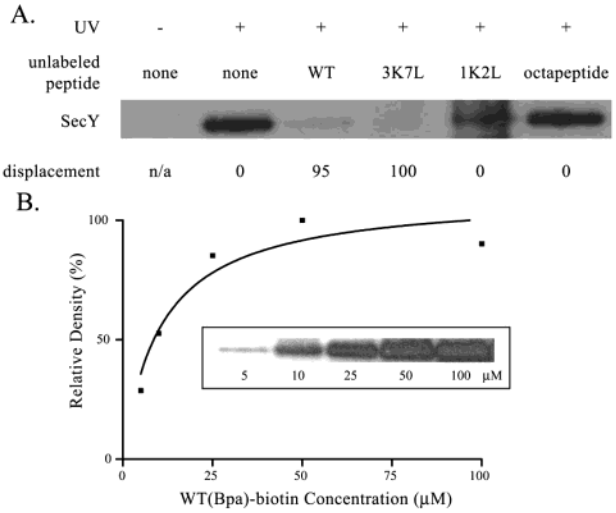
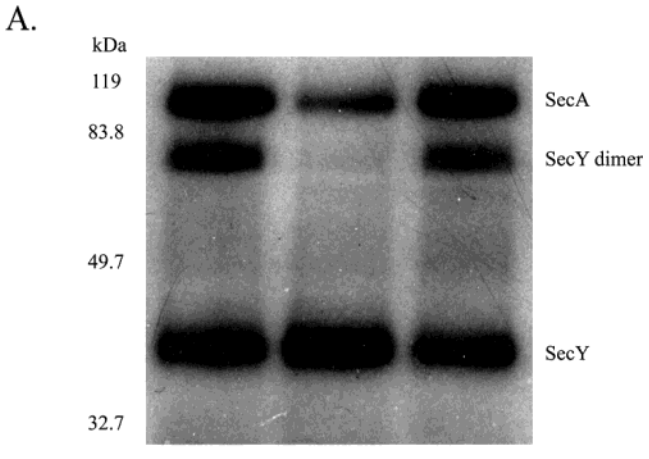


FIGURE 3: Specific cross-linking of SecYEG to WT(Bpa)-biotin. (A) SecYEG (1 μg protein/reaction) was cross-linked to 10 μM WT(Bpa)-biotin in the absence or presence of 150 μM nonbiotinylated peptide as indicated. All reaction mixtures were mixed on ice, followed by 60 min UV irradiation at room temperature. Reaction mixtures were supplemented with 6x loading cocktail, separated by 12.5% SDS−PAGE, electroblotted onto a PVDF membrane and detected via the biotin label as described in Experimental Procedures. The SecY−signal peptide bands migrated at approximately 36 kDa. The percent of biotinylated WT peptide displaced by the competing peptide is shown below each lane. (B) The relative intensities of the SecY−WT(Bpa)-biotin bands (shown in inset) were plotted against peptide concentration (0−100 μM). The intensity of the SecY band at 50 μM WT(Bpa)-biotin was set at 100%.

to detect the presence of the peptide. Given the position of the biotin attachment, the additional group is not expected to affect the characteristics of the signal peptide. This was confirmed by comparing the effect of the WT peptide and labeled analogues on SecA ATPase activity as shown in Figure 2. We found that all three peptides stimulated SecA to a similar degree, indicating that the Bpa and biotin labels do not alter the interaction. Pilot cross-linking experiments using amine specific homobifunctional cross-linkers, BS^3 and DSS, demonstrated close contact between the WT-biotin and SecY, but a high background was observed. The long linkage arm of these cross-linkers between the functional groups (11.4 Å) offers a large space in which neighboring entities can be detected. Furthermore, the need for external addition of these reagents renders the cross-linking inefficient. The requirement of amine groups for the cross-linking also introduces an artificial bias toward the interaction in which we are interested. The two lysine residues in the peptide reside at the N- and C-termini, yet the signature of a functional signal peptide is the hydrophobic core region. Bpa, a phenylalanine analogue which serves as a photoactivatable cross-linking initiator with a reaction radius of 6 Å, was incorporated within the signal peptide hydrophobic core, and the fact that it is photoactivated greatly improved our control over the cross-linking reaction, resulting in specific cross-linking relative to using homobifunctional cross-linkers.

