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Energetics of SecA Dimerization

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Summary

Transport of many proteins to extracytoplasmic locations occurs via the general secretion (Sec) pathway. In *Escherichia coli*, this pathway is comprised of the SecYEG protein conducting channel and the SecA ATPase. SecA plays a central role in binding the signal peptide region of preproteins, directing preproteins to membrane-bound SecYEG and promoting translocation coupled with ATP hydrolysis. Although it is well-established that SecA is crucial for preprotein transport and thus cell viability, its oligomeric state during different stages of transport remains ill defined. We have characterized the energetics of SecA dimerization as a function of salt concentration and temperature and defined the linkage of SecA dimerization and signal peptide binding using analytical ultracentrifugation. The use of a new fluorescence detector permitted analysis of SecA dimerization down to concentrations as low as 50 nM. The dimer dissociation constants are strongly dependent on salt. Linkage analysis indicates that SecA dimerization is coupled to the release of about five ions, demonstrating that electrostatic interactions play an important role in stabilizing the SecA dimer interface. Binding of signal peptide reduces SecA dimerization affinity such that K_d increases about 9-fold from 0.28 μ M in the absence of peptide to 2.68 μ M in the presence of peptide. The weakening of the SecA dimer that accompanies signal peptide binding may poise the SecA dimer to dissociate upon binding to SecYEG.

Keywords

SecA; signal peptide; analytical ultracentrifugation; thermodynamic linkage

A key issue in cell biology centers on how proteins that function in extracytoplasmic locations are correctly localized following their cytoplasmic synthesis. These proteins are typically synthesized as preproteins with an amino-terminal, cleavable signal peptide that earmarks the protein for transport and interfaces with components of the translocation machinery. In prokaryotes and eukaryotes, the general secretion (Sec) pathway is used for transport of many proteins and membrane translocation occurs via a conserved protein

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conducting channel.^{1;2} In *Escherichia coli*, the translocase comprises the integral membrane channel SecYEG and the SecA ATPase.^{3;4} SecA is a large, multifunctional protein that is found in cytoplasmic-, phospholipid-, and SecYEG-bound forms.⁵⁻⁷ For post-translationally transported proteins, a preprotein associates via its signal peptide with SecA in the cytoplasm often with its mature portion bound by the chaperone SecB.^{8;9} Subsequently, SecA docks with the membrane-bound SecYEG and guides preprotein translocation via this channel.^{4;10-15} Although the details of the membrane translocation steps are unknown, evidence suggests that the preprotein traverses the membrane by insertion and retraction cycles of SecA, coupled to the hydrolysis of ATP.^{4;16-18}

It is well-established that SecA plays a central role in the Sec pathway because of its direct interaction with SecB,^{19;20} preproteins,^{21;22} anionic phospholipids,^{23;24} SecYEG^{25;26} and nucleotide;²⁷ however, the functional oligomeric state of SecA during translocation is still in dispute. The view that SecA functions as a monomer in protein translocation was supported by the finding that a monomeric SecA mutant, SecAΔ11/N95 (lacking both ten amino-terminal residues and 70 carboxyl-terminal residues), sustained growth of the SecA deletion strain EO527;²⁸ yet this was at odds with the finding that the monomeric mutant SecAΔ11 was unable to complement the *secA* (*Ts*) amber mutant strain BL21.19.²⁹ Later, SecAΔ11/N95 was reported to be dimeric *in vivo* and able to complement BL21.19,³⁰ making this topic more complicated yet.

The cellular concentration of SecA is estimated to be about 5-8 μM^{31;32} with a dimer dissociation constant of 0.25-0.5 μM.³³ Therefore, the majority of this 102 kDa molecule (protomer molecular weight) is likely to exist as a dimer in the cytoplasm in the absence of ligands that might alter its oligomeric properties.^{33;34} Nonetheless, previous studies with purified components have obtained evidence for both monomeric and dimeric forms of SecA.^{13;20;32;35-38} For example, Fekkes and colleagues demonstrated that SecA binds as a dimer to SecB.²⁰ Or and co-workers observed that SecA dissociates to monomers in the presence of acidic phospholipids.³² SecA is also dissociated by detergents.^{32;35} Woodbury *et al.* used gel filtration with static light scattering to demonstrate that SecA exists in an equilibrium mixture of the monomeric and dimeric forms at micromolar concentrations.³³ Moreover, they found that the monomer-dimer equilibrium of SecA was sensitive to temperature and to salt concentration. Confounding this issue, X-ray crystallographic data of SecA from different bacterial sources reveals monomeric,^{10;39} dimeric,^{7;40-42} and even tetrameric⁴³ forms of the protein.

