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Binding of SecA to the SecYEG Complex Accelerates the Rate of Nucleotide Exchange on SecA*

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SecYEG translocase mediates the transport of preproteins across the inner membrane of Escherichia coli. SecA binds the membrane-embedded SecYEG protein-conducting channel with high affinity and then drives the stepwise translocation of preproteins across the membrane through multiple cycles of ATP binding and hydrolysis. We have investigated the kinetics of nucleotide binding to SecA while associated with the SecYEG complex. Lipid-bound SecA was separated from SecYEG-bound SecA by sedimentation of the proteoliposomes through a glycerol cushion, which maintains the SecA native state and effectively removes the lipid-bound SecA fraction. Nucleotide binding was assessed by means of fluorescence resonance energy transfer using fluorescent ATP analogues as acceptors of the intrinsic SecA tryptophan fluorescence in the presence of a tryptophanless variant of the SecYEG complex. Binding of SecA to the SecYEG complex elevated the rate of nucleotide exchange at SecA independently of the presence of preprotein. This defines a novel pretranslocation activated state of SecA that is primed for ATP hydrolysis upon preprotein interaction.

The SecA protein functions as a molecular motor to drive precursor proteins (preproteins) across the bacterial cytoplasmic membrane (1–3). It is a dimeric peripheral ATPase subunit of the preprotein translocase and binds with high affinity to the translocation channel formed by an oligomeric assembly of the SecYEG complex. The translocation channel allows membrane passage of preproteins in their unfolded state (4, 5). SecA couples the energy of ATP binding and hydrolysis to the preprotein translocation. ATP hydrolysis also drives the cycling of SecA between the membrane-bound and free cytosolic state (6, 7). This cycling reaction occurs on the same time scale as the overall preprotein translocation reaction (6), but it is unclear if cycling is a necessary step in catalysis. Evidence for the high affinity binding of SecA to the SecYEG complex is demonstrated by an increased number of SecA membrane binding sites upon overproduction of the SecYEG complex (7), the reduction of specific binding of SecA to SecYEG by an antibody directed against SecY (1), and the presence of a significant SecYEG-dependent fraction of SecA that cannot be membrane-extracted by urea, alkali, or high salt (1, 8). SecA can also bind to the lipid surface, where it associates with acidic phospholipids (1, 3) and seems to insert into the fatty acid acyl phase of the membrane (1, 2). This process of lipid association occurs with low affinity and is inhibited by ATP (9).

The translocation of preproteins across the membrane involves several rounds of nucleotide binding and hydrolysis by SecA (1, 10, 11). On the basis of mutational and functional studies and the use of photoactivatable nucleotide analogues (11–14), it was suggested that SecA contains a high and a low affinity nucleotide binding domain (NBD). The high affinity NBD corresponds to the catalytic site, whereas the low affinity NBD may act as a regulatory site. The structures of the Bacillus subtilis (15) and Mycobacterium tuberculosis SecA (16), however, show no evidence of a second catalytic NBD (17). The low affinity NBD may correspond to a nonfunctional nucleotide binding domain (11, 18).

The nucleotide binding characteristics of the soluble SecA protein have been studied extensively (13, 14, 19) and used as a model to describe the conformational states of SecA when actively involved in preprotein translocation. The translocation active state of SecA may, however, not be reflected by those studies, since this requires binding of SecA to the SecYEG complex (3, 12). On the other hand, nucleotide binding experiments with the SecYEG-bound SecA using natural membranes or membranes containing overexpressed SecYEG complex are complicated by the presence of many unrelated ATPases. Whereas purification and reconstitution provide a suitable functional alternative, high affinity binding of SecA to the reconstituted SecYEG complex has never been demonstrated for the purified proteins. This has been attributed to the very high nonspecific binding of SecA to the lipid surface (20). We have investigated the binding of nucleotides to SecA when bound to the SecYEG complex. For that purpose, we have devised a method that efficiently separates the high and low affinity-bound SecA fractions to SecYEG proteoliposomes. This method was combined with fluorescence resonance energy transfer (FRET) experiments that monitor the interaction between fluorescent ATP analogues as acceptors of the intrinsic SecA tryptophan fluorescence in the presence of a tryptophanless variant of the SecYEG complex. The data demonstrate that

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1 The abbreviations used are: NBD, nucleotide binding domain; FRET, fluorescence resonance energy transfer; MANT, 2-(or-3)-O-(N-methylanthraniloyl); PMF, proton motive force; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPEG, 1,2-dioleoyl-sn-glycero-3-phospho[acyl]ethanolamine; IMV, IMV, inner membrane vesicle.
Nucleotide Binding to SecA

