Phospholipid-induced monomerization and signal-peptide-induced oligomerization of SecA.

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Abstract

The SecA ATPase drives the processive translocation of the N-terminus of secreted proteins through the cytoplasmic membrane in eubacteria via cycles of binding and release from the SecYEG translocon coupled to ATP turnover. SecA forms a physiological dimer with a dissociation constant that has previously been shown to vary with temperature and ionic strength. We now present data showing that the oligomeric state of SecA in solution is altered by ligands that it interacts with during protein translocation. Analytical ultracentrifugation, chemical crosslinking, and fluorescence anisotropy measurements show that the physiological dimer of SecA is monomerized by long-chain phospholipid analogues. Addition of wild-type but not mutant signal sequence peptide to these SecA monomers re-dimerizes the protein. Physiological dimers of SecA do not change their oligomeric state when they bind signal sequence peptide in the compact, low temperature conformational state but polymerize when they bind the peptide in the domain-dissociated, high-temperature conformational state that interacts with SecYEG. This last result shows that, at least under some conditions, signal peptide interactions drive formation of new intermolecular contacts distinct from those stabilizing the physiological dimer. The observations that signal-peptides promote conformationally-specific oligomerization of SecA while phospholipids promote subunit dissociation suggest that the oligomeric state of SecA could change dynamically during the protein translocation reaction. Cycles of SecA subunit recruitment and dissociation could potentially be employed to achieve processivity in polypeptide transport.
Introduction

The SecA translocation ATPase mediates preprotein translocation through the cytoplasmic membrane of eubacteria via cycles of binding and release from the SecYEG translocon (1-5) coupled to its own ATPase cycle (6-8) (reviewed in (9)). Although the default translocation pathway is believed to involve an initial phase powered by the ATPase activity of SecA and a later phase powered by a transmembrane electrochemical potential coupled to SecYEG, the ATPase activity of SecA can mediate the translocation of an entire preprotein in the absence of a transmembrane potential (7). This observation has led to the conclusion that the SecA ATPase can mediate the processive translocation of polypeptide chains (10).

Each cycle of ATP binding and hydrolysis by SecA is believed to result in the translocation of about 40 residues of preprotein (7,11). There is substantial evidence that SecA interacts with both the N-terminal signal sequence (6,12-21) that targets preproteins for export from the cytoplasm as well as the mature region of the preprotein (22,23). These binding interactions presumably allow SecA to transfer polypeptide segments to SecYEG. However, the details of the preprotein binding and transfer reactions are not understood, so there is little information on how processivity is achieved in preprotein translocation.

Achieving efficient processive translocation is likely to involve complex interactions between SecA and the preprotein. Experimental evidence for such complexity comes from studies on the interaction of synthetic signal sequence peptides with SecA in different states. The binding of such peptides inhibits the ATPase activity of a 64 kDa N-terminal fragment of SecA with elevated basal activity (19,21) but stimulates the lipid-activated ATPase activity of intact SecA (17,18). The different functional consequences of signal peptide interactions in
these two assays suggest that SecA interacts with preproteins differently at different stages of its ATP-driven conformational reaction cycle.

Acidic phospholipids are required for efficient SecA-mediated preprotein translocation both \textit{in vivo} (24) and \textit{in vitro} (25,26). SecA inserts into phospholipid monolayers in a reaction that is enhanced by the presence of acidic phospholipids (27). SecA’s ability to interact with the hydrocarbon region of phospholipids in bilayer membranes is also supported by several experiments conducted using vesicles containing acidic phospholipids (26,28-30). Bilayer destabilizing lipids increase hydrocarbon exposure (29) and accelerate the rate of both preprotein translocation (26) and a conformational change that can be induced in SecA by interaction with vesicles (30). Moreover, SecA can be labeled by lipids containing photoactivatable groups in their hydrocarbon moieties (31,32) when such probes are incorporated into pure lipid vesicles, although experiments of this kind also indicate that SecA becomes shielded from such interactions when it binds to SecYEG (14,32).

SecA is believed to form a physiological dimer based on the preponderance of the dimeric form in hydrodynamic assays \textit{in vitro} (33-35). The monomer-dimer equilibrium is sensitive to temperature and to the ionic environment, and the dimer has a tendency to form higher-order oligomers as protein concentration is increased (34,36). Detailed analysis of hydrodynamic data indicates that two different forms of the SecA dimer are present in solution under some circumstances, differing either in their conformation or in the nature of their intersubunit interface (34). Different studies have come to differing conclusions regarding the oligomeric state of SecYEG in the active translocation complex, some supporting its functioning as a monomer (37) but others supporting its functioning as a dimer (38,39) or a tetramer (40). The possibility that SecYEG has a different oligomeric organization from SecA, combined with the general complexity of the processive protein translocation reaction, has raised the possibility
that SecA could change its oligomeric state during the functional translocation cycle (9,34,37,38,40). However, little evidence has been presented to support this possibility.

The present work shows that phospholipid and signal peptide ligands alter the complex equilibria between monomeric and oligomeric forms of SecA, supporting the possibility that changes in the oligomeric state of SecA could play a functional role in the protein translocation reaction.
Materials & Methods

Buffers and reagents. KET buffer contains 50 mM KCl, 1.0 mM Na-EDTA, 25 mM Tris-Cl, pH 7.6. Phospholipid analogues were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification.

Protein purification. E. coli SecA variants were purified as described (41). Strains overexpressing the PrlD suppressor mutants were obtained from D. B. Oliver of Wesleyan University.

Signal peptide synthesis and purification. The KRR-LamB signal peptide (\(\text{H}_3\text{N}-\text{MMITLRKKRLPLAVAVAAGVMSAQAMA-COO}^\prime\)) and the \(\Delta\ 78\) variant (\(\text{H}_3\text{N}-\text{MMITLRKKRPVAAAGVMSAQAMA-COO}^\prime\)) were synthesized and purified as described (13). The concentration of signal peptide in a concentrated stock solution in water was determined using quantitative amino acid analysis (conducted at the W.M. Keck Biopolymer Facility at Yale University). Some wild-type signal peptide preparations had a tendency to generate a precipitate when diluted into KET buffer, complicating fluorescence anisotropy measurements. However, these preparations produced equivalent re-dimerization of \(\ell\)-MPG-bound SecA monomers in sedimentation velocity assays where the precipitate is rapidly cleared from the cell so that it does not interfere with quantitation.

Tryptophan fluorescence anisotropy measurements. Excitation-corrected emission spectra were measured as described using 297 nm excitation (41). Total fluorescence was monitored at 340 nm, while the anisotropy values were averaged in a window from 320 nm to 380 nm in order to improve the signal-to-noise ratio of the data. The anisotropy curves changed uniformly throughout this spectral region and showed no fine structure. While background subtraction was
generally performed using spectra collected from a pure buffer sample, a fluorescent contaminant in the dicaproyl phospholipid stocks required protein-free solutions at equivalent phospholipid concentrations to be used for background subtraction of samples containing concentrations of these species in excess of 1 mM (i.e., for the fluorescence experiments summarized in Table II).