Our laboratory has preliminary evidence based on native gel analysis that the addition of signal peptide induces SecA monomerization.³⁷ The recent crystal structure of SecA bound to the SecY complex¹³ and analysis of SecA binding to SecY embedded in lipid nanodiscs⁴⁴ suggest that a SecA protomer binds to the protein channel, underscoring the possibility that oligomeric SecA dissociates prior to delivery of the preprotein to translocation sites. Other studies suggest that SecA remains dimeric during translocation.^{29;30;45-47} These data imply that the oligomeric state of SecA might change during different stages of protein transport. In order to define the role of SecA in preprotein transport it is important to characterize the energetics of SecA oligomerization and how the binding of the signal peptide region of the preprotein to SecA is coupled to SecA self-association.

As a first step in the quantitative analysis of SecA self-association during protein translocation, we have used analytical ultracentrifugation to define SecA dimerization in solution as a function of salt concentration and temperature. We have also characterized the thermodynamic linkage of SecA dimerization and binding of signal peptide. Analytical ultracentrifugation is a rigorous method to measure the size, shape and interactions of biological molecules in solution.⁴⁸⁻⁵⁰ We have employed a fluorescence detector for the

analytical ultracentrifuge^{51;52} to extend the sensitivity of this technique well below the detection limits obtainable using conventional biophysical instrumentation. The dissociation constants are extraordinarily sensitive to KCl concentration, indicating a substantial contribution of electrostatic interactions to SecA dimerization. Furthermore, signal peptide binding is thermodynamically linked to SecA dimerization such that peptide binding enhances dissociation of the dimer. Our results are consistent with the possibility that monomeric SecA is the form of SecA that binds to SecYEG to guide protein translocation.¹³

Results

Dimerization of SecA

Sedimentation velocity measurements were used to characterize the dimerization affinity of SecA and the hydrodynamic properties of the monomer and dimer. Initially, data were analyzed using the time derivative method⁵³ to generate $g(s^*)$ sedimentation coefficient distributions. This model-independent method is useful to define the distribution of different oligomeric states and to probe the effects of protein concentration and experimental conditions. Figure 1 shows the $g(s^*)$ distributions obtained at multiple concentrations of SecA (1-20 μ M) in 400 mM KCl (20 mM HEPES, pH 7.5, 400 mM KCl, 0.5 mM EDTA, and 0.1 mM TCEP) at 20°C using conventional interference optics. The peak of the normalized $g(s^*)$ distribution shifts to the right with increasing SecA concentration, indicating that SecA reversibly associates under these conditions. The increase in the weight-average sedimentation coefficient from ~5.5 to ~7.5 S over the range of 1-20 μ M SecA is consistent with a monomer-dimer equilibrium. In the analysis of reversible interaction by sedimentation velocity, cross-correlation between association constants and the sedimentation coefficients can reduce the precision in the fitted parameters. We have taken advantage of the strong dependence of SecA association on salt concentration to directly measure the sedimentation coefficients of the monomer and dimer and then fixed them when fitting for the association constants. The equilibrium is strongly shifted towards dimer at lower salt concentration such that in 100 mM KCl the normalized $g(s^*)$ distributions obtained for 0.5-10 μ M SecA overlay with a peak near 8.5 S corresponding to dimer (Supplementary Data, Figure S1). Thus, the SecA dimer does not dissociate over this concentration range. Fitting the data obtained at the highest protein concentration (10 μ M) to a single ideal species model in SEDANAL gives a corrected sedimentation coefficient of $s_{20,w} = 8.76 \pm 0.04$ S for the dimer. This value falls within the range of sedimentation coefficients previously reported for the SecA dimer of 8.44 S,⁵⁴ 8.65 S⁵⁴ and 9.06 S.⁵⁵

The sedimentation coefficient of the SecA monomer was determined at low protein concentration in higher salt. However, it is necessary to reduce the protein concentration below the limit of sensitivity using absorption or interference optics to completely dissociate the dimer. Therefore, we have used fluorescence detection of labeled SecA in order to perform sedimentation velocity measurements at SecA concentrations as low as 50 nM. At this protein concentration, the corrected sedimentation coefficient is independent for salt concentration ranging from 300-500 mM, indicating negligible dimerization. Fitting the data obtained at 50 nM SecA in 500 mM KCl to a single ideal species model gives a corrected sedimentation coefficient of $s_{20,w} = 5.83 \pm 0.10$ S for the monomer. Note that the mass of a single Alexa Fluor 488 label is insignificant relative to the mass of the SecA monomer and the label would not be expected to influence the sedimentation coefficient.