As shown in Figure 3, Bpa-mediated WT(Bpa)-biotin cross-linking to SecY is readily observed upon UV irradiation at 350 nm. The electroblot of the cross-linking products separated by SDS−PAGE and detected for the presence of biotin revealed SecY-cross-linked adducts in the UV-irradiated sample. To evaluate the specificity of the interaction, we ran parallel competitive displacement reactions with unlabeled peptide. The cross-linking was effectively competed by the addition of a 15-fold excess of functional signal peptides, WT and 3K7L, whereas the presence of a similar molar excess of nonfunctional 1K2L and an unrelated octapeptide had no effect (Figure 3A). Moreover, the cross-linking of SecY and WT(Bpa)-biotin was concentration dependent and saturable (Figure 3B). This provides strong evidence that the signal peptide interacts with a defined site on SecY not unlike a receptor−ligand interaction.

Preprotein reaches the translocon and the SecA ATPase initiates the translocation process by membrane insertion. This is followed by a conformational change that occurs upon ATP binding at the translocon, and concomitantly, polypeptide chain movement proceeds. Hydrolysis of ATP results in the membrane deinsertion of SecA and release of SecA from the membrane surface (*57*). Considering that the signal peptide is likely to be passed through the relay system from SecA to SecY as the preprotein is translocated, it is important to ask how the SecY−signal peptide interaction is affected by SecA and nucleotides. When SecA is included in the cross-linking mixture, WT(Bpa)-biotin is cross-linked to it in addition to SecY (Figure 4). Interestingly, the cross-linking of peptide to SecY increased when ADP was added with an accompanying decrease in cross-linking to SecA. When AMP-PCP, a nonhydrolyzable ATP analogue, was present, SecY cross-linking decreased, with an increase in that of SecA, similar to the level in the absence of nucleotide. In addition, there was a loss of cross-linking to a band corresponding to a SecY SDS-stable dimer when SecA and ADP were present. Protein staining (data not shown) verified that a comparable level of dimer was still present; however,



B.

SecA	36%	27%	39%
SecY dimer	28%	0%	26%
SecY	36%	73%	35%

FIGURE 4: Nucleotide effect on the cross-linking of WT(Bpa)-biotin to SecY and SecA. Cross-linking reactions were conducted as described in Figure 3 with SecYEG, SecA (0.66 μg), WT(Bpa)-biotin (10 μM), and 5 mM ADP or AMP-PCP as indicated. (A) Cross-linking products are shown after chemiluminescent detection. (B) The relative intensity of individual cross-linking products within each reaction was quantified by densitometry.

