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Domain Interactions of the Peripheral Preprotein *Translocase* Subunit SecA†

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ABSTRACT: The homodimeric SecA protein is the peripheral subunit of the preprotein *translocase* in bacteria. It binds the preprotein and promotes its translocation across the bacterial cytoplasmic membrane by nucleotide modulated coinsertion and deinsertion into the membrane. SecA has two essential nucleotide binding sites (NBS; Mitchell & Oliver, 1993): The high-affinity NBS-I resides in the amino-terminal domain of the protein, and the low-affinity NBS-II is localized at $^2/_3$ of the protein sequence. The nucleotide-bound states of soluble SecA were studied by site directed tryptophan fluorescence spectroscopy, tryptic digestion, differential scanning calorimetry, and dynamic light scattering. A nucleotide-induced conformational change of a carboxy-terminal domain of SecA was revealed by Trp fluorescence spectroscopy. The Trp fluorescence of a single Trp SecA mutant containing Trp775 decreased and increased upon the addition of NBS-I saturating concentrations of ADP or AMP-PNP, respectively. DSC measurements revealed that SecA unfolds as a two domain protein. Binding of ADP to NBS-I increased the interaction between the two domains whereas binding of AMP-PNP did not influence this interaction. When both NBS-I and NBS-II are bound by ADP, SecA seems to have a more compact globular conformation whereas binding of AMP-PNP seems to cause a more extended conformation. It is suggested that the compact ADP-bound conformation resembles the membrane deinserted state of SecA, while the more extended ATP-bound conformation may correspond to the membrane inserted form of SecA.

SecA, the peripheral subunit of the preprotein *translocase* in bacteria, is a homodimer of 102-kDa subunits (Driessen, 1993). It associates in a binary complex with preproteins or in a ternary complex with the cytosolic chaperone SecB bound preprotein, and guides them to the translocation sites at the cytoplasmic membrane (Hartl et al., 1990). SecA interacts with both the signal sequence and parts of the mature domain of preproteins (Akita et al., 1990; Lill et al., 1990) and associates with the cytoplasmic membrane due to its affinity for negatively charged phospholipids and the integral membrane subunits of the *translocase*: SecY, SecE, and SecG (see, for recent reviews: Wickner et al., 1991; Driessen, 1994). Together they form the minimal stoichiometric subunits required for the preprotein translocation (Brundage et al., 1990; Douville et al., 1994; Nishiyama et al., 1993, 1994). SecA is an ATPase and uses the energy of ATP binding to insert into the cytoplasmic membrane (Economou & Wickner, 1994) and to release the signal sequence domain of the bound preprotein (Schiebel et al., 1991). During preprotein translocation, SecA is intimately associated with SecY and with the preprotein (Joly & Wickner, 1993). The interaction between SecA and the SecYEG complex activates SecA for ATP hydrolysis. This process drives the release of the preprotein from SecA and may promote exclusion of SecA from the cytoplasmic membrane. SecA has two essential nucleotide binding sites (Matsuyama et al., 1990; Klose et al., 1993; Mitchell & Oliver, 1993; Van der Wolk et al., 1993, 1995): a high-affinity binding site ($K_D \approx 0.15$ μM) is confined to the amino-terminal domain of the protein (NBS-I),[1] and a low-affinity binding site ($K_D \approx 340$ μM) is located in the second half of the protein sequence (NBS-II; Mitchell & Oliver, 1993; Figure 1). Binding of the nonhydrolyzable ATP analog adenylyl imidodiphosphate (AMP-PNP) or the combined binding of ATP and preprotein to NBS-I is sufficient for SecYEG associated membrane insertion of SecA, but not for preprotein translocation (Economou et al., 1995). Little information is available concerning the conformational state of SecA or how this state might change during the nucleotide-modulated preprotein translocation. In the absence of nucleotides, SecA is readily digested by V8 protease, whereas

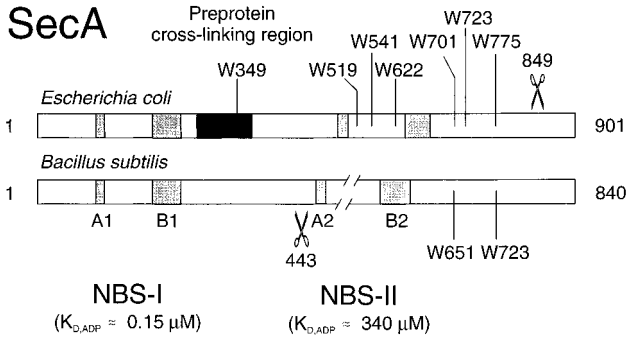

FIGURE 1: Schematic representation of the amino acid sequence of *E. coli* and *B. subtilis* SecA in which NBS-I, NBS-II, preprotein cross-linking domain, and the position of the tryptophans are indicated.

