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# Probing the Affinity of SecA for Signal Peptide in Different Environments†

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## Abstract

SecA, the peripheral subunit of the *Escherichia coli* preprotein translocase, interacts with a number of ligands during export, including signal peptides, membrane phospholipids, and nucleotides. Using fluorescence resonance energy transfer (FRET), we studied the interactions of wild-type (WT) and mutant SecAs with IAEDANS-labeled signal peptide, and how these interactions are modified in the presence of other transport ligands. We find that residues on the third α-helix in the preprotein cross-linking domain (PPXD) are important for the interaction of SecA and signal peptide. For SecA in aqueous solution, saturation binding data using FRET analysis fit a single-site binding model and yielded a $K_d$ of 2.4 $\mu$M. FRET is inhibited for SecA in lipid vesicles relative to that in aqueous solution at a low signal peptide concentration. The sigmoidal nature of the binding curve suggests that SecA in lipids has two conformational states; our results do not support different oligomeric states of SecA. Using native gel electrophoresis, we establish signal peptide-induced SecA monomerization in both aqueous solution and lipid vesicles. Whereas the affinity of SecA for signal peptide in an aqueous environment is unaffected by temperature or the presence of nucleotides, in lipids the affinity decreases in the presence of ADP or AMP-PCP but increases at higher temperature. The latter finding is consistent with SecA existing in an elongated form while inserting the signal peptide into membranes.

More than one-third of the proteins synthesized inside the cell must be exported to extracytoplasmic locations to perform their functions. In *Escherichia coli*, many preproteins are recognized and transported by the Sec transport machinery. This secretory pathway has been extensively studied and several of the key proteins involved have been identified and characterized; however, the mechanisms by which the preprotein interacts with the secretion machinery are not clearly understood.

In the cytoplasm, SecB, a chaperone, binds preproteins to keep them in an unfolded state and delivers them to the membrane-associated SecA for post-translational export (1, 2). SecA is a critical component of the Sec transport pathway; it recognizes and binds the preprotein and functions as an ATPase. Moreover, conformational changes resulting from the interaction of SecB with SecA are thought to result in the transfer of the preprotein from the chaperone to the ATPase (3, 4). The membrane proteins, SecG, SecD, and SecF, stabilize and stimulate SecA at the membrane, and as a consequence, SecA can deliver the preprotein through the SecYEG pore (5, 6). Some studies indicate binding of ATP causes the dissociation of SecB from the enzyme, and cycles of ATP hydrolysis (7, 8) and conformational changes lead to membrane insertion of the SecA–preprotein complex followed by deinsertion of SecA (9, 10). Meyer et al. (11) demonstrated, using electron

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microscopy, that oligomers of SecYEG form protein-conducting channels in lipid bilayers that are sufficiently wide to accommodate part of SecA with the preprotein, yet the recent X-ray structure of SecYEG (12) revealed that the channel is too small to allow insertion of SecA; rather, the SecYEG pore is large enough only for preprotein incorporation, consistent with signal peptide recognition by SecYEG (13).

In solution, SecA has been found to exist as an antiparallel (14, 15) homodimer (16–18) which folds via a dimeric intermediate (19). Monomeric SecA in an open conformation has also been crystallized (20), and has a fold different from that of the proposed closed dimeric form from *Bacillus subtilis* (14) or *Mycobacterium tuberculosis* (21). Recently, a monomeric mutant of SecA has been found to be active in vivo, indicating that monomers of SecA must be active at some point during the translocation (22); however, the oligomeric state preferred for interactions with signal peptide in aqueous solution and membranes is unclear.

One SecA dimerization site is located at its C-terminus (18), which also binds SecY, SecB, and phospholipids (23), and not surprisingly, these and other ligands have been found to influence its dimerization state. A rapid monomer–dimer equilibrium depends on temperature, concentration, and ionic strength (24). According to some experiments, SecA exists as a monomer with some dimer present when bound to SecYEG (25). Benach et al. (26) have observed that SecA is a dimer in solution with or without the signal peptide bound, while the protein monomerizes when bound to lipids and redimerizes with addition of peptide. Fluorescence studies also show that *E. coli* phospholipids monomerize SecA, but suggest that it remains monomeric upon peptide addition (27). SecA, with either AMP-PCP[1] or ADP bound, remains a dimer in solution (27, 28), whereas it monomerizes in lipids, with different conformations depending on the nucleotide bound (29). SecA with bound ADP is more compact, while that with bound ATP is more elongated (29, 30). The thermal transition of SecA has also been studied (14, 31, 32); the conformation of SecA at higher temperatures has a higher affinity for phospholipids and is different from the conformation induced by the exothermic reaction of ATP hydrolysis (30).

SecA has a preprotein cross-linking domain (PPXD) (33) that is separated from the ATP binding domain by a long α-helix. The signal peptide binding site is not resolved in the X-ray structure (14), and the residues which are critical for the interactions are unknown. Tyr-326 has been found to be critical in controlling SecA–preprotein interactions (34). An N-terminally truncated SecA, representing the activated state, has been found to have high ATPase activity, binds the signal peptide with significantly higher affinity than WT SecA, and exists in a monomeric form (8, 35). This suggests that the affinity of the signal peptide for SecA depends on its conformational state (36). The proposed intramolecular regulator of SecA may control signal peptide binding by changing its ATPase activity and conformation (37).

Although numerous studies have indicated a direct interaction of the signal peptide with SecA (36, 38) leading to stimulation of its ATPase activity (39), few assays characterize this interaction at the low SecA concentration that is necessary for the determination of its affinity for the signal peptide and for preventing nonspecific reactions. Here, we have developed a fluorescence binding assay for SecA and signal peptide using nanomolar concentrations of SecA. Using synthetic signal peptide labeled with the acceptor, IAEDANS, FRET with SecA tryptophans is used to monitor signal peptide binding (40, 41).

[1]Abbreviations: AMP-PCP, adenylylmethylenediphosphonate; BSA, bovine serum albumin; CD, circular dichroism; DMSO, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; HPLC, high-pressure liquid chromatography; IAEDANS, 5-({[(2-iodoacetyl)amino]-ethyl}amino)naphthalene-1-sulfonic acid; MS, mass spectroscopy; SP, signal peptide; SP–P, signal peptide labeled with the IAEDANS probe; SUV, small unilamellar vesicle; WT, wild type.