**CMC and micelle size measurements.** Detergent critical micelle concentrations (cmc’s) were determined using *in situ* elastic light-scattering measurements in the fluorimeter. Excitation and emission wavelengths were set to 297 nm and 298 nm, respectively, and the excitation-corrected 90˚ light scattering signal was measured as a function of detergent concentration (in KET buffer) using vertical excitation and emission polarizers with a 30 second averaging time for digital photon-counting and 4 nm slits. Linear regressions were used to fit the pre- and post- cmc regions in the plot of light-scattering vs. detergent concentration, and the cmc was calculated as the point at which these curves intersect. The molecular masses of the *l*-MPG and *l*-MPC micelles in KET buffer were determined to be 35 kDa and 65 kDa, respectively, from static light scattering and refractive index measurements performed using Dawn EOS and Optilab detectors (Wyatt Inc., Santa Barbara, CA). The details of these experiments will be published elsewhere.

**Analytical ultracentrifugation measurements.** Sedimentation velocity experiments were performed at 20˚C in KET buffer in a Beckman XL-A centrifuge using an 8 slot rotor at 20,000 rpm. Double-sector Epon centerpieces were used with 420 µl in the sample cell and 440 µl of buffer in the reference cell. Absorbance measurements at 280 nm were taken in 0.002 cm radial steps. Absorbance and refractive index scans were measured from each cell every 8 minutes over the course of 14 hours. Absorbance data were analyzed with the program SEDFIT using the continuous distribution c(S) and c(M) Lamm equation model (42,43). The partial specific volume of the protein was assumed to be 0.734 ml/g, and the density of the
solvent was calculated to be 0.998148 g/ml (44). An ensemble of ~90 scans was used for the final refinement of each c(M) distribution plot. The meniscus was identified by manual inspection and refined during fitting, and the data range was truncated near the bottom of the plateau region in the ensemble of sedimentation curves. The value of the frictional ratio f/f₀ was initially assumed to be 1.2 and refined by the program (Table I). S-values between ~0.5 and ~18 were considered and divided into 1000 steps. Maximum entropy regularization was used assuming a confidence level of 0.9. All fits yielded a root-mean-square deviation below 0.1% for the entire ensemble of scans (light gray traces in Fig. 1A).

**Evaluation of the effect of micelle binding on analytical ultracentrifugation results.** Data from the sedimentation velocity runs were analyzed using the program SEDFIT (42,43) as described above assuming 8 evenly-spaced values for the overall partial specific volume of the hydrodynamic particle between 0.734 and 0.905 cm³/gm. The first value represents the partial specific volume of the protein, while the second value represents an upper limit for the partial specific volume of the l-MPC and l-MPG micelles given that fact that they are more dense than D₂O buffer (as evidenced by their observed sedimentation rather than floatation in this environment). The resulting plot of molecular mass vs. the assumed partial specific volume of the hydrodynamic particle was empirically fit to a 3rd-order polynomial. This equation was used to calculate the total molecular mass of the particle for any given value of its overall partial specific volume (\( \bar{V}_{\text{overall}} \)), as calculated from the assumed values for the phospholipid-to-protein mass ratio in the complex (R) and the partial specific volume of the phospholipid (\( \bar{V}_{\text{PL}} \)): The molecular mass of the protein in the complex was then calculated based on the assumed phospholipid-to-protein mass ratio, yielding data of the kind shown in Fig. 1C.
Crosslinking experiments. 50 µM (20°C) or 46 µM (37°C) SecA monomer was preincubated for 10 minutes in crosslinking buffer (10 mM KCl, 20 mM MgOAc₂, 50 mM triethanolamine, pH 7.5) with signal peptides (at a 100 µM concentration) and/or phospholipids (at a 2 mM concentration). Crosslinking was initiated by the addition of 0.1% glutaraldehyde for 5 minutes (20°C) or 3 minutes (37°C) and stopped by adding 2× SDS gel-loading buffer and boiling for 3 min. Crosslinked proteins were analyzed by 4% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue.
Results

*Long-chain phospholipid analogs monomerize the physiological dimer of SecA.* Analytical ultracentrifugation (Figs. 1A - 1C), fluorescence anisotropy (Fig. 1D), and glutaraldehyde crosslinking (Fig. 2A) experiments show that the physiological dimer of SecA is monomerized by the long-chain phospholipid analogs *lyso*-myristoyl-phosphatidylglycerol (l-MPG) or *lyso*-myristoyl-phosphatidylcholine (l-MPC). Fluorescence anisotropy experiments (Fig. 1D) show that SecA remains a dimer in the presence of equivalent concentrations of short-chain phospholipid analogs with the same headgroup structures (dicaproyl-phosphatidylglycerol (DCPG) and dicaproyl-phosphatidylcholine (DCPC)).

Absorbance scans from sedimentation velocity experiments conducted either in the absence or presence of 150 µM l-MPG (Fig. 1A) show an obvious reduction in the sedimentation coefficient of SecA in the presence of this phospholipid analog. The program SEDFIT was used to fit these data and data from an equivalent experiment conducted in the presence of 150 µM l-MPC using the continuous distribution c(S) and c(M) Lamm equation model (42,43), with results summarized in Table I. The c(M) mass distributions inferred from these analyses (Fig. 1B) indicate that the molecular mass of SecA is reduced by approximately half in the presence of either phospholipid analog, suggesting that they monomerize the physiological dimer of SecA. Equivalent hydrodynamic results are obtained in the presence of a 300 µM or 500 µM concentration of either phospholipid analogue (data not shown).

Several details of these sedimentation velocity experiments deserve comment. The experiment conducted in the absence of phospholipid confirms that that 1 µM *E. coli* SecA is present primarily in the form of a dimer (33,34) at room temperature in a buffer containing 50 mM KCl, 1 mM EDTA, 25 mM Tris-Cl, pH 7.5 (Fig. 1B and Table I). Consistent with
previous observations (33), a high frictional ratio is observed for this dimer (Table I). Sedimentation velocity analyses of the kind used here give time-averaged molecular masses for hydrodynamic species undergoing rapid equilibration at the sedimentation boundary (42,43). Therefore, the minority population of SecA monomer observed in Fig. 1B indicates that a small fraction of the SecA molecules are incapable of forming the physiological dimer, probably due to N-terminal proteolysis (36). The molecular mass of the non-exchanging SecA monomer is slightly underestimated in Fig. 1B because a single frictional ratio must be assumed in analyzing the entire ensemble of hydrodynamic species (due to software limitations), and the high value required to model the sedimentation of the dimer overestimates the frictional ratio of the monomer.

Sedimentation velocity experiments conducted on pure phospholipid samples and monitored using refractive index measurements show that the sedimentation of both l-MPG and l-MPC micelles is substantially slower than that of the SecA monomer under these conditions (data not shown). Therefore, an approximately constant micelle concentration is present in the protein-containing regions of the cell during the sedimentation velocity experiments. Because the phospholipid species have no absorbance at 280 nm, they do not contribute to the optical absorbance profiles used to determine the protein’s molecular mass distribution. Therefore, the hydrodynamic properties of the protein / phospholipid complex can be assessed directly from the sedimentation absorbance profiles even in the presence of the micelles.