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[1] Abbreviations: DTT, dithiothreitol; DLS, dynamic light scattering; DSC, differential scanning calorimetry; IPTG, isopropyl 1-thio-β-D-galactopyranoside; NBS, nucleotide binding site; Δp, proton motive force; PMSF, phenylmethanesulfonyl fluoride.

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Table 1: Mismatch Primers Used for Oligonucleotide-Directed Mutagenesis, the Resulting Amino Acid Substitutions, and the Additional Endonucleotide Restriction Recognition Sites (Underlined) Are Shown

plasmid	introduced or deleted (Δ) restriction site	amino acid substitution	mismatch oligonucleotides[a]
pET104	Δ*Ava*II	W349F	CCGTCGCT**TC**TCCGATG
pET105	*Hae*II	W519F	TGGTAGCT**TC**CAGGCAGAAGTTGC**A**GCGCTGG
pET105	*Ava*II	W541F	AGCCGACT**TC**CAGGT**C**CGTCACG
pET109	*Pvu*I	W622F	ACACCCGT**TC**GTGACTAAAGCGAT**C**GCCAAC
pET109	*Sma*I	W701F	AGAAATGT**TC**GATATTCCCGGGCTGC
pET109	*Eco*RI	W723F	ATTGCCGAAT**TC**CTGGATAA
pET110	*Dra*I	W775F	CTCCCTGT**TT**AAAGAGC

[a] The nucleotides in bold mark the mismatches in the DNA sequence of the oligonucleotide.

saturation of both NBSs with nucleotides induces a V8 protease resistant SecA conformation (Shinkai et al., 1991; Van der Wolk et al., 1995). We used the intrinsic tryptophan (Trp) fluorescence of SecA and its susceptibility to trypsin digestion to access the nature of the conformational change upon binding of ADP or AMP-PNP to NBS-I of SecA. Differential scanning calorimetry (DSC) and dynamic light scattering (DLS) were used to investigate the domain structure of SecA and the conformational states of SecA with ADP or AMP-PNP bound to NBS-I or to both nucleotide binding sites. Our data indicate that ADP and AMP-PNP binding induce extensive conformational changes in SecA. We propose a compact conformation for the ADP-bound state and a more extended conformation for the ATP-bound state of SecA.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media. Unless indicated otherwise, strains were grown in Luria Bertani (LB) broth or on LB-agar (Miller, 1972) supplemented with 50 μg of ampicillin/mL, 0.5% (w/v) glucose, or 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), as required. Cloning and plasmid constructions were done in *Escherichia coli* JM101 or JM110 (Yanish-Perron et al., 1985), and SecA overproduction was done in JM109 (Yanish-Perron et al., 1985) and NO2947 (MC1061 Δ*lac*, *recA56* Srl::Tn10, Amersham International plc, U.K.). Uracil-containing single stranded template DNA for oligo-directed mutagenesis was obtained by growing the corresponding M13KO7 helper phage on *E. coli* CJ236 (Raleigh et al., 1988). SecA complementation experiments were done in *E. coli* strain MM66 (*geneX*^{am}, *supF*^{ts}) (Oliver & Beckwith, 1981).

DNA Techniques. All DNA techniques were essentially performed as described (Sambrook et al., 1989).

Oligonucleotide-Directed Mutagenesis. The procedure used for site directed mutagenesis was basically the same as described by Kunkel et al. (1987). The mismatch oligonucleotides, the resulting amino acid substitutions, and the introduction or deletion of restriction endonuclease digestion sites are shown in Table 1. Mutagenesis was done on single stranded DNA containing the *Sal*I−*Eco*RV, *Eco*RV−*Kpn*I, and *Kpn*I−*Pst*I restriction fragments of SecA, which were cloned in the pBluescript KS(+)/SK(+) series (Stratagene Cloning Systems, La Jolla, CA) yielding pET104, pET105, and pET109, respectively. Mutagenized DNA fragments of SecA were sequenced (Sanger et al., 1977) completely.