We demonstrate specific and saturable binding of the signal peptide to SecA in aqueous solution and lipid vesicles. Screening several mutants of SecA for signal peptide binding using FRET shows that the third α-helix found in the PPXD is important for that interaction. At room temperature, SecA in lipids has a reduced affinity for the signal peptide and a sigmoidal binding curve suggestive of recognition by two different SecA conformational states. The low-affinity state of SecA is lost at higher temperature which has been shown to bring about an endothermic transition in its conformation (31, 32). Moreover, we establish a preference of monomeric SecA and the conformation induced by high temperature for signal peptide binding.

## EXPERIMENTAL PROCEDURES

### Materials

Reactive blue 4 agarose, ADP, AMP-PCP, and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). The fluorescent probe IAEDANS was obtained from Molecular Probes (Eugene, OR). Ni–NTA agarose was purchased from Qiagen (Valencia, CA). The *E. coli* wild-type alkaline phosphatase signal peptide, MKQSTIALALLPLLFTPVTKAC-$NH_2$, was synthesized by Biomolecules Midwest Inc. (Waterloo, IL), purified by HPLC and its identity verified with electrospray MS at the Keck Biotechnology Resource Laboratory at Yale University (New Haven, CT). The nonfunctional 3K2L peptide, MKQKKAALAAAALAASSSASAC-$NH_2$, was synthesized and purified as described previously (39, 42). Both peptides incorporate a carboxyl-terminal cysteine for labeling and end in an amide to prevent an unnatural negative charge (39). Small unilamellar vesicles (SUVs) were generated using *E. coli* phospholipids as described previously (38).

### Protein Expression and Purification

Substitution mutagenesis on pZ52-SecAHis plasmid, encoding an N-terminal His-tagged SecA (43), was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutations were confirmed by DNA sequence analysis. SecA was overexpressed and purified from the S300 fraction of *E. coli* strain BLR(DE3) expressing the plasmid as described previously (38, 44). His-tagged SecA was purified further to separate mutants from chromosomal WT SecA under nondenaturing conditions according to the manufacturer's protocol (Qiagen). The protein was dialyzed against 1× translocation ATPase reaction buffer [50 mM HEPES-KOH (pH 7.0), 30 mM KCl, 30 mM $NH_4Cl$, 0.5 mM $(CH_3\text{-}COO)_2Mg$, and 1 mM DTT]. Protein concentrations were determined by the Bradford assay at 595 nm using BSA as the standard (45). Working stock solutions were stored at 4 °C; ammonium sulfate precipitates were kept at −70 °C for long-term storage.

### Signal Peptide Labeling

For signal peptide labeling with IAEDANS, the peptide was dissolved in DMSO to a concentration of 3 mM and IAEDANS was dissolved in the reaction buffer [50 mM $NaH_2PO_4$ (pH 6.8)] to a final concentration of 10 mM. Signal peptide was added to 10 mL of the reaction buffer to a final concentration of 200 $\mu$M, and the probe was added to the reaction dropwise to a final concentration of 800 $\mu$M. The reaction mixture was incubated at room temperature for 4 h with shaking and the reaction stopped by freezing the mixture at −70 °C. The labeled signal peptide was purified with HPLC, lyophilized, dissolved in DMSO to a final concentration of 3 mM, and stored at −70 °C until it was used. The degree of labeling was calculated according to the manufacturer's instructions (Molecular Probes) using the formula $A_x/\varepsilon \times$ MW of peptide/(milligram of peptide per milliliter) = moles of dye per moles of peptide, where $A_x$ is the absorbance of the dye at the absorbance maximum wavelength and $\varepsilon$ is the molar extinction coefficient of the dye at the absorbance maximum

wavelength. All experiments involving the fluorescent probe were performed in the dark, and the tubes were covered with aluminum foil.

### Fluorescence Measurements

SecA at 50 nM in 1× translocation ATPase reaction buffer was used in fluorescence measurements. The molar ratio of lipids to SecA was kept at 950:1 as established previously (38), with addition of 1 mM nucleotide where indicated. Samples were incubated at 22 °C for 30 min before each measurement, unless otherwise indicated. The samples were excited at 297 nm and the emission spectra observed at 320–530 nm with a 4 nm band-pass width and 0.1 increments every 0.5 s on a FluoroMax3 instrument (Jobin Yvon, Inc., Edison, NJ). The instrument was connected to a water bath and equipped with Glen-Thompson polarizers. Maximal suppression of scattering of samples in small lipid vesicles was obtained by setting the excitation polarizer to 90° and emission polarizer to 0° as discussed by Ladokhin et al. (46). Some increase in fluorescence was observed with the unlabeled peptide, but the change was minor relative to the change due to FRET with the labeled peptide. The transfer efficiency was determined as described by Lakowicz (47) using the equation $E = 1 - F_{DA}/F_D$, which is called $1 - F/F_O$ in this study. Results were analyzed with GraphPAD from Prism (San Diego, CA) using a nonlinear regression fit as previously described (48) to determine $K_d$ or $K_i$. The $K_i$ value was calculated using the Cheng–Prusoff equation with a $K_d$ value of 2.4 $\mu$M. The Hill coefficient was determined from fitting the data to the Hill equation, $\theta = m_1 m_0^{m3}/(m_0^{m3} + m_2^{m3})$, where $m_0$ = [SP–P], $m_1 = B_{max}$, $m_2 = K_d$, and $m_3$ is the Hill coefficient. For acrylamide quenching experiments, the samples were incubated at 22 °C for 30 min, followed by addition of an 8 M acrylamide stock to final concentrations as indicated. The sample was then mixed and the measurement taken. The Stern–Volmer constant ($K_{SV}$) was determined by fitting the data to the equation $F_o/F = 1 + K_{SV}[Q]$, where [Q] is the concentration of acrylamide (47).