EXPERIMENTAL PROCEDURES

Materials—SecA, SecB, pro-OmpA, and SecYEG and the tryptophan-less SecYEG mutant were purified as described (4). SecYEG was reconstituted into liposomes composed of Escherichia coli phospholipids or synthetic liposomes as described (21). Synthetic phospholipids 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) (Avanti Polar Lipids, Inc., Birmingham, AL) were mixed in a 60:40 (w/w) ratio, and liposomes were prepared as described (22). 2-(or-3’)-O-(N-methylanthraniloyl) (MANT)-labeled nucleotides were obtained from Molecular Probes, Inc. (Leiden, The Netherlands).

Binding of Nucleotides to SecA-SecYEG Proteoliposomes—Liposomes or SecYEG proteoliposomes (0.05 mg of SecYEG protein/m) were incubated with 50 mM [α-32P]ATP (Amersham Biosciences) in buffer A (50 mM HEPES-KOH, pH 7.6, 100 mM KCl, 5 mM MgAc2, 10 mM dithiothreitol, 1 mg/ml bovine serum albumin) with or without 250 μM SecA dimer. After a 20-min incubation at room temperature, proteoliposomes were spun (Beckman Airfuge, 30 p.s.i., 5 min) through a 15% (w/v) glycerol cushion, and the pellet fraction was recovered from the supernatant by resuspension in buffer containing 2.5 mM Tris-HCl, pH 8.0, 5 mM MgCl2, and 5 mM MgCl2. The pellet was resuspended in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 5 mM MgCl2, and the final proteoliposome-bound SecA concentration was determined to be 0.26 μM SecA dimer by comparative SDS-PAGE.

Binding of Fluorescent Nucleotides by SecA—Fluorescent nucleotides were used to monitor nucleotide binding to SecA. Binding of fluorescent nucleotides to SecA was monitored by FRET measurements using a temperature-controlled Aminco-Bowman Series 2 luminescence spectrometer (Aminco-Bowman, Urbana, IL) with Glan-Thompson polarizers. The shift widths for the excitation and emission beam were set open, the excitation beam polarizer was set to 90°, and the emission polarizer was set to 0° to reduce light scatter. Tryptophan fluorescence was measured in time at excitation and emission wavelengths of 295 and 350 nm, respectively. FRET to MANT-ATP or MANT-ADP was measured in time at excitation and emission wavelengths of 295 and 450 nm, respectively.

Kinetics of Fluorescent Nucleotide Binding of SecA—The kinetics of MANT-nucleotide binding and release was monitored over a wide range of nucleotide concentrations (0.01–2 μM). MANT-nucleotide was added to a solution containing either 0.165 μM SecA dimer or 0.07 μM SecA dimer bound to Trp-less SecYEG proteoliposomes in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 5 mM MgCl2, and the observed association rate (kassoc) was calculated from the progress curves. The dissociation rate (kdiss) was determined after the addition of 0.6 mM ATP or ADP. The kobs values obtained at various MANT-nucleotide concentrations were analyzed according to the following relationship (26):

\[ k_{\text{obs}} = k_{\text{aff}} + k_{\text{diss}} \times [\text{MANT-nucleotide}] \]  

Data were fitted by linear regression, whereby the slope of the reaction was used to calculate the kaff for MANT-nucleotide binding to SecA.

RESULTS

Nucleotide Binding to SecA-SecYEG Proteoliposomes—Binding of nucleotides to SecA free in solution has been studied extensively (13, 14), but no information is available on nucleotides binding to SecA associated with SecYEG complex (28). Inner membrane vesicles (IMVs) of E. coli bind SecA with high affinity at the SecYEG complex (1), but since this system contains many other ATPases that interfere with an accurate analysis of nucleotide binding, it is unsuitable for the analysis of nucleotide binding to SecYEG-bound SecA. Therefore, we have performed nucleotide binding experiments with purified SecA and reconstituted SecYEG proteoliposomes. Although the functional reconstitution of SecYEG into proteoliposomes is well established (29, 30), the high affinity SecA binding has

binding of SecA to the SecYEG complex stimulates nucleotide exchange at SecA. This defines a novel intermediate step in the activation of SecA for preprotein translocation.