However, the c(M) distribution (Fig. 1B) calculated for the complex depends directly on the overall partial specific volume of the hydrodynamic particle ($\overline{\nu}_{\text{overall}}$), which will change depending on how much phospholipid is attached to the protein molecule. In order to estimate the influence of this effect on the inferred molecular mass distributions, the sedimentation velocity data from the experiments conducted in the presence of phospholipid micelles were re-

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analyzed assuming varying lipid-to-protein ratios in the hydrodynamic particles (shown explicitly for \( l\)-MPG in Fig. 1C and summarized for both phospholipid species in Table I). Because the partial specific volumes of these phospholipid species are not known, this analysis was performed assuming all reasonable values for this parameter. Most phospholipids and detergents have partial specific volumes in the range between 0.85 and 0.89 cm\(^3\)/gm (45-47), but the analysis was performed assuming a considerably broader range of values up to the experimental upper limit of 0.905 cm\(^3\)/gm (see Methods) and down to 0.734 cm\(^3\)/gm (i.e., equivalent to the partial specific volume of the protein). This analysis shows that the sedimentation velocity data for SecA in the presence of 150 \( \mu \)M \( l\)-MPG are inconsistent with the hydrodynamic particle containing anything larger than a SecA monomer (Fig. 1C). Moreover, the calculated molecular mass closely matches that of the SecA monomer for the binding of amounts of lipid ranging from an \( l\)-MPG monomer to a complete micelle (which has a molecular mass of 35 kDa) assuming a partial specific volume in the range from 0.85 – 0.90 cm\(^3\)/gm. An equivalent analysis of the sedimentation data obtained in the presence of \( l\)-MPC yields a similar conclusion (Table II). However, in this case, assuming that a small number of \( l\)-MPC monomers are bound to the protein gives a molecular mass estimate slightly higher than that of the SecA monomer while a closer match is obtained assuming that one micelle (which has a molecular mass of 65 kDa) is bound to the protein molecule.

**Evaluation of the concentration-dependence of monomerization using fluorescence anisotropy spectroscopy.** Steady-state fluorescence anisotropy measurements offer a convenient means to monitor the oligomerization state of a fluorophore-containing protein due to their sensitivity to changes in rotational correlation time (48). The intrinsic tryptophan fluorescence of *E. coli* SecA can therefore be used to assess the concentration-dependence of the monomerization reaction induced by either \( l\)-MPG or \( l\)-MPC (triangles in Fig. 1D). A steep quenching in relative total
fluorescence (lower panel in Fig. 1D) is observed in the concentration range from 25 to 150 µM when either phospholipid analog is titrated onto SecA, coinciding approximately with the cmc’s of these micelle-forming lyso-lipids (~64 µM for l-MPG and 68 µM for l-MPC). The observed fluorescence quenching indicates that a protein conformational change occurs upon phospholipid binding (26,28-30). Because quenching decreases the excited-state lifetime of the fluorophore ensemble, it tends to cause a small increase in fluorescence anisotropy in the absence of a change in the rotational diffusion coefficient of the protein. Instead, a 30% decrease in anisotropy closely parallels the major change in relative total fluorescence in both the l-MPG and l-MPC titrations (upper panel in Fig. 1D), indicating that a substantial increase in the rotational diffusion coefficient of the protein accompanies the conformational transition. Based on the results of the sedimentation velocity experiments, most of this increase in rotational diffusion rate is attributable to monomerization of the SecA dimer.

A second apparent binding interaction is observed exclusively in the titration with the l-MPG micelles in the concentration range from 150 to 200 µM, causing an additional 20% quenching in relative total fluorescence (lower panel in Fig. 1D). However, this second binding event produces a small increase in anisotropy (upper panel in Fig. 1D) which could be caused either by the reduction in the lifetime of the fluorophore ensemble due to the quenching and/or by a slight reduction in the molecular rotation rate due to the binding of a second l-MPG micelle to the protein.

**Phospholipid-induced monomerization is also observed in chemical crosslinking experiments.**

In the absence of phospholipids, exposure of SecA to 0.1% glutaraldehyde (GA) for 5 minutes at 20˚C yields primarily a covalent dimer when samples are analyzed using SDS-PAGE (lane 5 in Fig. 2A). Addition of either l-MPC or l-MPG reduces the crosslinking of the protomers and
results in the protein running primarily as a monomer even after GA exposure (lanes 6 & 9 in Fig. 2A).

**Similar total fluorescence changes during the phospholipid-induced monomerization and the endothermic transition of SecA.** Thermal titrations can be used to induce an ATP-modulated endothermic conformational transition in *E. coli* SecA (8,28,49,50) (Fig. 1E) which produces an increase in the mobility of the α-helical wing domain in the protomer but not dissociation of the physiological dimer (41). This transition does produce a decrease in tryptophan anisotropy (*i.e.*, increase in reciprocal anisotropy in the Perin plot (48) in the upper panel of Fig. 1E), but the magnitude of this anisotropy change is significantly smaller than that produced by the phospholipid-induced monomerization reaction (uppers panel in Figs. 1D & 1E). Nonetheless, the change in relative total fluorescence that takes place during the endothermic conformational transition (20,28,50) (lower panel in Fig. 1E) is very similar to the change that takes place during the phospholipid-induced monomerization (lower panel in Fig. 1D), suggesting that there may be similarities in the conformational change that takes place within the SecA protomer in the two cases. Consistent with this possibility, phospholipid-monomerized SecA does not experience any additional change in relative total fluorescence upon subsequent thermal titration (Fig. 1E). Dissection of the contribution of the individual Trp residues in SecA to the fluorescence changes shown in Fig. 1D (manuscript in preparation) also supports the conclusion that a related conformational change takes place in the protomer during phospholipid-induced monomerization and during the endothermic conformational transition, even though the physiological SecA dimer does not monomerize in the latter case.
**Evaluation of different phospholipid and detergent species for the ability to monomerize SecA.**

In order to characterize the chemical features of the phospholipids responsible for monomerizing the physiological dimer of SecA, fluorescence anisotropy titrations were conducted with a variety of different phospholipid and detergent species (Table II). All of the long-chain lyso-lipid species that were tested trigger dissociation of the physiological dimer of SecA, with the concentration producing 50% monomerization being consistently at or slightly below the critical micelle concentration (cmc) of the phospholipid species except in the case of lyso-palmitoyl-PC. In contrast, the short-chain diacyl phospholipids DCPG and DCPC fail to induce monomerization of SecA at concentrations either below or above their cmc’s. While the non-ionic detergents β-octylglucoside, β-dodecylmaltoside (DDM), and C₁₂E₈ also fail to trigger monomerization at concentrations either below or above their cmc’s, lauryl-dimethylamineoxide (LDAO) does so at a concentration slightly below its cmc.