### Step Gradient Native Gel Electrophoresis

SecA (500 nM) was incubated at 25 °C for 30 min in the presence or absence of 1 or 20 $\mu$M wild-type alkaline phosphatase signal peptide in 1× translocation ATPase reaction buffer containing 10% glycerol. To examine the influence of lipids, SecA was incubated with SUVs at a lipid:SecA molar ratio of at least 950:1 as in the fluorescence measurements, prior to incorporation into the PAGE gel. Under these conditions, we expect some boundary lipid remains associated with SecA and changes in the state of SecA during electrophoresis are minimal, analogous to the results for detergent–membrane protein complexes run on native gels (49). All reactions were run on 4 to 12% native (50) step gradient gels followed by silver staining.

## RESULTS

### Binding of the Signal Peptide to SecA Monitored by Fluorescence Resonance Energy Transfer

The substantial overlap between tryptophan fluorescence and IAEDANS absorption spectra provides the basis for a fluorescence resonance energy transfer (FRET) assay (51–53) for assessing signal peptide–SecA interactions. A synthetic peptide corresponding to the alkaline phosphatase signal sequence with a C-terminal cysteine residue (39) was labeled with IAEDANS and purified by HPLC (Figure 1A). Labeling resulted in a reduction in the elution time (31.21 vs 31.82 min), indicating that the labeled peptide was slightly more hydrophilic than the unlabeled one. However, the extent of SecA ATPase stimulation (ref 39 and data not shown) and the dose-dependent binding to SecA by both peptides was comparable (see below), suggesting that the presence of the probe did not significantly impact SecA interactions. The final product was found to consist of >95% labeled peptide.

Since the emission of SecA tryptophans and the absorbance of IAEDANS-labeled signal peptide overlap in the region of 320–380 nm (Figure 1B), proximity (<60 Å) and a favorable orientation of donor and acceptor fluors (47) can result in FRET with a concomitant reduction in the intrinsic tryptophan fluorescence of SecA and an enhancement in signal peptide–IAEDANS fluorescence. This was observed in a manner that was dependent on signal peptide–IAEDANS concentration (Figure 1C). No FRET was observed if BSA, containing three tryptophans, was used in place of SecA (data not shown).

The equilibrium binding affinity of the IAEDANS-labeled signal peptide for SecA was measured from the labeled signal peptide concentration dependence on tryptophan fluorescence quenching due to FRET at 22 °C (Figure 2A). While *E. coli* SecA has seven tryptophans, three have been found to primarily contribute to its fluorescence (54); these provide sufficient fluorescence intensity that we were able to use SecA at a concentration as low as 50 nM and employ the standard assumptions regarding free and total ligand concentration used in derivation of $K_d$. A comparable scheme using multiple tryptophans as donors was previously used to study DEAD-box protein A (55). We found that the extent of FRET was signal peptide dose-dependent and saturable. At saturation, ~50% of the SecA fluorescence remained which could be contributed by tryptophans not sufficiently close to the bound acceptor for FRET or from incomplete transfer from more proximal donors. The best fit of the data was found with a single-site binding model (Hill coefficient, 1.1 ± 0.2) and yielded a $K_d$ of 2.4 ± 0.4 $\mu$M for signal peptide–IAEDANS binding (Table 1).

Separately, the binding affinity of the signal peptide for SecA was determined using the IAEDANS-labeled signal peptide at 3 $\mu$M as a tracer and competition with a concentration range of unlabeled signal peptide. The fluorescence of SecA lost due to FRET was restored with an increase in the concentration of the unlabeled peptide (Figure 2B). From these data, a $K_i$ of 2.5 ± 0.5 $\mu$M was calculated (Table 1), in agreement with the saturation binding analysis. In contrast, no significant change in FRET was observed when a synthetic peptide (3K2L) corresponding to a nonfunctional signal sequence (42) was used for competitive displacement of the labeled wild-type signal peptide (Figure 2B inset).

The three SecA tryptophans which fluoresce most substantially, W701, W723, and W775 (54), and a fourth, W349, are located in the preprotein translocation domain of SecA (21). This region includes the preprotein cross-linking domain (PPXD), the helical scaffold domain (HSD), and the helical wing domain (HWD). To assess the relative contribution of these tryptophans to FRET with the signal peptide, we progressively replaced them with phenylalanine. As shown in Figure 2C, substituting only W775 (in HSD) resulted in an 18% reduction in the level of FRET relative to that with wild-type SecA; the additional substitution of W701 and W723 (both in HWD) resulted in only an additional 6% loss in the level of FRET, while the substitution of W349 (in PPXD) in addition to the other three substitutions resulted in a further 39% loss in the level of FRET with the labeled signal peptide. Assuming the interactions between these tryptophans and any intramolecular quenching is negligible (54), of the tryptophans examined W349 appears to contribute most substantially to the observed FRET while W775 also plays a role.

## Residues on SecA Vital for Signal Peptide Binding

Site-directed mutagenesis and FRET were used to screen residues on SecA that are important for interaction with the signal peptide. I225N, M235N, V239N, I243N, I327N, and I335N/V336N SecA mutants are comparable (≤12% difference) to WT SecA in the ability to transfer energy to the IAEDANS-labeled signal peptide (Figure 3A). L314N and L319N elevate, while V310N decreases to some extent, the efficiency of transfer. These latter three are mutants found on an α-helix in the PPXD which is the third helix from the PPXD N-terminus, encompassing residues 292–319 (hereafter called the third α-helix in

PPXD). Residue 319 was used to further explore the importance of this region in signal peptide binding. The triple change of M305, A312, and L319, all to asparagine, led to a significant decrease in the level of FRET, as did individual substitutions of residue L319 (Figure 3A). The circular dichroism spectra for all mutants analyzed were found to be comparable to those of wild-type SecA (Figure 3B, data not shown), indicating no significant difference in their structure, although we cannot entirely rule out the possibility that small structural changes may have contributed to the change in FRET. Panels C and D of Figure 3 show the structure of *B. subtilis* SecA PPXD and the location of the corresponding *E. coli* substitution mutants. Note the most dramatic changes are observed when the residues on the third α-helix on the PPXD, highlighted in the box, are altered (14).