Materials—SecA, SecB, pro-OmpA, and SecYEG and the tryptophan-less SecYEG mutant were purified as described (4). SecYEG was reconstituted into liposomes composed of Escherichia coli phospholipids or synthetic liposomes as described (21). Synthetic phospholipids 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) (Avanti Polar Lipids, Inc., Birmingham, AL) were mixed in a 60:40 (w/w) ratio, and liposomes were prepared as described (22). 2-(or-3’)-O-(N-methylanthraniloyl) (MANT)-labeled nucleotides were obtained from Molecular Probes, Inc. (Leiden, The Netherlands).

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Kinetics of Fluorescent Nucleotide Binding of SecA—Fluorescent nucleotides were used to monitor nucleotide binding to SecA. Binding of fluorescent nucleotides to SecA was monitored by FRET measurements using a temperature-controlled Aminco-Bowman Series 2 luminescence spectrometer (Aminco-Bowman, Urbana, IL) with Glan-Thompson polarizers. The shift widths for the excitation and emission beam were set open, the excitation beam polarizer was set to 90°, and the emission polarizer was set to 0° to reduce light scatter. Tryptophan fluorescence was measured in time at excitation and emission wavelengths of 295 and 350 nm, respectively. FRET to MANT-ATP or MANT-ADP was measured in time at excitation and emission wavelengths of 295 and 450 nm, respectively.

To measure binding of MANT-nucleotide to free SecA or Trp-less SecYEG proteoliposome-bound SecA, 0.147 μM MANT-nucleotide was added to a solution containing 0.26 μM SecA dimer in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 5 mM MgCl2 (or 0.2 mg/ml 60% DOPG, 40% DOPG liposomes). Trp-less SecYEG proteoliposome-bound SecA was obtained by incubating the proteoliposomes (2.6 μM Trp-less SecYEG and 0.2 mg/ml of phospholipid) and 5.2 μM SecA dimer for 20 min at room temperature and spun (Beckman Airfuge, 30 p.s.i., 5 min) through a 15% (w/v) glycerol cushion in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 5 mM MgCl2. The pellet was resuspended in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 5 mM MgCl2, and the final proteoliposome-bound SecA concentration was equalized to 0.26 μM SecA dimer by comparative SDS-PAGE.

Dissociation of the MANT-nucleotide from SecA was measured after 30 min of binding and initiated by the addition of nucleotides (0.6 mM). Data were corrected for background and fitted using nonlinear regression on single exponential curves (SigmaPlot 2000, SPSS Inc.).

RESULTS

Nucleotide Binding to SecA-SecYEG Proteoliposomes—Binding of nucleotides to SecA free in solution has been studied extensively (13, 14), but no information is available on nucleotides binding to SecA associated with SecYEG complex (28). Inner membrane vesicles (IMVs) of E. coli bind SecA with high affinity at the SecYEG complex (1), but since this system contains many other ATPases that interfere with an accurate analysis of nucleotide binding, it is unsuitable for the analysis of nucleotide binding to SecYEG-bound SecA. Therefore, we have performed nucleotide binding experiments with purified SecA and reconstituted SecYEG proteoliposomes. Although the functional reconstitution of SecYEG into proteoliposomes is well established (29, 30), the high affinity SecA binding has

Table I

Mismatch primers used for site-directed mutagenesis of the secYEG genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amino acid substitution</th>
<th>Mismatch oligonucleotides*</th>
</tr>
</thead>
<tbody>
<tr>
<td>secY</td>
<td>W173F</td>
<td>5’-TTCTGATGTTCTGGGCGAAC</td>
</tr>
<tr>
<td></td>
<td>W300F/W302F</td>
<td>5’-GGGTTACGTGGTTCACTTCG</td>
</tr>
<tr>
<td></td>
<td>W256F</td>
<td>5’-GGGCACTCTGGTCCGTTT</td>
</tr>
<tr>
<td></td>
<td>W19F</td>
<td>5’-GGGATAGTCTGGTCTGGTG</td>
</tr>
<tr>
<td></td>
<td>W84F</td>
<td>5’-GTAAAGCGCATTTCCGACCTGG</td>
</tr>
<tr>
<td></td>
<td>W109F</td>
<td>5’-CAGCTATCCTGTCGGACTTGG</td>
</tr>
<tr>
<td></td>
<td>W84F</td>
<td>5’-CAGCTGAGTATTCAAATGCTGACCC</td>
</tr>
</tbody>
</table>