Biochemicals. E. coli SecA (Cunningham et al., 1989), SecB (Weiss et al., 1988), proOmpA (Crooke et al., 1988), and *Bacillus subtilis* SecA (Van der Wolk et al., 1993) proteins were purified from overproducing strains as described. AMP-PNP (Sigma, St. Louis, MO) was purified from contaminating ADP (10%) by MonoQ (Pharmacia, Upssala, Sweden) ion-exchange chromatography using a 10−60% H_2O/1M NH_4HCO_3 linear gradient. ADP and AMP-PNP eluted at 0.28 and 0.41 M, respectively. The AMP-PNP containing fractions were pooled and precipitated with 5 M NH_4Ac and 11 volumes of ice cold ethanol. The nucleotide was dissolved in 50 mM HEPES-KOH, pH 7.5, and its purity was confirmed by cel 300 PEI/UV254 (Polygram, MN, Düren, Germany) thin layer chromatography developed with 0.65 M KH_2PO_4, pH 3.5. Trypsin (specific activity of 11 000 U/mg of protein) and soybean trypsin inhibitor were purchased from Sigma, and hexokinase (93 U/mg) was purchased from Boehringer (Mannheim, Germany).

Expression and Purification of Mutant SecA Proteins. Plasmid bearing cells were grown overnight in LB containing glucose and ampicillin at 37 °C. Cells were diluted 100-fold in fresh LB containing ampicillin, and induced for protein expression with 1 mM IPTG at an OD_{660} 0.6. Cells were harvested by centrifugation after 2 h, and resuspended in an equal weight of 10% (w/v) sucrose/50 mM Tris-HCl, pH 7.5. The suspension was frozen as nuggets by rapid pipetting in liquid nitrogen and stored at −80 °C. The mutant SecA proteins were purified using Cibracon Blue Agarose 36A (Sigma) as described by Mitchell and Oliver (1993). For a purity standard required for fluorescence spectroscopy, protein samples were concentrated by centrifugation in a Centriprep 30 concentrator (Amicon, W. R. Grace & Co., Beverly, MA) and applied on a Hiload Superdex 200 gel-filtration column (Pharmacia) equilibrated in 50 mM Tris-HCl, pH 7.6/1 mM DTT supplemented with 1 mM PMSF and eluted with the same buffer. The SecA mutant proteins eluted at the position of the dimeric wild type SecA protein (Driessen, 1993). Pooled fractions were concentrated in Centriprep concentrators, aliquoted, and stored at −80 °C at 0.1−1 mg/mL. Purified proteins were analyzed by Coomassie-stained SDS−PAGE (Laemmli, 1970) and judged to be more than 95% pure. Protein concentration was determined as described (Bradford, 1976).

In Vitro Translocation. In vitro translocation of ^{35}S-labeled proOmpA (in 50 μL) was performed as described (Hartl et al., 1990) with 1.0 μg of SecA, 1.6 μg of SecB, 2 mM ATP, 10 mM phosphocreatine, and 2.5 μg of creatine kinase in buffer B (50 mM HEPES-KOH, pH 7.6, 30 mM KCl, 2 mM DTT, 0.5 mg/mL bovine serum albumin, 2 mM MgAc). ^{35}S-Labeled proOmpA was diluted 40-fold from a solution of 8 M urea and 50 mM Tris-HCl, pH 7.6, followed by the addition of urea-treated *E. coli* D10 inverted inner membrane vesicles at a final protein concentration of 0.32 mg/mL. Reactions were incubated for 30 min at 37 °C and

stopped by cooling on ice. Samples were treated with proteinase K (0.1 mg/mL) for 15 min on ice. Subsequently, the samples were precipitated with 6.7% (w/v) trichloroacetic acid, washed with acetone, and analyzed by SDS−PAGE and autoradiography.

Trypsin Digestion of E. coli SecA. Aliquots of 10 μL of SecA (200 μg/mL) or of *E. coli* KM9 inside-out (ISO) vesicles (0.44 mg/mL) were incubated at 4 °C with 0.09 or 0.5 mg/mL trypsin, respectively, in the presence of AMP, ADP, or AMP-PNP (0.05 μM−1 mM). After 15 min, the proteolysis was quenched with 0.18 mg/mL trypsin inhibitor for the soluble SecA or 0.9 mg/mL for the vesicles, and analyzed on SDS−PAGE. Immunoblotting and detection were carried out with a pAb against SecA and alkaline phosphatase conjugated anti-rabbit IgG (Boehringer). Blots were developed either with bromo-4-chloro-3-indolyl phosphate/*p*-nitroblue tetrazolium (BCIP/NBT, Boehringer) or with a chemiluminescence kit (Tropix, Bedford, MA). ISO vesicles were isolated as described by Kaback (1971). The trypsin digestion of the endogenous SecA present in the ISO vesicles was performed either under static conditions or after incubation for 15 min at 37 °C in the presence of all components used in the *in vitro* protein translocation reaction (see above), and then the nucleotide was studied. In case of ADP, 160 U/mL hexokinase and 10 mM glucose were added to dephosphorylate any ATP in the solution.