Dependence of SecA Dimerization on Salt Concentration

The dependence of the SecA dimerization on salt concentration was measured by global analysis of sedimentation velocity data. Interference detection was used at the higher salt concentrations (300-500 mM KCl) where dimerization is relatively weak. Fluorescence

detection was necessary to measure the strong association of SecA at lower salt concentration (100-300 mM KCl). Figure 2 shows a global analysis of SecA dimerization in 300 mM KCl collected using interference data. The data were subtracted in pairs to remove the time-invariant systematic background and the resulting difference curves were fit to a monomer-dimer model using SEDANAL. Systematic deviations were detected in the residuals using this model, indicating the presence of additional species. Because higher oligomers are not present (Figure 1), we considered models that incorporate non-equilibrating, incompetent monomer and dimers, as previously reported in analytical ultracentrifugation studies of self-associating systems.⁵⁶⁻⁵⁸ Good fits were obtained using a model that includes an incompetent dimer, which is not in equilibrium with the monomer. For the data obtained at 300 mM KCl (Figure 2), the RMS (root mean square) deviations decrease dramatically from 0.031 fringes to 0.019 fringes upon addition of the incompetent dimer to the fitting function, giving a best-fit K_d of $4.4 \pm 0.4 \mu\text{M}$. The amount of incompetent dimer corresponds to 30% by weight for the fit depicted in Figure 2 and ranged from 13 to 37% for all the fits we performed. The origin of this incompetent SecA dimer is not known but may correspond to the heterogeneity previously reported in SecA preparations.³³ Table 1 shows that K_d for SecA dimerization is highly dependent on salt concentration from 300-500 mM. Using fluorescence optics, we were able to extend these measurements down to 100 mM KCl. Figure 3 shows a global analysis for dimerization of Alexa Fluor 488-labeled SecA in 300 mM KCl using fluorescence detection. The two detection methods agree very well at 300 mM KCl for a sample of labeled SecA, with a best-fit K_d of $1.10 \pm 0.11 \mu\text{M}$ by interference and $1.13 \pm 0.11 \mu\text{M}$ by fluorescence.

Quantitative analysis of the salt dependence of SecA dimerization can provide insights into the role of electrostatic interactions. Figure 4 shows a thermodynamic linkage plot for the salt dependence of SecA dimerization. At moderate salt concentrations the slope of this plot is given by

$$\left(\frac{\partial \ln [K]}{\partial \ln [KCl]} \right)_{T,P} = -\Delta n_{K^+} - \Delta n_{Cl^-} \quad (1)$$

where Δn_{K^+} and Δn_{Cl^-} correspond to the number of K^+ and Cl^- ions released or taken up.⁵⁹ A linear fit to the data in Figure 4 yields a slope of -5.26 ± 0.30 , indicating that ~ 5.0 ions are released upon dimerization.

Dependence of SecA Dimerization on Temperature

We have examined the temperature dependence of SecA dimerization between 8 and 20°C at 300 mM KCl. SecA dimerization becomes more favorable as the temperature increases (see Table 2), indicating a positive enthalpy change. A van't Hoff plot of $\ln K$ vs. $1/T$ was used to estimate an enthalpy change of $\Delta H^\circ = 22.4 \pm 0.6 \text{ kcal/mol}$ (Supplementary Data, Figure S2). At 20°C, the free energy change for SecA dimerization is $\Delta G^\circ = -7.4 \pm 0.3 \text{ kcal/mol}$. Thus, SecA dimerization is enthalpically disfavored and is driven by a favorable, positive entropy change, ΔS° . The van't Hoff plot is linear, suggesting that the heat capacity change (ΔC_p) is near zero.