* The resulting amino acids (underlined) are shown.
Nucleotide Binding to SecA

never been demonstrated for this system. Such experiments are complicated by the high nonspecific binding of SecA to the lipid surface, which masks the specifically bound fraction (20). We have therefore devised a method that allows the detection of nucleotide binding to the high affinity SecYEG-bound SecA in proteoliposomes. For this purpose, liposomes and SecYEG proteoliposomes were incubated with $^{[32]P}$ATP with and without SecA (Fig. 1). The specific binding of $^{[32]P}$ATP to the (proteo)liposomes was determined after centrifugation through a glycerol instead of a sucrose cushion to separate the lipid-bound and free nucleotide fraction. In previous studies, sedimentation through a sucrose cushion was shown to be ineffective (20). Glycerol has been reported to preserve the native state of SecA (31), and therefore, sedimentation to a glycerol cushion more likely minimizes denaturation of SecA at the lipid surface. When $^{[32]P}$ATP was incubated with liposomes (Fig. 1, lane 3) or SecYEG proteoliposomes (lane 5) in the absence of SecA, no radioactivity was recovered in the pellet fraction after glycerol cushion centrifugation. In contrast, a high level of nucleotide binding was observed in the pellet fraction when SecYEG proteoliposomes were incubated with SecA (lane 6), whereas only a small amount of radioactivity was recovered when liposomes were incubated with SecA (lane 4; less than 10% of the control SecA-SecYEG proteoliposomes). During the incubation, even at 4 °C, all bound $^{[32]P}$ATP was hydrolyzed to ADP. These data suggest that the glycerol cushion effectively reduces the nonspecific binding of SecA to the lipid surface.

To determine the kinetic parameters of nucleotide binding to the SecYEG-bound SecA, SecA-SecYEG proteoliposomes were prepared with specifically bound SecA by the method described above. Subsequently, the SecA-SecYEG proteoliposomes were incubated with $^{[32]P}$ADP, and the bound and free nucleotides were separated by a second glycerol cushion centrifugation step. Fig. 2A shows a plot of the free ADP versus the ADP bound to the SecA-SecYEG proteoliposomes. The $K_d$ for ADP binding to SecYEG-bound SecA was 63 ± 9 nM. The presence of the preprotein pro-OmpA (and SecB) had a minor effect on the ADP binding affinity ($K_d$ 107 ± 14 nM), whereas the $B_{max}$ remained unchanged (56 ± 3 and 53 ± 3 nM, respectively). The number of ADP binding sites determined after the second glycerol cushion centrifugation was independent of the SecA concentration, provided that SecA was present in sufficient amounts to saturate all SecYEG binding sites (300 nM SecA dimer). The binding reaction was linearly dependent on the concentration of SecYEG in the range of 5–20 µg/ml.

To determine the number of nucleotide binding sites per SecA dimer bound to SecYEG, SecA binding experiments were performed with the SecYEG proteoliposomes. Since this assay involves only a single glycerol centrifugation step, biphasic SecA binding kinetics were observed (Fig. 2B) that can be attributed to a small fraction of remaining nonspecifically bound SecA. SecA binds SecYEG with a $K_d$ of 31 ± 5 nM. This value is similar to the one reported for IMVs (32). The background SecA binding appeared nonsaturable in the range tested. The $B_{max}$ of 24 ± 5 nM for specific SecA dimer binding corresponds to about half (0.45 ± 0.07) the number of high affinity ADP binding sites that were determined with SecYEG proteoliposomes. This is equivalent to 1.1 ± 0.1 mol of ADP/mol of monomeric SecA. Therefore, we conclude that each SecYEG-bound SecA dimer contains two high affinity nucleotide binding sites.

$$binding of Fluorescent Nucleotides by SecA—$$To analyze the kinetics of nucleotide binding to SecA, we employed fluorescence (MANT)-labeled nucleotides (33) in a nucleotide exchange assay. MANT-ATP was found to support the in vitro pro-OmpA translocation into SecYEG proteoliposomes (Fig. 3A), demonstrating that it functionally interacts with SecA and that it can drive protein translocation (27), albeit with an almost 3-fold lower efficiency as compared with ATP. Nucleotide binding and release of SecA can be monitored by changes in MANT-nucleotide anisotropy (19). Although anisotropy is a fast and easy way to monitor the association and dissociation of MANT-nucleotide to SecA, the presence of liposomes or SecYEG proteoliposomes severely interfered with the measurements (data not shown). A fraction of the MANT-ATP or ADP appears to intercalate with the lipid bilayer, which results in a huge rise in anisotropy. To circumvent this problem, we shifted to FRET measurements (34). Herein, the intrinsic protein fluorescence of SecA is used as a donor for the excitation of the bound MANT-nucleotide. The Förster distance ($R_0$) of tryptophan residues varies up to 40 Å (35), which lies within the dimensions of SecA (36). This makes FRET measurements insensitive to nonspecific background binding of MANT-nucleotides to lipids. Upon excitation of the Trp residues of SecA at 295 nm in the presence of MANT-ATP, the Trp fluorescence at 350 nm is decreased (Fig. 3B) with a concomitant increase in MANT fluorescence at 450 nm (Fig. 3C). The FRET could be reversed by the addition of an excess of nonlabeled ATP that chases the MANT-ATP from the SecA bound state (Fig. 3C). This chase reaction was accomplished by an increase in the Trp fluorescence (Fig. 3B). This effect was not observed when SecA was absent from the reaction or when Mg$^{2+}$ was excluded from the system. MANT-ATP binding is strictly dependent on the presence of Mg$^{2+}$ with an apparent dissociation constant ($K_d$) of 40 µM (data not shown).