## Comparison of SecA–Signal Peptide Interactions in Aqueous and Lipid Environments

Using SecA incorporated in small unilamellar vesicles composed of *E. coli* lipids at a 950:1 molar ratio shown to be critical for its lipid integration and peptide-stimulated activity (38), binding with the IAEDANS-labeled signal peptide was assessed using FRET analysis. In contrast to the hyperbolic relationship between reduction in SecA fluorescence and signal peptide concentration observed in aqueous solution, in lipids the binding isotherm adopts some sigmoidal character (Figure 4A). The data yielded a Hill coefficient of 1.9 ± 0.2, and a best fit was found with a two-site binding model giving a $K_{d1}$ of 0.9 ± 1.0 $\mu$M and a $K_{d2}$ of 11.2 ± 2.8 $\mu$M (Table 1).

We considered the possibility that at low concentrations the signal peptide might be sequestered into the lipid; however, we have previously determined that the affinity of the signal peptide for SecA is substantially higher than its affinity for lipid (39), making this unlikely. Moreover, we examined the accessibility of the IAEDANS probe on the signal peptide in the absence and presence of lipids by acrylamide quenching (Figure 4B). In either case, the quenching is less pronounced in the presence of SecA but not in the presence of BSA (data not shown), consistent with diminished accessibility of IAEDANS upon signal peptide binding.

## Signal Peptide-Induced Changes in the SecA Oligomeric State

Since the binding isotherm for SecA in lipid is suggestive of two different signal peptide binding species, we examined whether this reflected different oligomeric states of SecA. Using nondenaturing PAGE, we found that SecA was predominantly in the dimeric form in aqueous solution (Figure 5), consistent with earlier analyses (16, 17, 56). Maintaining the protein:lipid ratio used in the FRET experiments, we found that SecA remained dimeric in the presence of *E. coli* lipids. Interestingly, addition of the signal peptide induced SecA monomer in a manner that is dependent on the signal peptide concentration. No difference was observed at 10-fold higher or lower SecA concentrations (data not shown) or in the presence of lipid (Figure 5). In the latter circumstance, the integrity of the vesicle is likely lost during PAGE, and it remains possible that the oligomeric state of SecA is altered during this process. Nonetheless, the data suggest that the signal peptide preferentially binds monomeric SecA in aqueous solution and membranes.

## Influence of Nucleotide Binding and Temperature on SecA–Signal Peptide Interactions

Addition of nucleotides, ADP or AMP-PCP, had little influence on the signal peptide concentration dependence of the energy transfer between SecA and IAEDANS in aqueous solution, though the level of FRET at saturation of peptide binding was somewhat diminished (Figure 6A). In lipids, both nucleotides resulted in a shift of the binding curve to the right beyond that observed in lipids in the absence of nucleotides, indicative of weaker signal peptide binding and still yielding a Hill coefficient of 2 (Figure 6B). We estimate conversion to a $K_d$ of 4.5 $\mu$M for the high-affinity signal peptide binding site (from 0.9 $\mu$M

in the absence of nucleotide), while the determination for the low-affinity site is not feasible because the reduced binding affinity makes it difficult to fully saturate the SecA with the IAEDANS-labeled signal peptide. Nonetheless, the change was not nucleotide specific, indicating that ATP hydrolysis might affect more than signal peptide membrane insertion during the translocation reaction of SecA (14, 57).

Although our data (Figure 5) suggest that substantial differences in the stoichiometry of oligomers do not account for the weakened, sigmoidal binding relationship that we observe in lipid relative to aqueous environments, we considered that other conformational differences might be involved. Since an endothermic transition in the conformation of SecA occurs at 37 °C which involves cooperative changes in domain–domain interactions of the SecA promoter (14, 54, 57–60), including an unwinding of the HWD domain (14), we examined the impact of higher temperature on SecA and signal peptide–IAEDANS FRET. Remarkably, at 37 °C, the binding isotherm for SecA in lipid shows a shift toward that observed for SecA in aqueous solution at either 22 or 37 °C (Figure 6C). Furthermore, the binding data yielded a Hill coefficient of 1.5 ± 0.09, suggesting the partial loss of the population with lower signal peptide binding affinity and which is produced in lipid at 22 °C. Previous studies have indicated that under some conditions, dimers of SecA exist which differ in conformation (61). It may well be that such differences involved in signal peptide recognition are promoted in the lipid at 22 °C but not at 37 °C. A change in oligomeric state per se does not seem to be responsible for the enhanced signal peptide affinity observed in the lipid at 37 °C but rather a conformational change induced by temperature (14, 31, 32) that controls signal peptide binding and may correspond to the elongated form involved in interacting with SecYEG (32).

## DISCUSSION

The signal peptide is the critical feature that distinguishes proteins destined for extracytoplasmic locations from those that reside in the cell cytosol. Specific recognition of the signal peptide by one or more components of the translocation machinery provides one mechanism for ensuring the fidelity of the transport process. Since SecA plays a pivotal role in sieving signal peptide-containing proteins from others in the cytosol and in initiating the membrane translocation step, its interaction with signal peptides is of particular interest. Here we have investigated the affinity of wild-type and mutant SecA for the signal peptide in aqueous solution and lipid vesicles, in the presence of nucleotide, and upon induction of a key thermal transition, conditions which represent SecA at different stages of the translocation process.

Using a synthetic signal peptide, we demonstrate that the wild-type alkaline phosphatase signal sequence binds SecA in a dose-dependent and saturable manner. Using a sensitive FRET strategy that takes advantage of the intrinsic fluorescence of SecA, we were able to study the interaction at low (nanomolar) levels of SecA. For the interaction in aqueous solution, we find a $K_d$ of 2.4 $\mu$M. This is comparable to the value found for SecA and the model signal peptide, 3K7L, using a biosensor approach (37) and the $EC_{50}$ for wild type and model signal peptide-induced stimulation of SecA ATPase activity observed for both the wild type and model signal peptide (39). Although peptide binding does not require the presence of nucleotide (this study and refs 39 and 54), collectively the data suggest that in the presence of ATP, hydrolysis will occur concomitantly with peptide binding and that these processes are closely coupled. That the affinity of SecA for the signal peptide is relatively weak (micromolar) is not surprising in view of the wide variety of signal peptides with which it must interact and the necessity that the preprotein be readily released from SecA during translocation.