**Synthetic signal peptide re-dimerizes phospholipid-monomerized SecA.** A synthetic analogue of the signal sequence of the LamB outer membrane protein from *E. coli* has been used as model preprotein substrate in a variety of biochemical and biophysical studies (19). The “KRR-LamB” synthetic signal peptide contains three extra residues (KRR) compared to the original LamB sequence in order to enhance its solubility, but a modification like this has been demonstrated not to perturb LamB signal sequence function *in vivo* (13). Sedimentation velocity experiments show that SecA molecules monomerized by the binding of l-MPG are re-dimerized in the presence of 25 μM wild-type KRR-LamB signal peptide (Fig. 3A and Table I). A small population of higher-order oligomers is also observed in this experiment, but the dominant hydrodynamic species is a dimer (Fig. 3A & Table I).
A significant increase in molecular mass is also observed when 25 µM wild-type KRR-LamB signal peptide is added to SecA molecules monomerized by interaction \( l \)-MPC (Table I). In this case, the apparent molecular mass of the complex formed in the presence of the signal peptide is slightly lower than that expected for a protein dimer (~165 kDa vs. slightly greater than 200 kDa). This observation suggests that the re-dimerization of SecA may be incomplete under these conditions. Higher concentrations of signal peptide yield a complex of the same molecular mass (data not shown), indicating that the lack of complete dimerization in \( l \)-MPC is not due to incomplete saturation of the signal-peptide binding site. Therefore, the equilibrium constant for the re-dimerization of the SecA / signal peptide complex with \( l \)-MPC appears to be lower than that for the equivalent complex with \( l \)-MPG.

When the concentration dependence of the re-dimerization of \( l \)-MPG-bound SecA by the wild-type KRR-LamB signal peptide is assessed using tryptophan fluorescence anisotropy spectroscopy, a sigmoidal change in fluorescence anisotropy is observed with a \( K_d \) of 14 µM (Fig. 3B) and a Hill coefficient of 2 (Fig. 3C), consistent with the occurrence of a cooperative dimerization reaction involving the binding of one signal peptide per monomer. The \( \Delta 78 \) variant of the KRR-LamB signal peptide contains a 4 residue deletion that has been observed to severely impair the function of the corresponding signal sequence in vivo (51). The specificity of the signal-peptide-induced re-dimerization is demonstrated by the fact an equivalent titration of \( l \)-MPG-monomerized SecA with the KRR-LamB-\( \Delta 78 \) mutant signal peptide produces only minor changes in fluorescence anisotropy (Fig. 3B).

As opposed to the large fluorescence quenching that is observed upon phospholipid-induced monomerization of SecA, only a minimal increase in total fluorescence is observed upon re-dimerization by the wild-type KRR-LamB peptide (Fig. 3B). Moreover, the anisotropy level of the re-dimerized protein is lower than that of the physiological dimer prior to phospholipid
exposure. Because the binding of the KRR-LamB peptide to the physiological dimer of SecA produces only minimal changes in either total Trp fluorescence (data not shown) or anisotropy (Fig. 4B below), both of these observations suggest that the conformation of the KRR-LamB-bound SecA dimer might differ from that of the original physiological protein dimer.

GA crosslinking experiments support the conclusion that signal-peptide binding induces re-dimerization of SecA molecules monomerized by either l-MPG or l-MPC based on the increase in the level of dimer observed when crosslinking is conducted in the presence of the wild-type KRR-LamB signal peptide but not the Δ78 variant (lanes 6 - 11 in Fig. 2A).

**Conformationally-specific polymerization of SecA dimers by synthetic signal peptide.**

Addition of 25 µM wild-type KRR-LamB signal peptide to a solution containing physiological dimers of SecA at 20˚ C produces only a small increase in the apparent molecular mass of the protein, concomitant with an increase in the width of the molecular mass distribution, as determined by sedimentation velocity measurements (Fig. 4A). Therefore, signal peptide binding to SecA dimers in the compact conformational ground state induces a modest tendency to self-associate (that is also observed in the GA-crosslinking experiment in lane 4 in Fig. 2A) but without producing a significant shift of the population into the form of higher-order oligomers. Consistent with this conclusion, at most minor changes in total Trp fluorescence (lower panel in Fig. 4B and additional data not shown) or anisotropy (upper panel in Fig. 4B) are observed when 25 µM wild-type KRR-LamB signal peptide is added to SecA at 24˚ C (Fig. 4B). However, a precipitous decrease is observed in the Perin plot (48) of reciprocal anisotropy versus temperature when such samples undergo thermal titration. This increase in Trp fluorescence anisotropy coincides with the onset of the endothermic conformational transition as detected by quenching of total fluorescence (lower panel in Fig. 4B). Based on several lines of evidence, this
anisotropy change corresponds to the formation of higher-order oligomers by the SecA dimer, indicating that signal peptide induces protein polymerization when it binds to the domain-dissociated conformation adopted by SecA at temperatures above that of the endothermic transition.

The first line of evidence supporting the conformationally-specific polymerization of SecA derives from the detailed properties of the observed fluorescence changes. The change in total fluorescence observed in Fig. 4B derives almost entirely from the protein conformational change that occurs during the endothermic transition. When signal peptide is added to SecA samples after first heating them to a temperature high enough to induce the endothermic transition, the strong increase in anisotropy occurs in the absence of any significant change in total fluorescence (data not shown), establishing that the anisotropy change does not derive from an alteration in fluorescence lifetime and must instead derive from an increase in the rotational correlation time of the Trp ensemble (48). Theoretically, this increase could be caused either by a protein conformational change leading to slower rotation of the physiological dimer or by formation of higher-order oligomers of SecA, which would rotate more slowly than the physiological dimer. However, the large change in rotational correlation time required to produce the observed 60% increase in anisotropy seems unlikely to derive from a conformational change and much more likely to derive from higher-order oligomerization. This conclusion is supported by the observation of detectable turbidity indicative of light scattering in the samples exposed to elevated temperature in the presence of signal peptide but not in those exposed to the same temperature in the absence of signal peptide (data not shown). Light scattering requires the presence of particles that are large compared to the wavelength of visible light and therefore indicates the formation of higher-order protein complexes. This conclusion is also supported by crosslinking experiments, which yield primarily dimer when WT SecA is exposed to GA at 37°C in the absence of signal peptide but high-molecular weight species that do not enter the gel.
when crosslinking is performed in the presence of the wild-type KRR-LamB signal peptide (lanes 2 - 4 in Fig. 2B) at the same temperature. Finally, the polymerization of domain-dissociated SecA in the presence of signal peptide is also supported by analytical ultracentrifugation experiments, which show continued steady progression of the sedimentation boundary in SecA samples at 37°C but rapid clearance of the protein from the sample cell at the same temperature in the presence of signal peptide (data not show), indicating the formation of large protein complexes.

When thermal titration of SecA is conducted in the presence of an equivalent concentration of the KRR-LamB-Δ78 mutant signal peptide, polymerization is still observed but only at substantially higher temperatures than with the wild-type signal peptide (Fig. 4B). There is a latent kinetic component in thermal titrations of this kind, so that the higher temperature at which the major change in anisotropy is observed probably reflects slower and less efficient polymerization of SecA by the mutant compared to wild-type signal peptide. SecA’s ability to mediate processive preprotein transport suggests that its binding site for transport substrate is capable of recognizing and binding a great diversity of polypeptide sequences. In this context, some degree of interaction between SecA and a mutated signal peptide is not surprising. Tighter binding of wild-type signal sequences to Sec’s polypeptide transport site could help ensure efficient initiation of preprotein translocation.