Linkage of Signal Peptide Binding and SecA Dimerization

Several studies have reported that binding of signal peptide induces dissociation of the SecA dimer^{32;37} whereas others indicate that the dimer remains intact throughout the preprotein translocation cycle.^{29;45;46;60;61} It has also been reported that signal peptide induces dimerization of SecA.³⁵ These discrepancies may be related to the different methods and experimental conditions used to assay the oligomeric state of SecA. Here, we have directly

investigated the linkage of signal peptide binding and SecA dimerization at equilibrium using analytical ultracentrifugation. Given the hydrophobic nature of the signal peptide, we first examined the oligomeric state of the signal peptide at the high concentrations required for the SecA linkage analysis. Sedimentation velocity measurements were performed with the KAP25 peptide at 100-400 μM , at 100 mM and 300 mM KCl. Under these conditions, we observe a single peak in the $c(s)$ distributions near $s_{20,w} = 0.406 \pm 0.003$ S (Supplementary Data, Figure S3). These data were fit to a single species model to obtain a molecular weight of 3.8 ± 0.3 kDa which agrees well with the molecular weight for the monomer of 3.5 kDa predicted from the sequence. Thus, KAP25 remains monomeric, even at high concentration.

Sedimentation velocity analysis of SecA dimerization in the presence of signal peptide was carried out using fluorescence detection so that the peptide would not contribute to the observed signal. Measurements were performed at 200 mM KCl with signal peptide concentrations ranging from 1 to 100 μM . The apparent K_d for SecA dimerization increases about 9-fold from 0.28 ± 0.13 μM in the absence of signal peptide, up to about 2 μM at high signal peptide concentration, indicating that peptide binding is thermodynamically linked to SecA dimerization. Figure 5A shows the dependence of the K_d on signal peptide concentration. These data were fit to a classical model for ligand-linked association⁶² shown in Figure 5B. In order to reduce the number of adjustable parameters we make the simplifying assumptions that L_{21} is the geometric mean of L_{20} and L_{22} , and similarly

$$\beta_{21} = [\beta_{11} \sqrt{\beta_{22}}]^{1/2} \quad (2)$$

Then, the variation in the apparent K_d for SecA dimerization as a function of peptide concentration is given by

$$K_{d,app} = K_d \frac{(1 + \beta_{11} [P])^2}{1 + \beta_{22}^{1/4} \beta_{11}^{1/2} [P] + \beta_{11} [P]^2} \quad (3)$$

This model fits that data well, with best-fit values of $\beta_{11} = 0.158$ ($K_d = 1/\beta_{11} = 6.32$ μM) and $\beta_{22} = 0.00264$ ($K_d = \sqrt{1/\beta_{22}} = 19.5$ μM). Although it is difficult to directly compare these values with previous studies where it was not possible to distinguish signal peptide binding to monomeric and dimeric forms, the dissociation constants are in the range previously reported for signal binding to SecA.⁶³ The linkage free energy is given by

$$\beta G^0 = -RT \ln \frac{\sqrt{\beta_{22}}}{\beta_{11}} = -RT \ln \frac{L_{22}}{L_{20}} \quad (4)$$

and corresponds to $\Delta G^0 = 0.66$ kcal/mol.

Discussion

Although it is well-established that SecA can form dimers, the energetics of this reaction and the dependence of dimerization on experimental conditions have not been rigorously investigated. Furthermore, potential changes in SecA oligomeric state that accompany preprotein binding and translocation remain controversial. Here, we have measured the energetics of SecA dimerization in free solution and the thermodynamic linkage of this reaction to ion binding, temperature and signal peptide binding.

Our data indicate that SecA dimerization affinity is extraordinarily salt dependent, with K_d ranging from 40 μM in 500 mM KCl down to 14 nM in 100 mM KCl. These results support previous work demonstrating a salt effect on SecA dimerization³³ and our measurements at intermediate salt concentrations ($K_d = 3.1 \mu\text{M}$ in 300 mM KCl) are consistent with earlier estimates obtained using size exclusion chromatography and static light scattering. Woodbury *et al.* estimated K_d for SecA dimerization of 0.25-0.5 μM in 300 mM potassium acetate.³³ Das *et al.* reported $K_d = 2.2 \mu\text{M}$ in 300 mM KCl for a His-tagged SecA construct.⁶⁴ Potassium is the most abundant cation in *E. coli* and under low osmolarity growth conditions it has a concentration of about 200 mM.⁶⁵ At this salt concentration, the SecA dissociation constant is 0.28 μM (Table 1), which is well below the cellular SecA concentration of about 5-8 μM .^{31;32} Thus, our results indicate that dimer is likely the predominant cytoplasmic state of SecA in the absence of bound ligands. However, *E. coli* adapts to growth in higher osmolarity media by modulating the intracellular concentrations of water and solutes, including potassium,⁶⁶ which may affect SecA dimerization.