Construction and Functional Characterization of a Tryptophanless SecYEG Complex—To use FRET for measurements of nucleotide binding to SecA in the presence of the SecYEG complex, a tryptophanless (Trp-less) variant was constructed. The SecYEG complex contains eight tryptophan residues: four in SecY (Trp$^{179}$, Trp$^{203}$, Trp$^{306}$, and Trp$^{307}$), three in SecE (Trp$^{19}$, Trp$^{96}$, and Trp$^{109}$), and a single Trp in SecG (Trp$^{292}$). All tryptophan residues were mutated to phenylalanine by site-directed mutagenesis (Table I), and the Trp-less variant of SecYEG was cloned in an expression vector under the control of the trc promoter with an N-terminal hexahistidine tag on SecY. To check the overexpression and functionality of the Trp-less SecYEG complex, IMVs were isolated from the SecYEG-overproducing strain. The Trp-less SecYEG complex could be overexpressed to the same level as the wild-type SecYEG complex

![Fig. 1. Thin layer chromatography of the binding of $^{[32]P}$ATP to SecA associated with liposomes or SecYEG proteoliposomes after centrifugation through a glycerol cushion. Lanes 1 and 2, ATP and ADP standards.](image-url)
This result was verified for the individual subunits by immunoblot analysis (data not shown). Wild type and Trp-less SecYEG complex were purified by ion exchange and Ni\(^{2+}\)-nitrilotriacetic acid affinity chromatography and reconstituted into liposomes composed of synthetic lipids (DOPE/DOPG, 60:40, w/w) (Fig. 4A, lanes 3 and 4). The Trp-less and wild type SecYEG complexes were equally active in supporting pro-OmpA translocation (Fig. 4B), SecA translocation ATPase ac-

**Fig. 2.** Analysis of [\(\alpha\)-\(^{32}\)P]ADP (A) and [\(^{14}\)C]SecA (B) binding to SecYEG-proteoliposomes. A, ADP binding experiments (1 nM to 2 \(\mu\)M [\(\alpha\)-\(^{32}\)P]ADP) were performed with SecA-SecYEG proteoliposomes that were isolated by glycerol cushion centrifugation of SecYEG proteoliposomes co-incubated with excess SecA. After incubation with various [\(\alpha\)-\(^{32}\)P]ADP concentrations, the SecA-bound and free nucleotide was separated by a second glycerol cushion centrifugation step. Binding experiments were performed in the absence (●) and presence (○) of the SecB-pro-OmpA complex as described under “Experimental Procedures.” The inset shows the Scatchard analysis of the high affinity nucleotide binding after correction for nonspecific binding using nonlinear regression. B, SecA binding (2–650 nM [\(^{14}\)C]SecA dimer) to SecYEG proteoliposomes was performed in the presence of 1 mM ADP. After incubation, the bound and free SecA was separated by glycerol cushion centrifugation. The inset shows the Scatchard analysis of the binding data as analyzed by nonlinear regression assuming biphasic binding kinetics. The solid line shows the high affinity binding of SecA to the SecYEG proteoliposomes, whereas the dashed line represents the contribution of the nonspecific binding.