Because SecA retains a dimeric structure during the endothermic transition (41), the polymerization reaction indicates that an additional intersubunit interface is likely to be formed between protomers when they bind signal peptide in the domain-dissociated conformation. The formation of an equivalent interface between phospholipid-bound SecA monomers could be responsible for their signal-peptide-dependent dimerization given the evidence discussed above for the related conformational properties of the SecA protomer following either phospholipid-
induced monomerization or endothermic domain dissociation as well as the evidence for possible conformational differences between the physiological dimer and signal-peptide re-dimerized SecA.

The effect of prlD mutations in SecA on signal-peptide induced polymerization. PrlD mutations in SecA are selected based on their ability to suppress secretion defects caused by mutations in the signal sequence of a preprotein in vivo (52,53). These alleles generally facilitate the endothermic conformational transition of SecA and shift its $T_m$ to lower temperature (50). When thermal titrations are conducted on a series of prlD alleles of SecA in the presence of 25 µM wild-type KRR-LamB signal peptide, the onset of the polymerization reaction moves to lower temperature, tracking the onset of the endothermic conformational transition in each allele as monitored by total fluorescence spectroscopy (Fig. 4C). This correspondence establishes that the polymerization reaction is controlled by the conformational state of SecA rather than deriving from the thermodynamic properties of the signal peptide itself. When thermal titrations of this set of SecA variants are conducted in the presence of the KRR-LamB-Δ78 mutant signal peptide, the prlD alleles also shift the polymerization reaction to lower temperature so that its thermal dependence more closely resembles that of wild-type SecA with wild-type signal peptide (data not shown). Therefore, the prlD alleles enhance the efficiency of SecA’s interaction with a defective signal peptide in this in vitro assay.

Lyso-lipids reverse the signal-peptide induced polymerization of SecA. To verify that the signal-peptide induced polymerization does not represent some form of irreversible aggregation, the ability of different lipid species to reverse the polymerization was evaluated using steady-state tryptophan fluorescence anisotropy experiments (Fig. 4D). After heating a SecA sample to
a temperature high enough to induce the endothermic conformational transition and domain dissociation, the addition of wild-type KRR-LamB signal peptide causes a rapid increase in anisotropy indicating polymerization. Addition of a 250 µM sub-cmc concentration of DCPG produces no change in Trp anisotropy (upper trace in Fig. 4D). However, addition of an equal concentration of l-MPG, which is above its cmc, reduces the anisotropy to a similar level to that observed prior to signal peptide addition (lower trace in Fig. 4D), consistent with reversion to a dimeric form. Thus, dissociation of the signal-peptide-induced polymers is achieved by introduction of a phospholipid species at a concentration that induces monomerization of the physiological dimer of SecA but not by the introduction of an equivalent concentration of a phospholipid species with the same headgroup structure and similar hydrocarbon content that is not capable of monomerizing SecA. Additional experiments show that the anisotropy level of signal-peptide-induced SecA polymers is reduced by the introduction of l-MPC at a concentration above its cmc that induces monomerization of SecA but not by an equivalent concentration of DCPC that does not induce monomerization (data not shown). However, while l-MPC fully reverses modest signal-peptide-induced anisotropy changes (reflecting lower degrees of polymerization), it only produces partially reverses stronger anisotropy changes of the kind shown in Fig. 4D. Therefore, the lyso-PG species seems to be somewhat more effective that the lyso-PC species in mediating SecA subunit dissociation under these conditions.

GA crosslinking experiments yield similar results, showing reversal of the signal-peptide induced polymerization of SecA by lipids specifically when they are used under conditions that induce monomerization of the physiological dimer. A 2 mM concentration of l-MPG that is above the threshold required to induce monomerization produces a strong increase in the amount of monomeric SecA entering the gel, while an equivalent concentration of DCPG that does not induce monomerization has little effect on the signal-peptide-induced polymerization (lanes 5 & 6 in Fig. 2B). The failure to obtain GA-crosslinked dimers in this experiment in the presence of
l-MPG and signal peptide, even though such dimers were observed after GA crosslinking in the presence of these ligands at 20°C (lane 7 in Fig. 2A), must be attributable to the different conditions used in this experiment (i.e., either the higher temperature or the shorter cross-linking time). However, the efficient crosslinking of the physiological dimer under identical solution conditions provides further evidence that there are differences in the structure of the intersubunit interface in the SecA dimer when both l-MPG and signal peptide are bound.

The reversibility of the signal-peptide-induced polymerization of SecA makes it unlikely that this phenomenon is caused by protein aggregation, especially given the data showing that this reversal only occurs when phospholipid analogues are used under conditions that produce monomerization of the physiological dimer of SecA.
Discussion

The SecA translocation ATPase is known to have complex oligomeric behavior in aqueous solutions (33-36). Although a dimer is the predominant species present in purified SecA samples in the absence of other proteins, the protein has a tendency to form higher-order oligomers or dissociate into monomers that depends on protein concentration, temperature, and the composition of the buffer. In this paper, we show that ligands that will be encountered by SecA in the course of protein translocation strongly modulate its oligomeric behavior.

The protein translocation reaction is believed to be initiated by the binding of SecA to the N-terminal signal peptide that targets proteins for export from the cytoplasm (6,9,12-21). Because the signal peptide is cleaved by a protease with a periplasmic active site, at least its C-terminal segments must be translocated across the cytoplasmic membrane, a process which presumably occurs when SecA binds to SecYEG. The data presented in this paper show that signal peptide binding tends to produce oligomerization of SecA in a manner that is modulated by the conformational state of the protein, with the tendency being weak in the conformational ground state but strongly enhanced in the high-temperature domain-dissociated state (8,28,41,49,50) of the physiological dimer or after monomerization by lyso-lipids. In this context, changes in oligomeric interactions could occur at specific steps in the conformational reaction cycle of the SecA-SecYEG complex (1-9) in a manner controlled by the presence or absence of the preprotein transport substrate. The results presented in this paper therefore raise the possibility that ligand-triggered changes in oligomeric interactions between SecA protomers may play a role in the complex dynamics of the preprotein translocation reaction (9,10).

SecA appears to be shielded from the hydrophobic region of the bilayer when stably inserted into SecYEG (14,32). However, it does interact directly with phospholipid bilayers
including their hydrophobic cores in the absence of SecYEG (27). SecA’s ability to interact with the hydrocarbon region of phospholipids in bilayer membranes is also supported by several experiments conducted using vesicles containing acidic phospholipids (26,28-30), and these interactions are enhanced (28) in the high temperature domain-dissociated conformation that is the product of the endothermic conformational transition in SecA. Because preprotein translocation requires cycles of SecA insertion and retraction from SecYEG (1-5), SecA will not be stably inserted into SecYEG at all stages of the productive translocation cycle. While it is retracted from SecYEG, SecA could interact directly with the phospholipid bilayer, which would then have the opportunity to drive conformationally-specific changes in SecA that could contribute to a carefully-controlled progression of the overall conformational reaction cycle. Such interactions could account for the acceleration in the rate of preprotein translocation in the presence of bilayer destabilizing lipids (26) which increase the solvent exposure of the hydrocarbon moieties of the phospholipids (29). The monomerization of SecA by certain phospholipid and detergent species that is established in this paper is of uncertain physiological relevance.