The strong dependence of SecA dimerization affinity on salt concentration indicates a significant electrostatic contribution to dimer stability. Quantitative linkage analysis demonstrates dimerization is coupled to release of ~5 ions. This large number of ions released is unusual for a protein-protein interaction; for example, dimerization of the protein holoBirA results in the release of only 1-2 ions.⁶⁷ Assuming that ion release is localized at the dimer interface, these results imply that SecA dimerization is associated with formation of several salt bridges.

Several different dimer interfaces have been detected in different crystal structures of the SecA dimer from multiple species^{7;40-43} and it is not yet clear which one mediates SecA dimerization in solution. Figure 6 shows potential SecA dimer interfaces derived from two representative SecA structures. The dimer interface from a crystal structure of *E. coli* SecA (2FSF)⁷ is comprised of a discontinuous surface totaling 1,370 $\text{\AA}^2$ lying within the DEAD box domain and contains a single salt bridge (Figure 6A). The dimer interface in a structure of full-length *B. subtilis* SecA (1M6N)⁴⁰ lies on the opposite face of the monomer and includes portions of the DEAD box, scaffold and C-terminal domains. This interface is considerably larger (2,780 $\text{\AA}^2$) and contains ten salt bridges. SecA proteins of *B. subtilis* and *E. coli* are highly homologous (52% identity) and we have mapped the interface for the *B. subtilis* dimer onto the *E. coli* structure for comparison (Figure 6B). The dimer interface observed in cryo-electron microscopy studies of full-length *E. coli* SecA dimer⁶⁸ differs from the *E. coli* crystallographic dimer interface⁷ and is similar to that observed in *B. subtilis*.⁴⁰ The interaction at this interface has a strong electrostatic component, which is compatible with the large number of ions released upon dimerization.

In addition to being highly dependent on salt concentration, SecA dimerization is also highly temperature dependent. Dimer stability is enhanced at higher temperatures and van't Hoff analysis yields a large positive enthalpy change of $\Delta H^\circ = 22.4 \pm 0.6 \text{ kcal/mol}$. Thus, SecA dimerization is opposed by an unfavorable enthalpy change and is driven by a favorable, positive entropy change. These results point toward a contribution of hydrophobic interactions in stabilizing the dimer interface. However, a negative heat capacity change, ΔC_p , is now considered a more reliable signature of the hydrophobic effect than a positive ΔS .⁶⁹ We were unable to measure ΔC_p for SecA dimerization over the limited temperature range that was examined.

Association of the signal peptide region of the preprotein with SecA is required for translocation via the SecYEG channel. Dissociation constants of ~1-10 μM have been reported based on fluorescence anisotropy assays.⁶³ Two studies reported that signal peptide binding dissociates SecA dimers^{32;37} while another indicates that peptide binding does not

perturb SecA self-association.⁷⁰ We have used sedimentation velocity measurements to directly probe the influence of signal peptide interaction on SecA dimerization in free solution. Binding of signal peptide shifts the SecA dimerization equilibrium towards monomer, with a moderate linkage free energy of $\Delta G^\circ = 0.66$ kcal/mol. This linkage may have biological significance. A crystal structure of the complex of SecA with SecYEG¹³ and analysis of SecA binding to SecYEG in lipid nanodiscs⁴⁴ suggest that monomeric SecA binds to the channel. The weakening of the SecA dimer that accompanies signal peptide binding may poise the SecA dimer to dissociate upon binding to SecYEG. However, the oligomeric state of SecA bound to the channel remains controversial and other studies suggest that SecA remains dimeric during translocation.^{29;30;45-47}