**Fig. 3.** Binding, hydrolysis, and release of MANT-nucleotides by SecA. A, SecA-dependent translocation of pro-OmpA into SecYEG proteoliposomes in the presence of ATP (lane 1), MANT-ADP (lane 2), and MANT-ATP (lane 3). B, time-dependent changes in the fluorescence intensity of the endogenous tryptophans of SecA upon the addition of 0.15 mM MANT-ATP and 0.6 mM ATP. Excitation and emission wavelengths were set at 295 and 350 nm, respectively. C, time-dependent changes in the fluorescence intensity of MANT-ATP upon the addition to SecA and the subsequent addition of 0.6 mM ATP. Excitation and emission wavelengths were set at 295 and 450 nm. In the absence of SecA, the MANT-ATP fluorescence level was equal to zero when an excitation wavelength of 295 nm was used.
FIG. 4. Functional expression of a tryptophanless variant of the SecYEG complex. A, Coomassie Brilliant Blue-stained SDS-PAGE of IMVs derived from *E. coli* SF100 overexpressing the wild-type (lane 1) and Trp-less (lane 2) SecYEG complex and proteoliposomes reconstituted with the purified wild-type (lane 3) and Trp-less (lane 4) SecYEG complex. B, ATP-dependent translocation of pro-OmpA into IMVs containing overproduced wild-type (lane 3) or Trp-less (lane 4) SecYEG complex and into proteoliposomes reconstituted with the purified wild-type (lane 5) or Trp-less (lane 6) SecYEG complex. Lane 1 corresponds to 10% of the input pro-OmpA. C, SecA ATPase activity of soluble SecA, liposome-bound SecA, and Trp-less SecYEG-bound SecA in the presence (filled bars) and absence of pro-OmpA (open bars). D, tryptophan fluorescence emission spectra of DOPG/DOPE liposomes (dotted and dashed line) reconstituted with the purified wild-type (solid line) or Trp-less (dashed line) SecYEG complex. Excitation was set at 295 nm. The inset represents tryptophan fluorescence spectra of the reconstituted wild-type (solid line) and Trp-less SecYEG (dotted and dashed line) complexes after correction of the light scatter and background fluorescence of liposomes. E, Coomassie Brilliant Blue-stained SDS-PAGE of Trp-less SecYEG proteoliposomes with bound-SecA as isolated by a glycerol cushion as described under “Experimental Procedures.”
activity (Fig. 4C), and SecA binding (27) (data not shown). Taken together, these data demonstrate that none of the Trp residues of the SecYEG complex are essential for activity.

Fig. 4D shows the fluorescence emission spectra at an excitation at 295 nm of the purified and reconstituted wild type and Trp-less SecYEG complexes. As expected, the Trp-less SecYEG variant showed no significant fluorescence above that of liposomes, which makes the Trp-less SecYEG complex suitable for our FRET measurement.

Binding of Fluorescent Nucleotides to the SecYEG-bound SecA—To measure the nucleotide binding characteristics of SecA bound to SecYEG, SecA was incubated with Trp-less SecYEG proteoliposomes, and the complex was recovered by sedimentation through a glycerol cushion (Fig. 4E). FRET measurements on nucleotide binding to SecYEG-bound SecA protein showed a very fast rate of MANT-ATP binding and release upon the addition of an excess of ATP (Fig. 5, dark line). No FRET was observed when the Trp-less SecYEG proteoliposomes were prepared in the absence of SecA (dark gray line). Moreover, the rate of MANT-nucleotide release was nearly identical when, instead of ATP (dark line), ADP was used for the chase (light gray line).

Next, the kinetic parameters of MANT-ATP binding were determined for SecA associated with the Trp-less SecYEG complex and compared with soluble SecA and SecA bound to DOPE/DOPG liposomes. In the presence of DOPE/DOPG liposomes, SecA readily associates with the lipid surface, and the majority of the SecA co-sediments with the liposomes after ultracentrifugation (20) (data not shown). The kinetics of MANT-ATP binding and release with SecA in solution (Fig. 6, A and D) or in the presence of liposomes (Fig. 6, B and E) were indistinguishable within the experimental error. Remarkably, substantial higher rates of MANT-ATP binding and release were observed with SecA bound to SecYEG proteoliposomes (Fig. 6, C and F). The enhanced rate is in particular pronounced with the nucleotide release. Whereas free or lipid-bound SecA only slowly releases the nucleotide, a 6–7-fold increase in the nucleotide dissociation rate, $k_{\text{off}}$, is observed with the SecYEG-bound SecA. These data suggest that SecA is stimulated for nucleotide binding and exchange when it associates with the SecYEG complex.

To determine whether the nucleotide binding kinetics are influenced by the presence of a preprotein, pro-OmpA was added to the reaction that contained the SecYEG-bound SecA complex. The wild-type pro-OmpA harbors five tryptophan residues (Trp 7, Trp 15, Trp 57, Trp 102, and Trp 143) (37), but no change in MANT-ATP fluorescence intensity was observed upon the addition of pro-OmpA (data not shown). Therefore, the Trp residues of pro-OmpA do not contribute to the FRET, probably because they are more than 40 Å away from the nucleotide binding site on SecA. Although the presence of pro-OmpA markedly stimulated the ATPase activity of SecA bound to Trp-less SecYEG proteoliposomes (Fig. 4C, lane 6), it only marginally affected the MANT-nucleotide binding and release kinetics (data not shown). Likewise, pro-OmpA did not significantly affect the nucleotide exchange kinetics of the soluble and lipid-bound SecA.