We believe that monomerization is mediated by the interaction of SecA with hydrophobic moieties exposed on the surface of the micelles formed by the active species. Because DCPG and DCPC fail to induce monomerization of SecA even at super-cmc concentrations up to 35 mM, the phospholipid headgroups cannot be responsible for inducing the monomerization of SecA. While LDAO induces monomerization of SecA, two other detergents with equivalent 12-carbon aliphatic chains (DDM and C_{12}E_8), do not, indicating that having a hydrocarbon chain of a given length is not sufficient for an amphiphile to be active. While this pattern could be attributable to a complex interplay of requirements for headgroup and hydrocarbon structures, it could also be explained by requirements for the physiochemical properties of the micelle formed by the amphiphile.
Based on two lines of evidence, the active species responsible for subunit dissociation in SecA are likely to be micelles and/or proto-micellar aggregates. First, there is a strong correlation between the cmc of the amphiphile and the concentration required to produce monomerization of SecA (Table II), which is readily explained if some kind of micellar aggregate is the active species. Second, the apparent cooperativity of the monomerization reaction varies from ~3 to ~8 for the different active species (Table II). This number gives the minimum number of amphiphile molecules bound during the reaction (54), so the data indicate that more than one amphiphile molecule per monomer is required to drive monomerization in all cases. While these data do not exclude the possibility that a small but variable number of molecules could drive the monomerization of SecA for the different amphiphiles, they could also be explained based on variations in the apparent cooperativity of the micellization reaction (55-57) for the different amphiphiles if micelle formation is involved in driving monomerization. In the case of a micelle containing a large number of monomers (as is the case for all of the species examined here), an ideal micellization process would involve a very high degree of cooperativity associated with the formation of micelles starting at the cmc. However, high-sensitivity titration calorimetry studies show that real micellization reactions show very substantial deviations from ideality (55-59), including the formation of proto-micellar aggregates of heterogeneous structure at concentrations in the vicinity of the nominal cmc (i.e., both below and above it). Because of these species, the apparent cooperativity of the micellization process is generally considerably lower than the aggregation number of the micelle (55-57) and can be in the range observed for the apparent cooperativity of the monomerization of SecA by the different amphiphiles (Table II). Monomerization of SecA by proto-micellar aggregates formed at concentrations below the cmc could therefore explain the results observed with lyso-lauryl-PC and LDAO, which trigger the dissociation reaction with different apparent cooperativities at slightly sub-cmc concentrations.
The calorimetric studies indicate that the proto-micellar aggregates formed near the cmc expose more hydrocarbon to the aqueous environment than the fully-formed micelles present at limiting concentrations above the cmc (55,57,58). The likelihood that such species are involved in inducing the monomerization of SecA by some of the amphiphiles suggests that surface exposure of hydrocarbon groups on the aggregates could be an important molecular parameter in mediating the effect. In this context, it is noteworthy that the nonionic detergents that fail to induce monomerization have comparatively larger headgroups that will tend to shield the hydrocarbon chains more thoroughly in the corresponding micelles, giving further support to the hypothesis that the level of static or dynamic exposure of hydrophobic moieties on the surface of the micelle could potentially be a critical parameter in determining the efficacy of the species in driving monomerization of SecA.

This requirement would echo the activity of bilayer-destabilizing phospholipids in stimulating the conformational reaction cycle of SecA (26,29). Therefore, the micelle-induced monomerization of SecA characterized in this paper might reflect what happens to SecA when interacting with destabilized regions of the phospholipid bilayer between successive rounds of insertion into SecYEG. Thus, the ability of certain phospholipid and detergent micelles to drive subunit dissociation of SecA could reflect the ability of phospholipid membranes in the correct microenvironment to drive changes in the interactions between SecA protomers at a specific stage of its conformational reaction cycle when engaged with SecYEG. If this hypothesis is correct, oligomeric interactions of SecA could be reciprocally controlled by signal peptide interaction and phospholipid interaction at different stages of the translocation cycle.

Each cycle of ATP-dependent binding and release of SecA from the SecYEG translocon is believed to drive the translocation of about 40 residues of preprotein (11). Therefore, in order
to achieve processive translocation of an entire preprotein, there must be some mechanism by which the translocation of each successive 40-residue polypeptide segment is efficiently coordinated (10). It is possible that a single SecA dimer could re-bind to C-terminal segments of a translocating preprotein molecule following delivery of an N-terminal segment of the same preprotein to SecYEG. In this case, the probability of re-binding to a proximal segment of the preprotein could be enhanced by keeping SecA partially bound to SecYEG between the pumping cycles. However, the data presented in this paper raise the possibility that processivity could be achieved using a different mechanism. The observation of conformationally-specific, signal-peptide-dependent higher-order oligomerization of SecA suggests that a SecA protomer with bound preprotein could recruit additional SecA molecules to mediate translocation of the C-terminal segments of the same preprotein molecule. Subunit recruitment would likely be temporally coupled to the delivery of the currently bound preprotein segment to the translocon because entry into the high-temperature domain dissociated conformation where subunit recruitment occurs is likely to gate the binding of SecA to SecYEG (20,50,60). The phospholipid-induced monomerization of SecA reported in this paper could then mediate subunit release and recycling.

The tandem motor domains in SecA bear some sequence homology (61) and strong structural homology (8,41) to those in ATP-dependent superfamily I & II helicases, with the closest relationship being observed with the DEAD-box family of RNA helicases (62,63). These enzymes mediate the processive unwinding of nucleic acid duplexes, and two competing models have been advanced to explain how processivity is achieved in this reaction. The “inchworm” model proposes that unwinding is mediated by the unidirectional translation of a helicase protomer along a single strand of a nucleic acid polymer, which pushes the duplex open at its leading edge (64,65). The “rolling” model proposes that unwinding is mediated when one protomer bound to a single-stranded segment of the nucleic acid polymer recruits a second
protomer to bind to the upstream segment of the strand in an equivalent manner, thereby stabilizing it in the single-stranded state (66-68). The first model assumes that a helicase monomer is the functionally active species, while the second model assumes that oligomerization plays a fundamental role mediating processivity. Proponents of the inchworm model cite a 1 base displacement of a bound oligonucleotide observed in comparing nucleotide-free and ATP-bound structures of the PcrA superfamily I helicase (64). They also point out that no consistent pattern of oligomerization has been observed in the existing helicase crystal structures, including multiple representatives from both superfamilies I & II; (65,69). Proponents of the rolling model cite the fact that both the Rep (68) and NS3 (70) helicases have both been shown to dimerize upon binding specific substrate DNA structures and therefore in a conformationally-specific manner. The results presented in this paper suggest that SecA could achieve processivity in polypeptide transport using a subunit recruitment mechanism similar to that invoked in the rolling model for the mechanism of the structurally homologous ATP-dependent helicases.