Although the structures of *B. subtilis* (14) and *M. tuberculosis* (21) SecA have been determined by X-ray crystallography, the signal peptide binding site remains unclear. The preprotein cross-linking domain (PPXD) was originally proposed on the basis of cross-linking with the purified preprotein and nested truncations of SecA (33); however, a similar study using synthetic signal peptide indicated the site was amino-terminal to the PPXD (37), while molecular modeling indicates that a groove between HSD/HWD and the PPXD is a potential site of signal peptide interaction (20). By examining the extent to which different SecA tryptophans contribute to FRET, we gain insight into this issue. Residues W701, W723, and W775 contribute most to SecA fluorescence (54) and consequently have the potential to contribute most substantially to FRET with the IAEDANS-labeled signal peptide. While we have not accounted for differences in quantum yield among the three, of these substitutions W775 had the strongest effect, and this is consistent with its location close to the third helix in the PPXD (see Figure 3C,D). Residues W701 and W723 are more peripheral, located outside the PPXD on the HWD. Moreover, the conformational change concomitant with SecA monomerization suggested by Rapoport and colleagues (20) puts residues 701 and 723 even farther from the PPXD. In contrast, although W349 has a substantially lower quantum yield, its substitution nonetheless has the most profound effect on FRET, underscoring the fact that its location and orientation are well suited for efficient transfer with the IAEDANS-labeled signal peptide. This is consistent with its location in the PPXD and the possibility that it is proximal to the IAEDANS-labeled signal peptide. While we cannot discount the possibility that these mutations resulted in structural changes that impacted signal peptide binding and thus FRET indirectly, the relatively conservative nature of the tryptophan for phenylalanine exchange and the finding that the peptide-stimulated ATPase activities of the mutant SecAs and the wild type were comparable (data not shown) suggest the primary impact is on the extent to which the residues contributed to FRET with the bound signal peptide.

Several SecA mutants were also generated involving exchange, in a possible peptide binding region, of naturally occurring hydrophobic residues for the small polar residue, asparagine. The FRET strategy was used to assess the impact on signal peptide interaction because it provides a relatively fast assay for screening a variety of mutations. We did not detect any change, relative to the wild type, in the FRET of mutants involving residues I225, M235, V239, and I243. These residues are in the substrate specificity domain (SSD) encompassing amino acids 219–244 and previously identified as a possible signal peptide interaction site (37). Mutants I327N and I335N/V336N also had little effect on signal peptide binding. In contrast, mutations found to most profoundly affect the interaction are on the third helix of the PPXD. Interestingly, the L314N and L319N mutants reproducibly yielded enhanced FRET. These residues are positioned on the same face of helix 3, are close together in the three-dimensional structure (see Figure 3C,D), and are oriented such that they may impact the extent to which SecA is in the open and closed forms observed via X-ray crystallography (14, 20) and thus influence the accessibility of the signal peptide binding site. It is tempting to speculate that the individual substitution for a small, polar but nonobtrusive (not charged) residue at this location might serve to weaken the interaction, rendering the signal peptide binding site more accessible. Several other residues were individually exchanged with L319, and these all perturbed the interaction with the signal peptide as did the asparagine substitution in combination with others (e.g., M305N/V312N/L319N). These data point to the sensitivity of signal peptide binding to alterations in this region (see Figure 3A,C,D).

The binding isotherm at 22 °C for the interaction of the signal peptide with SecA in lipid vesicles yields a sigmoidal relationship that best fits a two-site binding model. In other systems, a sigmoidal relationship for the interaction of a protein with its ligands, in the absence of cooperativity, is due to the presence of a mixture of isoforms that have different affinities for the ligand (62). Our data are consistent with the existence in lipid of two

different SecA conformations with different affinities for the peptide. Previously, using anisotropy measurements, a sigmoidal relationship for binding of the signal peptide to SecA in detergent was observed under conditions in which peptide induced dimerization of SecA (26). Under the conditions used in this study (discussed below), we find that signal peptide induces SecA monomerization in aqueous and lipid environments (Figure 5), and therefore, multisubunit cooperativity does not appear to be involved. At 37 °C with SecA in lipid vesicles, the data suggest the population of SecA becomes enriched in the conformation that has a higher affinity for the signal peptide. We conclude that with respect to signal peptide interaction, the endothermic conformational transition of SecA is different from the conformation induced by lipid binding at 22 °C; the former has a higher affinity for the peptide. In addition, the presence of nucleotide reduces further the affinity of lipid-bound SecA for the signal peptide, consistent with ADP binding inhibiting the endothermic conformational transition of SecA (30). In the context of translocation, this may help to ensure that one consequence of SecA–signal peptide membrane insertion is release of the signal peptide for interaction with SecY (13).

Using native gels to analyze the oligomeric state of SecA, we find SecA exists primarily as a dimer in aqueous solution and vesicles of *E. coli* lipids in the absence of signal peptide consistent with earlier findings (17, 29) but that the signal peptide induces formation of the monomeric state of SecA in both environments. A comparison of our experimental setup with that of other studies, in which water-soluble lipids are found to promote SecA monomers but signal peptide induces dimers (26) or lipid induces monomers and signal peptide maintains that state (27), reveals a few key elements become apparent. Monomeric SecA is found in the presence of the signal peptide when the lipid:SecA ratio is at least 950:1 and with the signal peptide concentration lower than 100 $\mu$M (this study and ref 27). Indeed, we have examined the importance of the lipid:SecA ratio on signal peptide-induced ATPase activity (38) and have observed that at 100 $\mu$M synthetic signal peptides often aggregate and induce SecA aggregation that withstands SDS–PAGE (our unpublished observations). Interestingly, within the range that was employed, the concentration of SecA played less of a factor; Or et al. (27) at 1.5 $\mu$M used the highest concentration of SecA yet observed evidence of monomers using FRET and cross-linking with SDS–PAGE.