The region of SecA that interacts with the signal peptide region of the preprotein has been mapped using biochemical and biophysical approaches.^{21;22;38;63;70} Figure 6A also illustrates the peptide binding portion of SecA based on an NMR structure of a SecA-peptide complex (2VDA).⁷⁰ This surface does not overlap with the dimer interfaces derived from the crystal structures of either *E. coli* or *B. subtilis* SecA (Figure 6) or with dimer interfaces found in other SecA structures. Thus, the binding of the signal peptide must allosterically modulate dimer affinity. It is known that SecA adopts both open and closed conformations^{39;40;68;70;71} and these states may exist in equilibrium.⁷⁰ This conformation transition may be associated with allosteric coupling of signal peptide binding and dimerization and function to convert SecA between low- and high-affinity dimerization states. Note that signal peptide binding is also known to allosterically activate the translocase.¹⁴

The high affinity of SecA dimerization, particularly at lower salt concentrations, requires that measurements be performed at low SecA concentrations, well below the range where conventional biophysical measurements are feasible. Therefore, we have used a newly developed fluorescence detector for the analytical ultracentrifuge to measure SecA dimerization at concentrations as low as 50 nM. In previous applications of fluorescence-detected sedimentation velocity, association constants have been obtained by fitting the isotherm of weight-average sedimentation coefficients.^{72;73} Statistically, it is desirable to directly fit sedimentation velocity data to obtain association parameters. However, this approach requires accurate extinction coefficients for each data channel, which can be difficult to obtain for fluorescence data recorded at different photomultiplier voltages. Therefore, we have developed a protocol using a fixed concentration of labeled macromolecule mixed with a variable concentration of the unlabeled protein such that a single voltage can be used for all channels. This approach should be generally applicable to studies of self-associating systems using fluorescence-detected analytical ultracentrifugation.

Materials and Methods

Protein and Peptide Preparation

The vector pSecA encoding the full-length wild-type *E. coli* SecA was generated from pIMBB7⁷⁴ by removing the N-terminal His₆-tag and inserting the first four residues following the start codon. *E. coli* BL21(DE3) harboring pSecA were grown in Lina medium⁷⁵ with 100 µg/ml ampicillin at 37°C until OD₆₀₀ reached ~0.6. Expression of SecA was induced with 0.5 mM IPTG, and cell growth proceeded for 2 hours. SecA protein was purified by stepwise salt gradient elution,^{54;76} containing 25 mM Tris, pH 7.6, 0.5 mM EDTA, 0.5 mM PMSF, 1 mM DTT and NaCl at concentrations ranging from 10 mM to 1.3 M. Briefly, the lysate prepared by the French pressure cell was spun for 20 min at 12,000 rpm in a Sorvall SS-34 rotor and then for 1 hr at 38,000 rpm in a Beckman Ti-60 rotor. The supernatant was applied to 6 ml Affix-gel blue gel (Bio-Rad) column equilibrated with low-

salt buffer (10 mM NaCl). After rinsing the column with 30 ml of low-salt buffer and 60 ml of medium-salt buffer (0.5 M NaCl), SecA was eluted with 30 ml of high-salt buffer (1.3 M NaCl). Further purification was performed on a Sephacryl S-300 HR column (Amersham Biosciences). SecA fractions were pooled and precipitated with 80% ammonium sulfate for storage at -80°C . Before using, the ammonium sulfate-precipitated SecA was dissolved in the working buffer containing 20 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.1 mM TCEP and KCl at concentrations ranging from 100 to 500 mM and desalted on BioGel P-6 spin columns (Bio-Rad) equilibrated in the same working buffer. SecA concentration was determined by absorbance at 280 nm using an extinction coefficient of $\epsilon_{280} = 75,750 \text{ M}^{-1}\text{cm}^{-1}$ calculated with Sednterp.⁷⁷

SecA was labeled at the N-terminus with Alexa Fluor 488 carboxylic acid, succinimidyl ester (Invitrogen) for fluorescence-detected analytical ultracentrifugation experiments. The conjugation reaction was carried out in 10 mM potassium phosphate, 0.15 M NaCl, pH 7.0 for two hours at 4°C using a 5-fold molar ratio of dye to SecA. Note that the N-terminus is preferentially labeled at neutral pH and below because the pK_a of the α -amino group is considerably lower than that of the ϵ -amino group of lysine.⁷⁸ The free dye was separated from the labeled SecA using a BioGel P-6 desalting spin column. The extent of labeling was quantified by absorbance measurements and ranged from 0.92 to 0.98 Alexa Fluor 488/SecA monomer, consistent with specific labeling at the α -amino group of the N-terminus. In control experiments, we compared the sedimentation velocity of unlabeled and labeled SecA over a concentration range of 0.5–10 μM in 300 mM KCl using interference optics. Under these conditions, the dimer dissociation constants of unlabeled and labeled SecA are $4.3 \pm 0.9 \mu\text{M}$ and $1.1 \pm 0.1 \mu\text{M}$, respectively, indicating that labeling slightly enhances dimerization affinity.