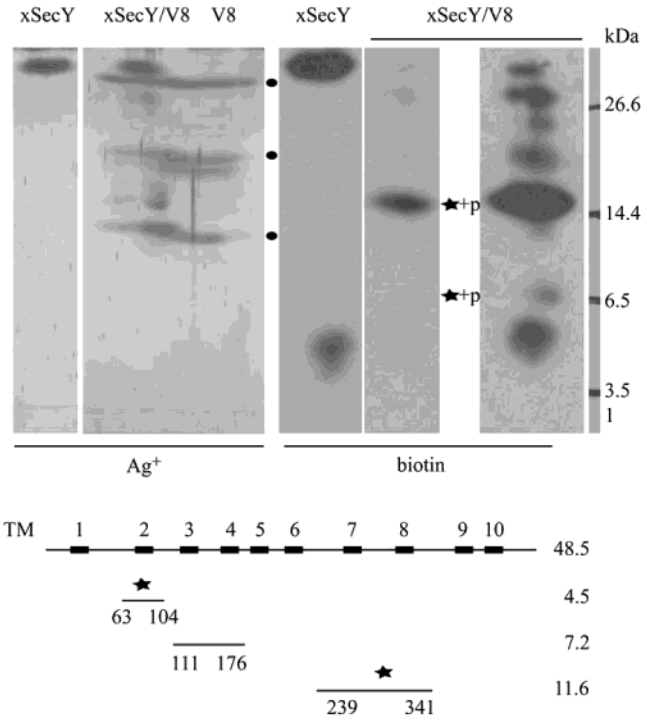


FIGURE 5: V8 proteolysis of SecY−WT(Bpa)-biotin complex. In-gel V8 digestion was conducted as described in Experimental Procedures. The fragmentation pattern, upon protein staining (Ag^+) and chemiluminescent detection (biotin) before (xSecY) and after proteolysis (xSecY/V8), shows the major bands resulting from SecY and the fragments resulting from autolysis of V8 protease (filled circles). The filled star depicts the fragments detected on the electroblot as well as that predicted from the V8 protease digest; +p indicates the signal peptide adduct. Shown below is the linear arrangement of SecY TM domains with the predicted cleavage fragments above 4 kDa estimated from the SecY sequence.

the biotin detection revealed no signal peptide cross-linking to this species. Our results are consistent with a signal peptide−SecA-ATP complex that is key for membrane insertion of the preprotein. Nucleotide turnover at SecA then facilitates the transfer of the signal peptide to SecY.

As the major component of the protein conducting channel, SecY has been shown to be in close contact with translocating polypeptide chains (*32*). Now a specific interaction between SecY and the signal peptide is also established (Figure 3A). To address where on SecY this interaction occurs, a scheme to cleave the SecY−signal peptide complex, followed by identification of the signal peptide containing protein fragments, was developed. To best retain the authenticity of the interaction and complex formation, we chose a scheme involving the intact wild-type SecY in complex with SecEG and we employed the Bpa photo-cross-linker within the signal peptide sequence as described above. The analysis is not trivial considering the highly hydrophobic nature of the SecY protein. Following covalent protein−peptide complex formation by UV irradiation, our strategy involved separation of the SecY−signal peptide adduct on SDS−PAGE and subsequent excision of the gel band for proteolysis. V8 protease was chosen for the cleavage because of the absence of acidic amino acid residues in the peptide, which ensures that the peptide portion of the complex remains intact. Initial attempts to retrieve the complex and perform proteolysis in solution resulted in little cleavage. However, modification of an in-gel digestion (see Experimental Procedures) turned out to be very effective, and V8 cleavage after incubation at 37 °C overnight was significant. It should be noted that under these conditions some proteolysis of the V8 protease itself also occurs. Using biotin detection, remarkably one major band was observed with an apparent molar mass of about 14.4 kDa (Figure 5, fourth panel) in a Tris-tricine SDS gel (*53*). On the basis of the amino acid sequence of SecY, four large V8 proteolytic fragments are predicted with one corresponding to residues Arg239−Glu341, encompassing TM7 and 8. This gives a calculated molecular mass of 11.6 kDa for the fragment alone and corresponds to the size of the detected band minus the mass of the attached WT(Bpa)-biotin (2.8 kDa). Upon increased exposure of the X-ray film, a second band with a molecular mass of 6.5 kDa was also apparent (Figure 5, far right), which may correspond to the adduct with fragment Met63−Glu104 (TM2). The high molecular weight bands that appear upon longer exposure are too large to represent fully digested protein; these partial digestion products likely contain the 14.4 kDa fragment.

To further define the localization and identification of the peptide binding site, we evaluated the chemical cleavage of SecY using BNPS-skatole which cleaves on the C-terminal side of tryptophan residues. Since SecY contains four Trp residues, complete cleavage would yield three major fragments: Met1−Trp173 (18.7 kDa), Leu174−Trp293 (13.0 kDa), and Leu303−Arg443 (15.9 kDa), and two minor fragments of 0.68 kDa and 0.2 kDa. Fragments with apparent molecular masses of approximately 21 kDa and 15 kDa were easily detected after in-gel chemical cleavage, electroblotting, and chemiluminescent detection of the biotinylated peptide (Figure 6). Considering that the cross-linked peptide adds an additional 2.8 kDa to the size of the fragment, the