Using nondenaturing gels, we have established the preference of the signal peptide for the monomeric form of SecA. Recently, the function of a SecA mutant which does not dimerize was evaluated in *E. coli*, and it was found that preprotein transport proceeded unabated (22). Our earlier work indicating that SecB interacts preferentially with SecA monomers (2) and the finding of others (26, 27) that lipids can enhance monomerization all underscore the notion that SecA functions as a monomer during translocation.

A recent model suggests that nucleotide free SecA binds to the translocon, and upon interaction with SecYEG, SecA inserts the preprotein into the channel (25, 63). Binding of ATP leads to deinsertion and ATP hydrolysis to dissociation of SecA from the membrane (30, 64). Our data suggest that SecA has a high affinity for the peptide in its temperature-induced elongated state in the absence of nucleotide. This species could correspond to the open conformation observed in the crystal structure (20). The elongated form inserts the signal peptide into the membrane; once the peptide is inserted, its affinity for SecA must be weakened before SecA retracts to the membrane surface so as not to take the preprotein with it. Binding of ATP leads to a decreased affinity for the signal peptide, consistent with our observations, and retraction of only SecA follows. The signal peptide is free to interact with SecYEG (13), and ATP hydrolysis leads to the release of deinserted SecA for exchange with another in the cytoplasm (9).

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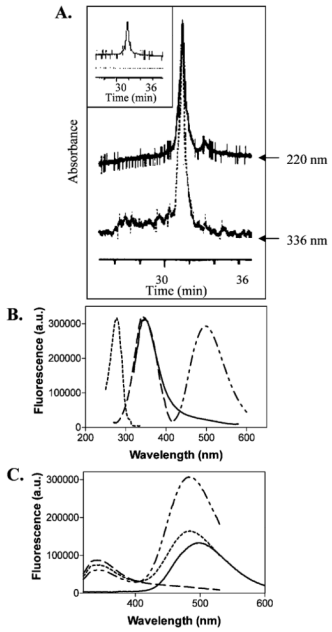


**Figure 1.**

Design of the fluorescence resonance energy transfer assay. (A) HPLC spectra of the WT signal peptide labeled with IAEDANS (SP–P). Signal peptide absorbs at 220 nm (__), and the probe absorbs at 336 nm (- - -). In the inset, nonlabeled peptide absorbs at 220 nm but not at 336 nm. The peptide was eluted from a C4 analytical column with 61% acetonitrile in 0.1% trifluoroacetic acid. (B) Spectral overlap of SecA tryptophan emission fluorescence and SP–P: absorption spectrum of SecA (- - -), emission spectrum of SecA (__), absorption spectrum of SP–P (– – –), and emission spectrum of SP–P (– - - –). (C) SecA tryptophan fluorescence quenching and IAEDANS fluorescence enhancement: SecA alone (– – –), SP–P alone (__), and SecA with 1 (- - -) and 4 $\mu$M (– - - –) SP–P. Fluorescence emission maxima are observed at 340 and 480 nm for SecA and IAEDANS, respectively.

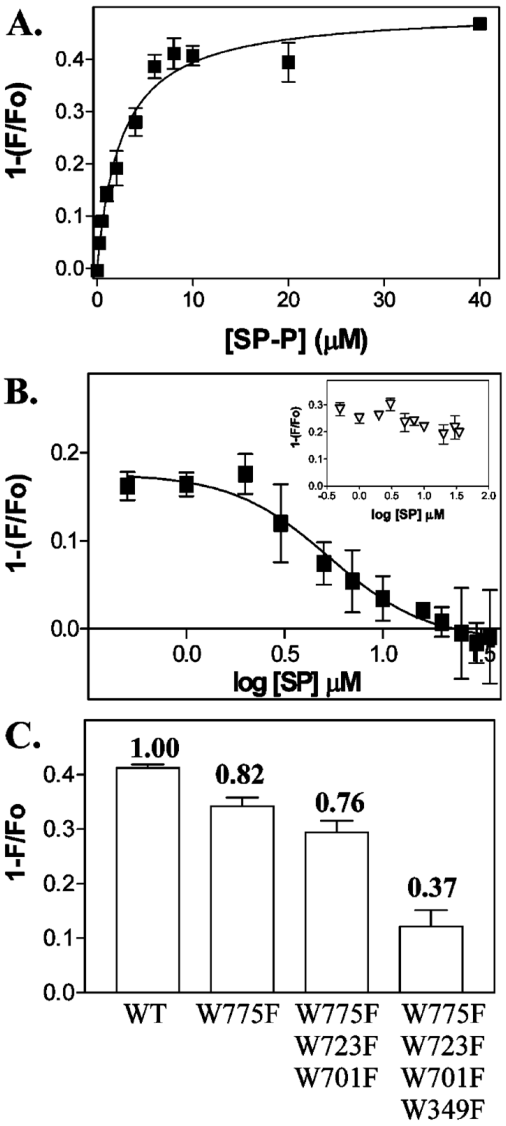


**Figure 2.**

Equilibrium binding of IAEDANS-labeled signal peptide (SP–P) and SecA monitored via fluorescence resonance energy transfer. (A) Saturation binding of SP–P to SecA. The fluorescence of SecA with each addition of SP–P was normalized to the SecA fluorescence without SP–P. The curve was fit to a one-site binding model. $F_o$ is the fluorescence of SecA alone, and $F$ is the fluorescence of SecA after addition of SP–P at 345 nm. (B) Competition binding of functional (WT) and nonfunctional (3K2L) signal peptides (SP) to SecA. SecA was incubated with 3 $\mu$M SP–P prior to addition of nonlabeled wild-type (■) or 3K2L (▽) signal peptide (shown in the inset). (C) Contribution to FRET of specific tryptophans (indicated below the bar) in the region of the proposed peptide-binding site. Numbers in bold, above the bars, indicate the fraction of FRET remaining for each mutant relative to WT SecA. Each data point represents an average of at least three separate experiments performed in duplicate.