SecYEG Lowers the Activation Energy for Nucleotide Binding to SecA—The stimulatory effect of the SecYEG complex on the nucleotide exchange kinetics on SecA was observed over a wide temperature range as shown in the Arrhenius plot for the binding (Fig. 7A) and release (Fig. 7B) kinetics of MANT-ATP. The activation energy ($E_a$) for MANT-ATP binding to free, liposome-bound, and SecYEG-bound SecA was 22.5, 20.8, and 15.2 kJ/mol, respectively. For nucleotide release, these values were equal to 44.2, 42.1, and 22.5 kJ/mol, respectively. This demonstrates that the SecYEG-bound SecA has a lower activation energy barrier for the nucleotide binding as well as for the nucleotide release.

To obtain an estimate of the association rate ($k_{\text{on}}$) of MANT-ATP and MANT-ADP, the rate of nucleotide binding ($k_{\text{obs}}$) was measured at different concentrations of the MANT nucleotides. As shown in Fig. 8A, the $k_{\text{obs}}$ for nucleotide binding to soluble SecA increased linearly with the nucleotide concentration. The data were fitted to the relationship $k_{\text{obs}} = k_{\text{off}} + k_{\text{on}} \times [\text{MANT-nucleotide}]$, with a $k_{\text{off}}$ value that was measured directly by monitoring the rate of MANT-nucleotide release upon the addition of 0.6 mM ATP. For soluble SecA, the $k_{\text{on}}$ for MANT-ADP ($7.8 \times 10^8$ M$^{-1}$ s$^{-1}$) was significantly higher as compared with...
Here we have investigated the kinetics of nucleotide interaction to the SecYEG-bound SecA protein. Nucleotide binding to the soluble form of SecA has been studied extensively (3, 10–12, 17, 38–40); however, the active form of SecA in protein translocation is represented by the SecYEG-bound state. Since nucleotide binding has not been analyzed kinetically for the SecYEG-bound SecA, it is unclear how SecYEG alters the SecA catalytic cycle except that it activates SecA for preprotein-stimulated ATP hydrolysis. Our data now demonstrate that the binding reaction to SecYEG alters the nucleotide binding kinetics to SecA. Unlike the SecA free in solution or bound to liposomes, the SecYEG-bound SecA appears to be primed for nucleotide binding and exchange. Both the rates of nucleotide binding and release are markedly stimulated. Therefore, we conclude that SecA undergoes a conformational switch upon binding to SecYEG, which completes its high affinity nucleotide binding fold and/or changes the accessibility of this domain to externally added nucleotide. This study defines a pretranslocation activated state of SecA that is primed for high rates of ATP hydrolysis upon accepting preproteins for translocation. The data further indicate that preproteins do not stimulate the nucleotide exchange activity of the SecYEG-bound SecA but instead activate SecA for ATP hydrolysis (12) by a mechanism that is largely unknown.

Based on the higher $k_{on}$ values with MANT-ADP, it appears that the soluble SecA prefers to bind to ADP over ATP (Fig. 8). This difference in binding kinetics seems to be maintained with the SecYEG-bound SecA, but the nucleotide association rates are almost 3-fold higher as compared with soluble SecA. Overall, it seems that the soluble form of SecA adopts a conformation that does not favor nucleotide binding and release, even in the presence of a translocation-competent preprotein. In effect, this prevents futile cycles of ATP hydrolysis when SecA is not associated with the translocation channel. It is well established that soluble SecA has only a low basal ATPase activity that is not stimulated by preprotein (3) (see also Fig. 4C). Also, the SecYEG-bound SecA is equipped with a low ATPase activity, but the activity is dramatically stimulated upon the binding of a translocation-competent preprotein, an activity termed SecA “translocation ATPase.” This process probably involves further conformational changes among which is the relief of the suppressing activity of IRA1 on the catalytic NBD (18). The initial acceleration of the nucleotide release kinetics upon SecYEG binding, independent of the presence of a preprotein, primes SecA for a high turnover rate of bound ATP.