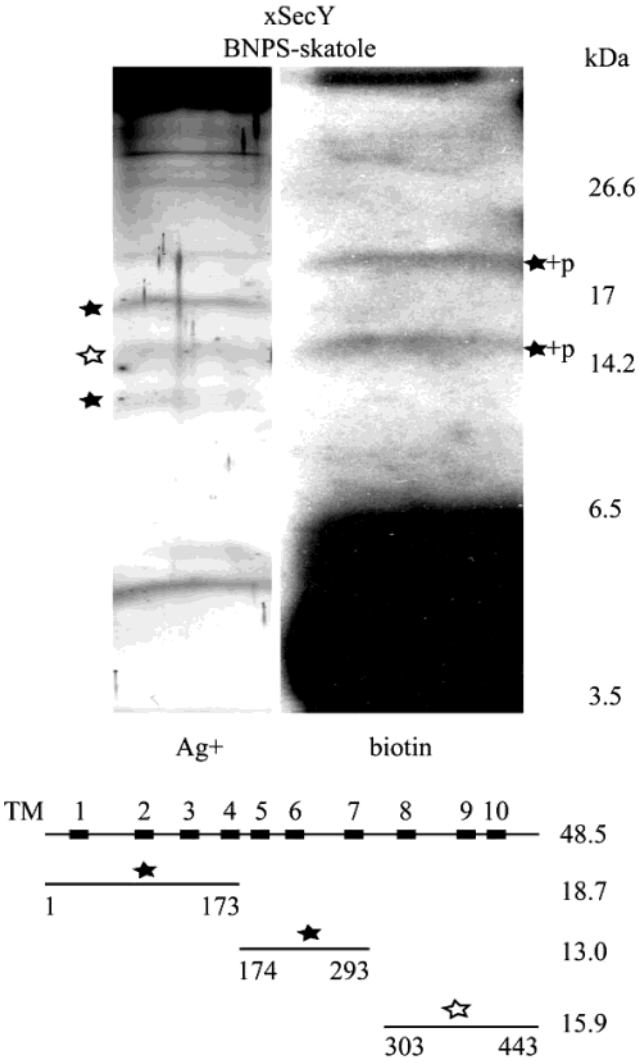


FIGURE 6: BNPS-skatole cleavage of the SecY−WT(Bpa)-biotin complex. BNPS-skatole cleavage was performed as described in Experimental Procedures. The SecY−WT(Bpa)-biotin fragments detected from protein staining (open and filled stars) and from the electroblot (filled stars +p) are marked. Shown below is the linear arrangement of SecY TM domains with the predicted cleavage fragments estimated from the SecY sequence.

observed fragments correspond to the regions containing Met1−Trp173 (TM1−TM4) and Leu174−Trp293 (TM5−TM7), respectively. The TM1−TM4 adduct was also detected when the same electroblot was probed with SecY antisera raised against the amino terminus (data not shown). The pronounced bands found in the very high and low molecular weight regions of the gel also appeared in control samples without BNPS-skatole, suggesting that they are not cleavage products.

The overlapping signal peptide cross-linked fragments detected from both V8 digestion and BNPS-skatole cleavage point to the primary SecY regions for signal peptide interaction as those corresponding to residues Arg239−Trp293 (TM7 plus the adjacent N-terminal cytoplasmic loop, C4) and residues Met63−Glu104 (TM2) of SecY. Significant peptide binding to any of the remaining TMs could not be demonstrated using our methods of detection, suggesting that they may be more structurally involved in the formation and orientation of the SecYEG channel.