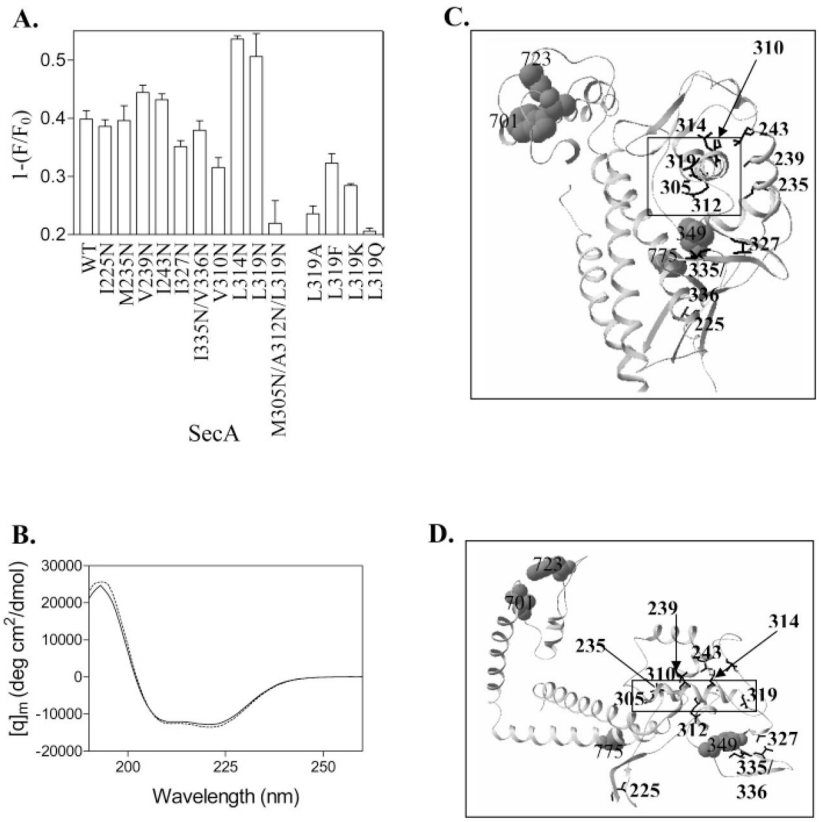


**Figure 3.**

Screening for the signal peptide binding site on SecA using FRET and mutants of SecA. (A) Effect of SecA residue substitutions on the efficiency of FRET. $F_o$ is the fluorescence of SecA alone, and $F$ is the fluorescence of SecA after addition of 6 $\mu$M SP–P at 345 nm. Each data point represents an average of at least two separate experiments performed in duplicate. (B) CD spectrum of WT SecA (__) and a representative mutant SecA (- - -) spectrum. (C and D) Structures of *B. subtilis* SecA translocation domain in closed [PDB entry 1M6N (14)] and open [PDB entry 1TF5 (20)] conformations, respectively, shown at different angles. Positions of mutants examined are marked in bold, and the third α-helix (residues 292–319) of the PPXD domain is highlighted in the box. Space-filled amino acids are tryptophans analyzed in Figure 2C and are marked by the residue number corresponding to the *E. coli* sequence.

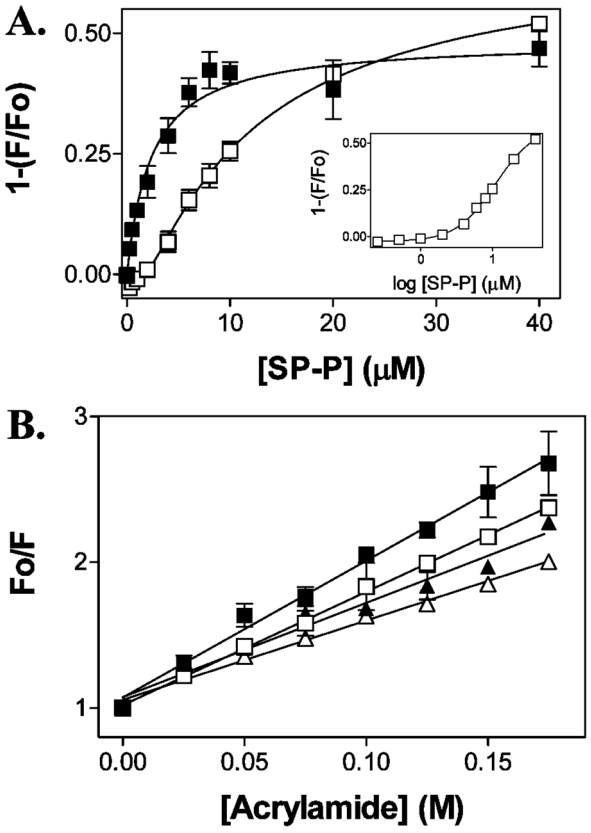


**Figure 4.**

Comparison of binding of the signal peptide to SecA in aqueous solution and lipid vesicles. (A) Saturation binding of the IAEDANS-labeled signal peptide (SP–P) to SecA in an aqueous environment (■) and in *E. coli* phospholipids (□). The inset shows a magnification of the binding curve at low signal peptide concentrations for SecA in lipid. $F_o$ is the fluorescence of SecA alone, and $F$ is the fluorescence of SecA after addition of SP–P at 345 nm. (B) Stern–Volmer plots of acrylamide quenching of SP–P. The fluorescence of the labeled peptide without the quencher ($F_o$) and fluorescence of the sample after the addition of acrylamide ($F$) at 500 nm were monitored in the absence of SecA in aqueous solution (■) and in the presence of lipid vesicles (□). Parallel measurements were taken for SP–P with 50 nM SecA in aqueous solution (▲) and in lipid vesicles (△). Each data point represents an average of at least three separate experiments performed in duplicate.