The low basal ATPase activity of soluble SecA is also elevated when SecA binds to the lipid surface. However, this effect is smaller than the preprotein stimulation of the SecA ATPase activity in the presence of the SecYEG complex (3). The exact role of the lipid-stimulated SecA is unknown. SecA is thermolabile in the presence of lipids and is readily inactivated unless a preprotein is present. This phenomenon has been attributed to a protective effect of the preprotein on the heat-induced inactivation of the SecA when bound to the lipid surface (3). Lipid ATPase activity is observed only at low Mg^{2+} concentration and cannot be detected with IMVs. The latter probably relates to a shielding of the lipid surface in IMVs due to the high protein-to-lipid ratio. Our experiments further suggest that the lipid activation of SecA differs mechanistically from the “translocation ATPase” of SecA. In contrast to the SecYEG-bound SecA, no acceleration of the nucleotide exchange kinetics is observed when SecA binds to lipids. Recent studies on the lipid-bound SecA suggest that nucleotide binding induces the conversion of the dimeric form of SecA into a monomeric form (41–43), whereas signal sequence binding has been reported to reassemble the SecA in its dimeric form (41). Apparently, monomerization of SecA does not suffice to activate the enzyme for nucleotide exchange. Thus, the activation must relate to the specific interaction of SecA with SecYEG, which apparently induces a conformational state of SecA that is not observed in
solution or when SecA is bound to the lipid surface.

To examine nucleotide binding kinetics by SecA, we have utilized the method of FRET with the endogenous tryptophans of SecA as donor and MANT-nucleotides as acceptor. The method of MANT-nucleotide fluorescence anisotropy to measure binding was found to be incompatible with the (proteoliposomal) system (19). The addition of liposomes to MANT-ATP already resulted in a dramatic increase in fluorescence anisotropy indicative of a (nonspecific) binding reaction (34). Since liposomes did not interfere with the FRET measurements and because MANT-ATP can drive the protein translocation, albeit inefficiently, we conclude that our method monitors an authentic nucleotide binding and release reaction even under conditions in which protein translocation occurs. Bound MANT-ATP (or ATP) is immediately converted into MANT-ADP (or ADP) in line with previous observations on nucleotide binding (28,44). In contrast to the nucleotide binding reaction, the rate of fluorescent nucleotide release was indistinguishable when ADP or ATP was used for the chase. This strongly suggests that ADP release is rate-limiting in the productive cycle of SecA. Previously, it has been reported that ADP release is a rate-limiting step in the SecA catalytic cycle, and it has been suggested that the presence of an ATP-generating system or the proton motive force (PMF) accelerates this step (45). The latter was based on observations that the apparent $K_m$ value of the translocation reaction for ATP is reduced by 2 orders of magnitude by the PMF. The PMF, however, does not affect the SecA translocation ATPase activity at saturating concentrations of ATP. On the other hand, the PMF has been shown to affect the conformation of SecA during its cycling reaction (11,40). Direct measurements of a kinetic effect of the PMF on the ADP release have not been reported and will be the subject of future studies.

Although the protein translocation was reconstituted with the purified translocase components more than a decade ago (29,30), high affinity binding of SecA to the purified SecYEG has not been demonstrated previously. Our data using the glycerol cushion now for the first time demonstrate that SecA can bind the purified SecYEG complex in proteoliposomes with an affinity nearly identical to that reported for intact IMVs. This supports the long existing notion that SecYEG suffices to form an authentic binding site for SecA. These experiments also allowed a quantitative estimate of the number of nucleotides bound per SecA dimer associated with the SecYEG complex. Our experiments indicate that in the steady state, 2 mol of ADP are bound per 1 mol of SecA dimer. This number deviates from the value reported for the soluble E. coli SecA by Schmidt and co-workers (28), who observed the binding of 1 mol of ADP per 1 mol of SecA dimer using a gel filtration-based analysis technique. On the other hand, isothermal titration calorimetry experiments on the soluble B. subtilis SecA support the binding of 2 mol of ADP per 1 mol of SecA dimer (14). The kinetic experiments did not reveal any cooperativity in the nucleotide binding; therefore, the presence of two high affinity NBD on the SecA dimer seems mostly likely. The question of whether, in addition to the high affinity nucleotide binding site, a second low affinity binding site is present in SecA remains to be answered.

Taken together, we conclude that SecA undergoes a conformational change upon binding to the SecYEG complex. This conformational change elicits an elevated rate of nucleotide binding and release. This study defines a preactivated state in which the SecA is primed for preprotein-stimulated ATP hydrolysis, a process that ultimately drives the translocation cycle.

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**REFERENCES**