DISCUSSION

SecY, in association with SecE and SecG, constitutes the primary component of the membrane-embedded channel for transporting proteins to extracytoplasmic sites (*5*). Due to its location at the core of the transport machinery, it has been the focus of considerable research aimed at understanding its structure and function. Previous studies have shown that *E. coli* SecY comes into close contact with the translocating polypeptide chain (*33*) and, in yeast, the signal peptide forms a helix in the process (*34*). We have now shown that the interaction is saturable and specific for functional signal peptides; neither a nonfunctional signal peptide nor an unrelated peptide effectively competes for binding. This parallels our previous findings for signal peptide−SecA interactions (*39*, *40*), suggesting that both components are integrally involved in signal peptide recognition; interactions with SecA promote targeting and membrane insertion of the preprotein while subsequent interactions with SecY ensure the translocation of a preprotein. That the signal peptide can be directly bound by SecA and SecY provides two points for quality control and underscores the importance of the selection of only secretory proteins for transport. Furthermore, a mechanism must exist for clearing the signal peptide from SecA following its release to ensure directional movement of the preprotein and to prohibit rebinding to SecA. Transfer to SecY provides one such mechanism. For preproteins which do not utilize SecA, SecY signal peptide recognition provides an especially critical proofreading step. The interaction may also serve to anchor the amino terminus of the preprotein at an early stage of translocation while more distal segments traverse the channel and/or promote a SecY conformational change that leads to channel opening (*18*).

In a manner consistent with the transfer of the signal peptide from SecA to SecY, cross-linking to these components is affected by the presence of nucleotides. When AMP-PCP is present, SecA is in a more extended conformation (58, *59*) and we observe higher levels of signal peptide−SecA cross-linking. Together with SecYEG, this may represent a SecA inserted state of the translocase (*44*) and corresponds to the initial delivery of the signal peptide into the membrane. Hydrolysis of ATP yields the more compact ADP-bound form of SecA (*58*, *59*). Concomitantly, SecA deinserts with transfer of the signal peptide to the translocon, and this is reflected in our observation of reduced cross-linking to SecA with an accompanied increase of cross-linking to SecY. Interestingly, we also see marked nucleotide-dependent differences in the level of signal peptide cross-linking to an SDS-stable SecY dimer (Figure 4). The distinct presence and absence of this dimeric signal peptide adduct, in the presence of AMP-PCP and ADP, respectively, suggests a possible SecA−signal peptide induced change in SecYEG dimerization at the time of the initial SecA membrane insertion step. This is consistent with the observations of Manting et al. (*22*), using scanning transmission electron microscopy, that the membrane insertion of SecA induces tetramerization of SecYEG. Retraction of SecA upon ATP hydrolysis leaves the signal peptide bound to the monomeric SecYEG, in agreement with the reported crystal structure of SecYEG, in the absence of SecA, which suggests that the translocating polypeptide is likely held by monomeric SecYEG (*24*). It should also be noted that the presence of

an SDS-stable SecY dimer that we observe is only suggestive of the presence of a SecYEG dimer and these cannot be strictly equated. Since SecYEG complexes exist in dynamic equilibrium (*21*) and translocation involves transient interactions among SecA, SecYEG, and preprotein, it is difficult to extrapolate from a defined set of experimental conditions to the biological process.

Specific cross-linking of the signal peptide to SecY was primarily through a region including TM7 and the adjacent cytoplasmic loop, and through that involving TM2. Considering the highly positively charged nature (+8) of the C4 loop region and the fact that it has been shown to interact with SecE (*27*), it is unlikely to be the direct contact site for the hydrophobic signal peptide core. Our results are consistent with previous reports on the signal sequence interaction with Sec61p in the ER membrane (*34*). Also in agreement with our data, the crystal structure of the channel revealed that TM7 and TM2 of SecY are both located at the lateral opening of the SecYEG with part of TM2 (designated TM2a) serving as a plug for the channel (*24*). Interestingly, the signal peptide cross-linking site is at the outside edge, and not the central core, of the channel. Collectively, the data are consistent with a model involving a conformational change in the orientation of SecY in the presence of an ATP loaded, SecA−signal peptide complex. This conformation change may involve a rotation of the polypeptide binding cleft to face that of the adjacent protomer. This would bring the signal peptide in contact with a dimeric form of SecYEG and provide the basis for the signal peptide cross-linked dimers we observe. Subsequent nucleotide turnover would power the translocation of the adjacent nascent chain through the central pore of the channel.

In bacterial systems such as *E. coli*, 25% or more of the total protein synthesized is finally localized in noncytoplasmic compartments. Understanding the movement of these proteins through membranes remains a pressing issue in biology. Use of a synthetic signal peptide harboring a photoaffinity label enabled us to trap the translocase in an early stage of translocation and dissect the interactions of the signal sequence in this process.

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