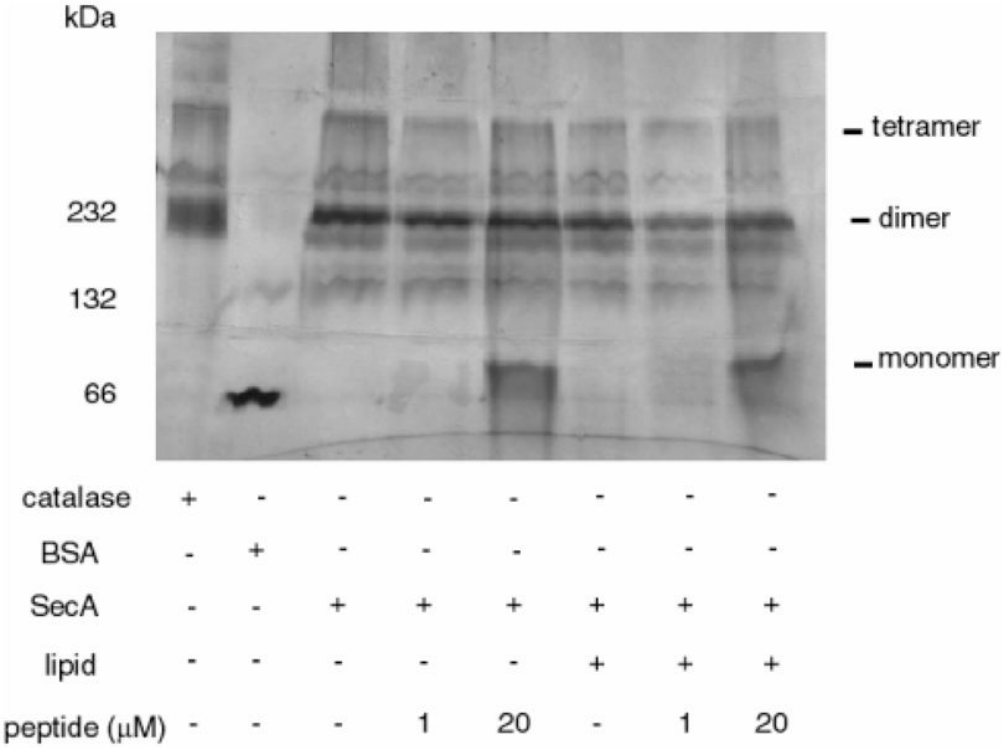


**Figure 5.**
Effect of the signal peptide on the oligomeric state of SecA. SecA (500 nM) was incubated in the presence or absence of vesicles composed of *E. coli* phospholipids and WT alkaline phosphatase signal peptide as indicated (see Experimental Procedures). Molecular weight standards are as follows: catalase (232 kDa), BSA dimer (132 kDa), and BSA monomer (66 kDa). The location of SecA in its monomeric, dimeric, and tetrameric forms is indicated.

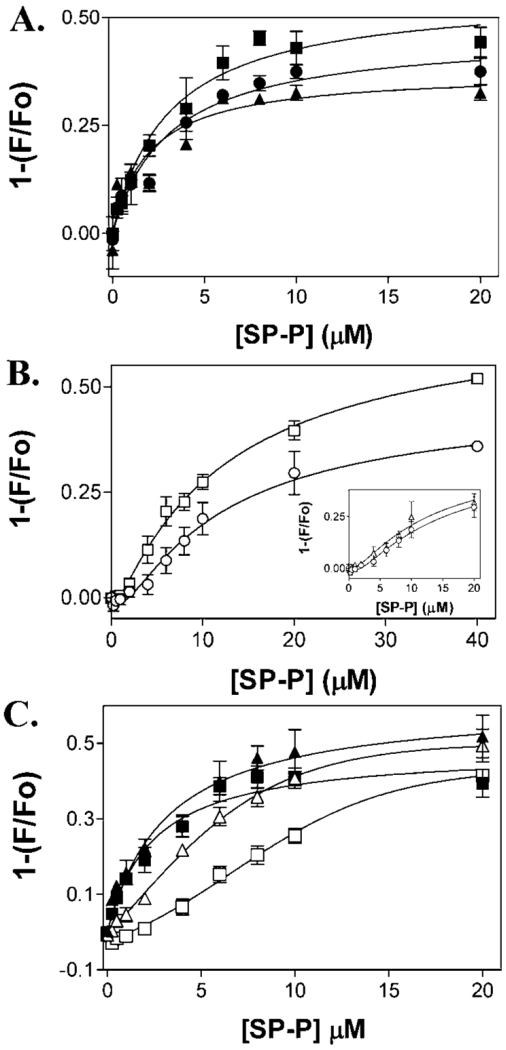


**Figure 6.**

Factors affecting the binding of the signal peptide to SecA. (A) Effect of nucleotide on signal peptide–SecA binding in an aqueous environment: without nucleotide (■), with 1 mM ADP (●), and with 1 mM AMP-PCP (▲). $F_o$ is the fluorescence of SecA alone, and $F$ is the fluorescence of SecA after addition of the IAEDANS-labeled signal peptide (SP–P) at 345 nm. (B) Effect of nucleotide on SecA–signal peptide binding in lipid vesicles: no nucleotide (□) and with 1 mM ADP (○). The curve generated in the presence of 1 mM AMP-PCP (△) is shown in the inset in comparison with that of ADP. (C) Effect of temperature on the concentration dependence of binding of the signal peptide to SecA in aqueous solution at 22 °C (■), lipid vesicles at 22 °C (□), aqueous solution at 37 °C (▲), and lipid vesicles at 37 °C (△). Each data point represents an average of at least two separate experiments performed in duplicate.

**Table 1**

Binding Constants for Signal Peptide–SecA Interactions under Different Conditions[a]

| | aqueous | | | lipid vesicles | |
|---|---|---|---|---|---|
| **temp (°C)** | **$K_d$[b] ($\mu$M)** | **$K_i$[c] ($\mu$M)** | **Hill[d] coefficient** | **$K_d$[b] ($\mu$M)** | **Hill[d] coefficient** |
| 22 | 2.4 ± 0.4 | 2.5 ± 0.5 | 1.1 ± 0.2 | 0.92 ± 1.0, 11.2 ± 2.8[e] | 1.9 ± 0.2 |
| 37 | 3.2 ± 0.6 | | 0.7 ± 0.2 | 10.1 ± 1.9 | 1.5 ± 0.1 |

[a] Values were obtained from at least three independent experiments and are reported ± the standard error.

[b] $K_d$ for binding of the peptide to SecA determined from a best fit of the FRET data.

[c] $K_i$ determined by competition of 3 $\mu$M labeled peptide with nonlabeled WT signal peptide.

[d] The Hill coefficient was determined as described in Experimental Procedures.

[e] Two $K_d$ values were obtained from fitting the data to a two-site binding model.