Intrinsic Fluorescence Measurements. Protein at a final concentration of 0.05−0.2 μM (dimer) in 50 mM HEPES, pH 7.5/50 mM KCl/5 mM $MgCl_2$ was titrated with ADP or with AMP-PNP. The fluorescence emission between 320 and 360 nm (slit width of 4 nm) was monitored in a quarz cuvette equipped with a magnetic stirrer at an excitation wavelength of 297 nm (slit width of 2 nm) and a scan rate of 1 nm/s at 25 °C on an SLM-Aminco 4800C spectrofluorophotometer (SLM-Aminco, Urbana, IL). Spectra were corrected for the volume increase and background fluorescence. The inner filter effect of the nucleotide was negligible at the used concentrations and excitation wavelength.

Calorimetric Measurements. DSC experiments were performed using a microcalorimeter (Microcal MC-2, Microcal, Amherst, MA), keeping an additional constant pressure of 2 atm over the liquids in the cell. A differential scanning rate of 1 °C/min was employed if not stated otherwise. The DSC experiments were carried out with a *B. subtilis* SecA concentration of 1−2 mg/mL in 50 mM potassium phosphate buffer (pH 7.5)/50 mM KCl or SecA in the same buffer which was suplemented with 5 mM $MgCl_2$ and ADP or AMP-PNP. The reversibility of the DSC transitions was checked by reheating the solution in the calorimetric cell after cooling (1 °C/min) from the first run. A thermogram corresponding to a water against water run was used as the instrumental base line.

Analysis of Calorimetric Results. The dependence of molar heat capacity on temperature was analyzed using the ORIGIN software (Microcal Ltd.). Analysis of the data involved fitting and subtraction of instrumental base line data as described (Blandamer et al., 1994). The data were normalized, assuming that the SecA dimer dissociated after or during the most stable transition.

Light Scattering Experiments. Light scattering experiments were performed using a DynaPro-801-TC molecular sizing instrument (Protein Solutions Inc., High Wycombe, G.B.). DLS of *B. subtilis* SecA (2 mg/mL) in 50 mM HEPES, pH 7.5/50 mM KCl was measured in the absence or presence of 5 mM $MgCl_2$ and/or 5 mM nucleotide at 25 °C. Ten or more independent measurements were made from each sample, and the reported values are calculated arithmetic means. The output from the dynamic light scattering is the translational diffusion coefficient D_T of the particles in solution. Under the assumption of Brownian motion and spherical particles, this coefficient is converted to the hydrodynamic radius R_H, in m, of the particles using the Strokes−Einstein equation:

$$R_H = \frac{k_b T}{6\pi \eta D_T} \tag{1}$$

where k_b is Boltzmann's constant, T is the temperature in kelvin (K), and η is the solvent viscosity and assumed to be 1.019×10^{-3} Ns/m^2. A measure of the molecular asymmetry is the ratio R_H/R_S, where R_S is the geometric radius for a sphere, i.e.,

$$R_S = \left(\frac{3}{4\pi}\frac{\bar{\nu}_p M_p}{N_A}\right)^{1/3} \tag{2}$$

$\bar{\nu}_p$ is the volume of the protein, which is 0.74 mL/g for an average protein, M_p is the molecular mass of the protein, and N_A is Avogadro's number. The ratio R_H/R_S may be greater than unity for spherical particles that are solvated, i.e.,

$$\frac{R_H}{R_S} = \left(\frac{\bar{\nu}_p + \delta\bar{\nu}_0}{\bar{\nu}_p}\right)^{1/3} \tag{3}$$

where $\delta\bar{\nu}_0$ accounts for the volume of the solvation shell (mL/g) (Schmitz, 1990).