While this paper was under review, Or et al. reported related results (71). In this work, chemical cross-linking and fluorescence resonance energy transfer results are presented showing that the physiological dimer of E. coli SecA is dissociated by interaction with phospholipids and some analogs. These results are mostly consistent with the results reported here. However, Or et al. also present chemical cross-linking data suggesting that SecA monomerizes upon binding the KRR-LamB signal peptide (71), directly contrary to the results reported here. This discrepancy could possibly reflect differences in the behavior of SecA in the crosslinking buffer used by these authors, although consistent behavior is observed in our fluorescence and crosslinking experiments in spite of the fact that there are greater differences in the compositions of the buffers used in these experiments. On the other hand, the intersubunit crosslinking efficiency is very low in the SecA dimer in the experiments reported by Or et al. (71), and its reduction in the
presence of signal peptide could potentially be explained by effects unrelated to a change in the oligomeric state of SecA. For instance, a protein conformational change upon signal peptide binding could reduce the efficiency of reaction of the crosslinking reaction even in the absence of dissociation of the physiological dimer.

Or et al. also report isolation of a mutant form of *E. coli* SecA with a strongly reduced tendency to form the physiological dimer (71). Based on the fact that this variant retains a small fraction of the activity exhibited by the wild-type protein in an *in vitro* preprotein translocation assay, they argue that SecA is likely to function fundamentally as a monomer and to use an inchworm mechanism to mediate processivity in preprotein transport (71). However, they do not characterize the quantitative change in the equilibrium constant for dimerization so that some degree of dimerization of this variant is still possible at specific stages of the transport reaction. Furthermore, it is unclear whether the *in vitro* assay employed in their studies would be sensitive to defects in the processivity of the SecA-mediated component of the preprotein translocation reaction, which is coupled jointly to the ATPase activity of SecA and also to the proton-motive force (7) (in the absence of uncoupling reagents that block the formation of transmembrane electrochemical potential gradients). Most importantly, the monomeric variant of SecA exhibits only a few percent of the protein translocation mediated by wild-type dimers under equivalent conditions with wild-type SecYEG, suggesting to us that the reduced ability to form the physiological dimer could be causing a severe defect in translocation activity. In this context, we believe that additional studies will be required to determine whether subunit recruitment plays a role in mediating processivity in preprotein transport.
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Table I. Hydrodynamic properties of SecA in the presence of model translocation ligands †.

| [Phospholipid] | [Signal Peptide] | 0 Micelles | + 1 Micelle $\bar{V}_{pl} = 0.8 \text{ cm}^3/\text{g}$ | + 1 Micelle $\bar{V}_{pl} = 0.9 \text{ cm}^3/\text{g}$ | Peak $S_{20,w}$ (S)$_n$ | f/f$_o$ | $D_{20,w}$ (cm$^2$s$^{-1}$) |
|----------------|-----------------|-------------|---------------------------------|---------------------------------|-------------------|---------|---------------------|
| 0              | 0               | 234,000     | 7.6                             | 1.8                             | 2.9 x 10$^{-7}$   |
| 0              | 25 µM KRR-wt    | 249,000     | 8.0                             | 1.8                             | 2.9 x 10$^{-7}$   |
| 150 µM l-MPG   | 0               | 101,000     | 85,000                          | 102,000                         | 5.2               | 1.5     | 4.6 x 10$^{-7}$    |
| 150 µM l-MPG   | 25 µM KRR-wt    | 235,000     | 205,000                         | 243,000                         | 7.9               | 1.7     | 3.0 x 10$^{-7}$    |
| 150 µM l-MPC   | 0               | 120,000     | 83,000                          | 108,000                         | 5.1               | 1.7     | 3.6 x 10$^{-7}$    |
| 150 µM l-MPC   | 25 µM KRR-wt    | 183,000     | 140,000                         | 165,000                         | 5.2               | 2.2     | 2.6 x 10$^{-7}$    |

† Sedimentation velocity experiments were conducted at 20,000 rpm on a Beckman XL-A analytical ultracentrifuge using 1 µM *E. coli* SecA in KET buffer at 20°C. Parameters were determined by the program SEDFIT (42,43) based on modeling an ensemble of ~90 absorbance profiles using the Lamm equation. Equivalent sedimentation results were obtained when the concentrations of the lyso-lipids were increased to 300 µM (data not shown). For samples run in the presence of lyso-lipids, protein masses are given assuming that either 0 or 1 micelle is bound to the SecA monomer, for reasonable limiting values of the partial specific volume of the micelle ($\bar{V}_{pl}$). See the Methods section for additional details on these experiments and calculations. Static light-scattering measurements give molecular masses of 35 kDa and 65 kDa for l-MPG and l-MPC micelles, respectively, in KET buffer.
Table II. Efficacy of different detergent species in inducing monomerization of SecA †.

| Phospholipid / Detergent | SecA Monomerization | Apparent Cooperativity | [Detergent]_{1/2} | Detergent CMC |
|--------------------------|---------------------|------------------------|-------------------|--------------|
| Lyso-lauryl-PC           | +                   | 7.7                    | 660 µM            | 750 µM       |
| Lyso-myristoyl-PC        | +                   | 3.6                    | 72 µM             | 68 µM        |
| Lyso-palmitoyl-PC        | +                   | 4.0                    | 34 µM             | 12 µM        |
| Lyso-myristoyl-PG        | +                   | 3.3                    | 62 µM             | 64 µM        |
| Dicaproyl-PC             | − a                 |                        | 22 mM             |              |
| Dicaproyl-PG             | −                   |                        | 24 mM             |              |
| β-octylglucoside         | − b                 |                        | 32 mM             |              |
| β-dodecylmaltoside       | −                   |                        | 100 µM            |              |
| C_{12}E_{8}              | −                   |                        | 39 µM             |              |
| Lauryl-dimethylamineoxide| +                   | 5.9                    | 1.5 mM            | 1.9 mM       |

† The monomerization of SecA was assayed at 24°C in KET buffer using fluorescence anisotropy titrations equivalent to those shown in Fig. 1C (terminating at a phospholipid or detergent concentration above the cmc), and the results were verified using sedimentation velocity experiments equivalent to those reported in Table I. The parameter [Detergent]_{1/2} represents the concentration of detergent giving 50% monomerization and was determined using non-linear curve fitting of the anisotropy data to a standard cooperative binding equation. The apparent cooperativity was determined using a Hill plot of the data in conjunction with the parameters determined from non-linear curve-fitting. The cmc values were determined in KET buffer in situ in the fluorimeter for all of the detergent and phospholipid species reported here (as described in the Methods section).
a Some degree of SecA tetramer formation was observed in analytical ultracentrifugation experiments conducted in the presence of concentrations of DCPC above its cmc.

b In both fluorescence and ultracentrifugation experiments, SecA showed a pronounced tendency to aggregate in the presence of β-octylglucoside concentrations above its cmc; protein aggregation could potentially have prevented the observation of SecA monomers in these experiments.
Figure Legends

Figure 1. Long-chain phospholipid analogues monomerize the physiological dimer of SecA.