The KAP25 peptide (KKKMKQSTIALALLPLL**FTPVT****KARTPE****KKK**-NH₂) was synthesized and purified by NEO-Peptide. This sequence includes the *E. coli* alkaline phosphatase signal peptide (underlined) and four residues of the mature region (boldface). Three lysines were added at each terminus to enhance solubility of the peptide. Addition of these lysines does not perturb the ability of this peptide to interact with the signal peptidase.⁷⁹ KAP25 stimulates SecA ATPase activity, indicating that it binds to SecA in a functionally-relevant manner.

Analytical Ultracentrifugation

For analytical ultracentrifugation measurements using interference optics, samples were loaded into synthetic boundary aluminum-epon cells equipped with sapphire windows. The sample and reference menisci were matched as previously described⁴⁸ and sedimentation velocity analysis was carried out at 40,000 rpm using an An-50 Ti rotor in a Beckman-Coulter XL-I analytical ultracentrifuge. The temperature was 20°C unless otherwise indicated. Measurements at low SecA concentrations were performed using fluorescence detection in a Beckman-Coulter XL-I analytical ultracentrifuge equipped with an AU-FDS detector (AVIV Biomedical). Samples containing 50 nM SecA labeled with Alexa Fluor 488 and variable concentrations of unlabeled SecA were loaded into charcoal-epon double sector cells equipped with quartz windows. Sedimentation velocity measurements were carried out at 20°C and 36,000 rpm using an An-50 Ti rotor.

Initial analysis of sedimentation velocity data was performed using the program DCDT+⁸⁰ to obtain $g(s^*)$ distributions for visual inspection. Molecular masses, partial specific volumes, solvent densities and viscosities were calculated using Sednterp.⁷⁷ Multiple datasets were globally fit to self-association models using SEDANAL⁸¹ to obtain the monomer-dimer equilibrium constants. The menisci for interference data were determined from the raw camera images. The meniscus position was not readily apparent in

fluorescence scans. We obtained this parameter from absorbance intensity scans taken after the fluorescence scans were completed. An alternative approach has recently been described,⁷² but requires the addition of a fluorescence dye suspended in light oil that may perturb the sample. A mass extinction coefficient of 3.3 fringes/ (mg/ml) was used for interference data. The interference data were analyzed using a monomer-dimer equilibrium model. As described in the Results section, good fits required the addition of an incompetent dimer species.

It is difficult to calculate extinction coefficients for fluorescence analytical ultracentrifugation data recorded at multiple gain settings over a broad concentration range of labeled macromolecule. Therefore, we developed a protocol using a fixed concentration of labeled macromolecule mixed with variable concentrations of the unlabeled macromolecule such that a single gain setting can be used for all channels. An effective extinction coefficient was assigned to the labeled species based on the plateau signal obtained from labeled SecA. The fluorescence data were analyzed using a monomer-dimer model which allows for reversible dimerization of labeled and unlabeled SecA as well as interaction between these two components to form a mixed heterodimer. Given the complexity of this model, we did not consider the presence of incompetent dimer. For data acquired with the fluorescence detector, the amplitude of the stochastic noise depends strongly on the signal intensity. Therefore, the data were weighted by the standard deviation stored in the third column of the raw data files. This also served to convert the RMSD reported by SEDANAL from arbitrary fluorescence intensity units to χ , where a value of 1.0 corresponds to a perfect fit. Error limits were calculated using F-statistics to define the 95% joint confidence intervals.

SecA structures were analyzed and presented using PYMOL (Schrödinger, LLC). Protein interface parameters were determined using PISA.⁸²

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Sedimentation velocity analysis of SecA self-association in 400 mM KCl
Normalized $g(s^*)$ distributions of 1 μM (red), 3 μM (yellow), 5 μM (green), 10 μM (blue), and 20 μM (purple) SecA in a buffer containing 400 mM KCl. The distributions are normalized by amplitude. Conditions: rotor speed, 40,000 RPM; temperature, 20°C, interference optics.