Calculation of the Nucleotide Occupancy of NBS-I and NBS-II. Assuming the following binding equilibrium: SecA + nucleotide $\rightleftharpoons$ SecA·nucleotide (or S + L $\rightleftharpoons$ SL) and $S = (1 - x)S_0$, $L = L_0 - xS_0$, and $SL = xS_0$, then,

$$x = \frac{S_0 + L_0 + K_D}{2S_0} - \frac{[(S_0 + L_0 + K_D)^2 - 4S_0L_0]^{1/2}}{2S_0} \tag{4}$$

where S_0 and L_0 are the initial concentrations of S and L, respectively. K_D is 0.15 μM and 340 μM for NBS-I and NBS-II, respectively. In the case of an excess of ligand, the following equation can be applied:

$$SL = SL_{max}\left(\frac{L_0}{L_0 + K_D}\right) \tag{5}$$

Assuming an enthalpy of ligand dissociation ($\Delta H°$) of 20 kcal/mol (Hu & Sturtevant, 1987), it follows from:

$$\ln\left(\frac{K_{D,T_2}}{K_{D,T_1}}\right) = \frac{-\Delta H°}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \tag{6}$$

that the change in K_D can be calculated to be negligible in the temperature range used.

RESULTS

Tryptophan Fluorescence of SecA Decreases upon Mg^{2+}/ADP Binding. In the presence of ATP, or ADP, the amino-

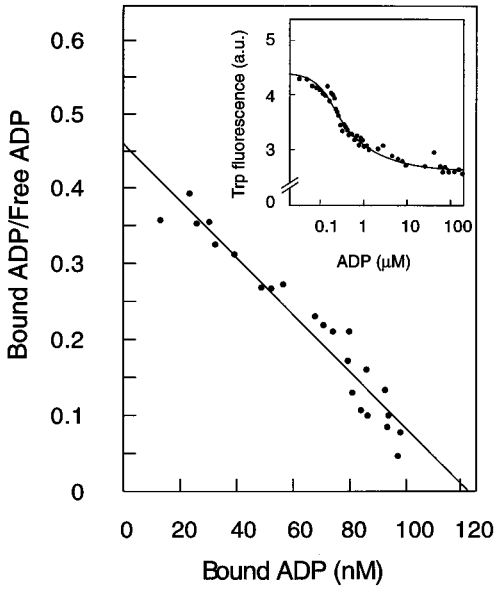

FIGURE 2: Tryptophan fluorescence of *E. coli* SecA in the presence of increasing ADP concentrations. Scatchard plot with a K_D of 0.3 μM for ADP. Insert, decrease in Trp fluorescence as the percentage of Trp fluorescence in the absence of ADP plotted against log scale ADP concentration.

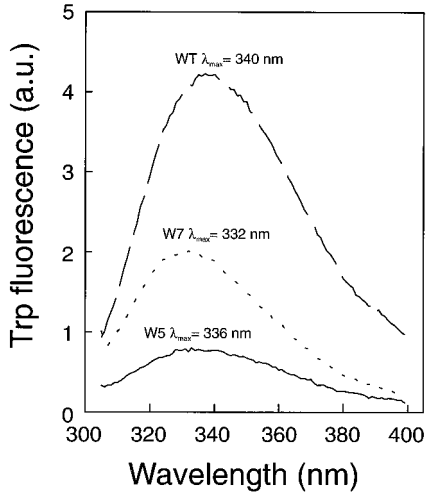

FIGURE 3: The relative tryptophan fluorescence spectra of *E. coli* wild type SecA, SecAW5, and SecAW7 mutants excited at 297 nm in 50 mM HEPES, pH 7.5/50 mM KCl.