The interaction of wild-type *E. coli* SecA with various water-soluble phospholipid analogues is characterized in KET buffer using analytical ultracentrifugation and tryptophan fluorescence spectroscopy.  (A) Four representative absorbance scans from equivalent time points are shown from sedimentation velocity experiments conducted at 20˚ C on 1.0 µM SecA either in the absence (upper panel) or presence (lower panel) of 150 µM *l*-MPG.  The gray traces at the bottom of each panel show the curve-fitting residuals for the complete ensemble of ~90 absorbance scans used for molecular mass calculations.  (B) Molecular mass distribution c(M) profiles were calculated by SEDFIT (42,43) from sedimentation velocity experiments like those shown in panel (A) conducted either in the absence of phospholipid (——) or in the presence of 150 µM *l*-MPG (————) or 150 µM *l*-MPC (- - - -).  The analysis assumes a partial specific volume of 0.734 cm³/gm for all hydrodynamic species.  Equivalent results were obtained when the lyso-lipid concentrations were increased to 300 µM or 500 µM (data not shown).  (C) Systematic analysis of the effect of phospholipid binding to SecA on the molecular mass of the protein as inferred from the sedimentation velocity data.  Because neither the partial specific volume of the phospholipid (νₚₗ expressed in cm³/gm) nor the amount of phospholipid bound to SecA is known with certainty, the analysis was performed assuming all reasonable values for these parameters.  The graph shows the mass of the protein component of the protein/detergent complex excluding the mass of the bound lipid.  See Methods section for details of the analysis.  The curves for which the partial specific volume of the lipid is not indicated in the figure assumed values of 0.758, 0.783, 0.807, and 0.832 cm³/gm.  Solid and dashed lines are used in alternation to facilitate visualization.  (D) Titrations of 0.25 µM SecA with *l*-MPG (▲), *l*-MPC (△), DCPG (■), or DCPC (□) were conducted at 24˚ C and monitored by tryptophan fluorescence spectroscopy.  Steady-state anisotropy is shown in the top panel, while relative total
fluorescence at 330 nm is shown in the bottom panel. (E) Thermal titrations of 0.25 µM SecA were conducted in the absence (-----) or presence of a 1.0 mM concentration of \( l \)-MPG (-----), \( l \)-MPC (------), DCPG (--------), or DCPC (----------). Reciprocal steady-state anisotropy is shown in the top panel, and relative total fluorescence at 330 nm is shown in the bottom panel. Reciprocal anisotropy is plotted here because of the linear relationship between this parameter and temperature for a particle of constant size and shape as described by the Perin equation (48).

**Figure 2.** Ligand-dependent changes in chemical cross-linking of SecA. The effects of phospholipid analogues (at 2 mM) and signal peptides (at 100 µM) on the oligomeric state of SecA were analyzed using glutaraldehyde cross-linking experiments evaluated by SDS polyacrylamide gel electrophoresis. See Materials & Methods section for experimental details. (A) Crosslinking conducted for 5 minutes at 20˚ C. (B) Crosslinking conducted for 3 minutes at 37˚ C. The crosslinking time was reduced in this experiment because a larger amount of background crosslinking is observed as temperature is increased.

**Figure 3.** Synthetic signal peptide re-dimerizes phospholipid-monomerized SecA. The experiments were conducted on wild-type *E. coli* SecA in KET buffer either in the presence or absence of the synthetic KRR-LamB signal sequence peptide (13). (A) Molecular mass c(M) distribution profiles were calculated by SEDFIT (42,43) from sedimentation velocity experiments on 1.0 µM SecA in the presence of 150 µM \( l \)-MPG either without (-----) or with 25 µM wild-type KRR-LamB signal peptide (--------). The experiments were performed at 20˚ C. Equivalent results were obtained when the lyso-lipid concentration was increased to 300 µM (data not shown). (B) After monomerizing 2.0 µM SecA by exposure to 250 µM \( l \)-MPG, titrations of either wild-type KRR-LamB signal peptide (squares) or the \( \Delta \)78 mutant peptide (circles) were monitored using tryptophan fluorescence spectroscopy. Steady-state anisotropy is shown in the top panel, while relative total fluorescence at 330 nm is shown in the bottom panel.
The arrows indicate the values observed for the physiological dimer of SecA prior to the addition of l-MPG. (C) Hill plot for the titration of wild-type KRR-LamB signal peptide presented in panel (B) based on the steady-state anisotropy data.

**Figure 4. Synthetic signal peptide induces polymerization of the high-temperature domain-dissociated conformation of SecA.** Experiments were performed on *E. coli* SecA in KET buffer. (A) Molecular mass distribution profiles were calculated by SEDFIT from sedimentation velocity experiments conducted on 1.0 µM wild-type SecA either in the absence (—) or presence of 25 µM wild-type KRR-LamB signal peptide (-----). The experiments were performed at 20˚ C. (B) Thermal titrations of 0.25 µM wild-type SecA were monitored using tryptophan fluorescence spectroscopy in the absence of signal peptide (——) or in the presence of a 25 µM concentration of either wild-type KRR-LamB signal peptide (-----) or the Δ78 mutant peptide (---). Perin plots (48) of reciprocal anisotropy are shown in the top panels, while relative plots of relative total fluorescence at 330 nm are shown in the bottom panels. (C) Thermal titrations were conducted in the same manner as in panel (B) on wild-type SecA (—) as well as the Y134C (------), A373V (-.-.-), and A507V (-..-.-.-.-.--) prlD suppressor mutants (52,53) in the presence of 25 µM wild-type KRR-LamB signal peptide. (D) Steady-state tryptophan fluorescence anisotropy data showing dissociation of signal-peptide-induced SecA polymers specifically by phospholipids producing monomerization of the physiological dimer (*i.e.*, by l-MPG above its cmc but not by DCPG). The A373V variant of SecA was heated to 37.5˚ C to induce domain-dissociation and allowed to equilibrate. This variant was used because the lower temperature of the endothermic transition gives efficient signal-peptide induced polymerization at a reduced temperature. Addition of wild-type KRR-LamB signal peptide (arrows on the left) causes a rapid increase in anisotropy reflecting protein polymerization. After 5 minutes, phospholipids were added at the indicated concentrations (arrows on the right). To facilitate visualization of the results, an arbitrary offset has been added.
to the data from the DCPG experiment but not the l-MPG experiment. There were minimal changes in the total fluorescence during these experiments other a small amount of photo-oxidation (data not shown).
A  

| Protein  | 20°C |  
|----------|------|  
| SecA     | +    |  
| GA       | -    |  
| KRRwt    | -    |  
| KRRΔ78   | -    |  
| LMPG     | -    |  
| LMPC     | -    |  

MW  

- Tetramer (406 kDa)  
- Dimer (204 kDa)  
- Monomer (102 kDa)  

B  

| Protein  | 37°C |  
|----------|------|  
| SecA     | +    |  
| GA       | -    |  
| KRRwt    | -    |  
| LMPG     | -    |  
| diCapPG  | -    |  
| LMPC     | -    |  
| diCapPC  | -    |  

MW  

- Dimer (204 kDa)  
- Monomer (102 kDa)
Phospholipid-induced monomerization and signal-peptide-induced oligomerization of SecA

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