Figure 2. Sedimentation velocity analysis of SecA self-association in 300 mM KCl using interference optics

Global analysis of sedimentation velocity difference curves obtained with interference optics. The data were subtracted in pairs to remove the time-independent noise. SecA concentrations: A, 0.5 μM ; B, 1 μM ; C, 3 μM ; D, 10 μM . Conditions: rotor speed, 40,000 RPM; temperature, 20°C. The data were fit to a monomer-dimer equilibrium plus incompetent dimer model. The best-fit $K_d = 4.4 \pm 0.4 \mu\text{M}$ with RMSD = 0.019 fringes.

Figure 3. Sedimentation velocity analysis of SecA self-association in 300 mM KCl using fluorescence detection

Global analysis of sedimentation velocity difference curves obtained with fluorescence optics. The data were subtracted in pairs to remove the time-independent noise. SecA concentrations: A, 0.1 μM ; B, 0.5 μM ; C, 1 μM ; D, 5 μM . Conditions: rotor speed, 36,000 RPM; temperature, 20°C. The data were fit to a monomer-dimer equilibrium model. The best-fit $K_d = 1.13 \pm 0.11 \mu\text{M}$ with RMSD = 1.06.

Figure 4. Dependence of SecA dimerization on salt concentration

KCl linkage plot ($\partial \ln K$ versus $\partial \ln [\text{salt}]$) for the monomer-dimer equilibrium of SecA. Red, data acquired with interference optics; blue, data acquired with fluorescence optics. The error bars correspond to the 2 SD (standard deviation) confidence intervals for each measurement of K . The dimer dissociation constants measured at 20°C range from 40 μM in 500 mM KCl to 14 nM in 100 mM KCl. The line is a linear fit to the data with a best-fit slope of -5.26 ± 0.30 .

Figure 5. Dissociation of the SecA dimer by signal peptide

A) Plot of apparent dimer dissociation constants ($K_{d,app}$) vs. signal peptide concentration. SecA dimer dissociation constants were obtained at 200 mM KCl by sedimentation velocity measurements using fluorescence optics. Numerical values are reported in Table 3. The solid line is a fit of the data to equation 3 performed as described in the text. The best-fit parameters are: $\beta_{11} = 0.158$ and $\beta_{22} = 0.00264$. B) Model for the thermodynamic linkage of SecA dimerization and signal peptide binding. A is Sec A and P is signal peptide. The binding constants are defined as follows: L_{20} is the dimerization constant for unliganded SecA, L_{22} is the dimerization constant for the AP complex, L_{21} is the dimerization constant for interaction of A and AP, β_{11} is the constant for P binding to A, β_{21} is the constant for P binding to A_2 , and β_{22} is the overall constant for interaction of two P monomers with A_2 to produce P_2A_2 .

Figure 6. Location of two potential SecA dimer interfaces and the signal peptide binding region
 The structure of *E. coli* SecA (2FSF)⁷ is depicted in two orientations to illustrate the relevant interfaces. A) The SecA dimer interface derived from the *E. coli* SecA structure is shown in green and the signal peptide binding region derived from NMR structure of a complex of *E. coli* SecA and signal peptide (2VDA)⁷⁰ is colored in magenta. B) The SecA dimer interface from *B. subtilis* (1M6N)⁴⁰ was mapped onto the *E. coli* structure and is shown in gold.

Table 1

Dimer dissociation constants of SecA as a function of KCl concentration. Data were obtained at 20°C. The K_d values represent the average of two to eight global fits

[KCl] (mM)	K_d (μ M)
100	0.014 ± 0.009
200	0.283 ± 0.131
300	3.14 ± 1.81
400	18.6 ± 0.29
500	40.4 ± 3.51

Table 2

Dimer dissociation constants of SecA in 300 mM KCl at different temperatures

Temperature (°C)	K _d (μM)
8	16.2 ± 0.82
14	7.30 ± 0.38
20	3.14 ± 1.81

Table 3

The effect of signal peptide binding on the dimer dissociation of SecA at 20°C in 200 μ M KCl. The K_d values represent the average of three to six global fits

[Peptide] (μ M)	K_d (μ M)
0	0.28 ± 0.13
1	0.86 ± 0.05
10	0.87 ± 0.03
20	0.99 ± 0.19
40	1.98 ± 0.10
70	2.13 ± 0.27
100	2.05 ± 0.10