terminal 95-kDa portion of SecA becomes resistant to V8 proteolytic digestion (Shinkai et al., 1991). This was interpreted as the result of a conformational change in SecA. To analyze the nature of the conformational change, we have studied the intrinsic tryptophan fluorescence in *E.coli* and in *B. subtilis* SecA. Both proteins share an overall homology of 65% (Overhoff et al., 1991; Sadai et al., 1991) and have been subject to intensive studies which show that their function is very similar (Takamatsu et al., 1992; Klose et al., 1993; Van der Wolk et al., 1993, 1995; McNicolas et al., 1995). *E. coli* SecA (0.05 μM, dimer) was titrated with ADP in the range of 0.02−10 μM. The change in Trp fluorescence was monitored at an excitation wavelength of 297 nm and a slitwidth of 2 nm. This combines a relatively high emission signal with the exclusion of any contribution of the tyrosine fluorescence in SecA by resonance energy transfer to the detected Trp fluorescence spectra. The Trp fluorescence decreased with increasing ADP concentrations and saturated at about 1 μM (Figure 2, insert). Of the two essential ATP binding sites of SecA (Mitchell & Oliver, 1993; Van der Wolk et al., 1995), NBS-I has a high affinity ($K_D \approx 0.15$ μM), and NBS-II has a low affinity ($K_D \approx 340$ μM). The observed decrease in Trp fluorescence occurs in an ADP range which saturates NBS-I and which will not occupy NBS-II. Therefore, we assumed one ADP bound per monomer in the analysis of the fluorescence data by Scatchard plot (Scatchard, 1949). This yielded an apparent K_D of 0.3 μM, which is indeed within the range of the high-affinity ATP binding site (Figure 2). As a control protein, the non-ADP-binding cytosolic preprotein chaperone SecB was titrated with ADP. The fluorescence intensity of the unique solvent exposed Trp36 of SecB (Fekkes et al., 1995) did not decrease in the presence of ADP (results not shown). This shows that the decrease in Trp fluorescence of SecA is due to specific binding of ADP. A similar phenomenon was observed with the *B. subtilis* SecA, although the Mg^{2+}/ADP involved decrease in fluorescence was only ≈15% as compared to ≈33% for the *E. coli* SecA. Addition of $MgCl_2$ without any nucleotides also affects the Trp fluorescence (Van der Wolk et al., 1995). It is not possible to determine if the changes in Trp fluorescence of $MgCl_2$ or Mg^{2+}-nucleotide are additive because addition of nucleotide in the absence of $MgCl_2$ appeared to destabilize SecA (not shown). Therefore, we only determined the combined effect by titrating the nucleotide into a $MgCl_2$ containing protein solution. Binding of ADP to NBS-I apparently changes the environment of Trp residues in both SecA proteins.

The Carboxyl Terminus Senses the Binding of ADP to the High-Affinity ATP Binding Site. *E. coli* SecA has seven tryptophans, of which all but one are located in the carboxy-terminal half of the protein (Figure 1). *B. subtilis* SecA has two tryptophans, Trp651 and Trp723, which correspond with Trp701 (or Trp5) and Trp775 (or Trp7) of *E. coli* SecA, respectively (Figure 1). Since the Trp fluorescence of both *B. subtilis* and *E. coli* SecA proteins decreases upon binding of MgADP, we speculate that at least Trp5 or Trp7, or both tryptophans of *E. coli* SecA, are involved in this phenomenon. To determine the contribution of Trp5 and Trp7 to the decrease in Trp fluorescence of *E. coli* SecA, a mutant was made (SecAW5) in which all Trp residues apart from Trp5 were exchanged for the similar amino acid phenylalanine. A second SecA mutant was made (SecAW7) with a unique Trp at the amino acid residue position of Trp7. Since phenylalanine has a very weak fluorescence intensity, which is not excited at 297 nm, it will not contribute to the fluorescence spectrum of the SecA mutants. The decision to make *E. coli* SecA mutants instead of *B. subtilis* mutants was based on the availability of an *in vitro* protein translocation assay for *E. coli*, but not for *B. subtilis*. *E. coli* strain MM66 (*geneX*^{am}, *supF*^{ts}), which does not synthesize SecA at 42 °C, was used to test whether the SecAW5 and the SecAW7 mutants could functionally replace the wild type *E. coli* SecA. The plasmids pET175 and pET177 encoding the SecAW5 and SecAW7 mutants, respectively, supported normal growth of *E. coli* MM66 cells at the nonpermissive temperature. This indicated that both mutants were able to substitute for the wild type SecA protein. The mutants SecAW5 and SecAW7 were purified, and their fluorescence spectra were compared with the wild type SecA at an excitation wavelength of 297 nm (Figure 3). The Trp emission maxima of SecAW5 and SecAW7 were 336 and

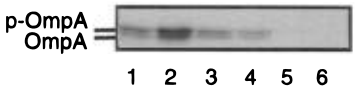

FIGURE 4: In vitro translocation of proOmpA by wild type *E. coli* SecA and the SecAW5 and SecAW7 mutants. Wild type SecA (lanes 1, 2, and 5), SecAW5 (lane 3), SecAW7 (lane 4), without ATP (lane 5), and no SecA added (lane 6). SecA proteins in lane 1, 3, 4, and 5 were isolated as described by Mitchell and Oliver (1993), and SecA in lane 2 was isolated as described by Cunningham et al. (1989).

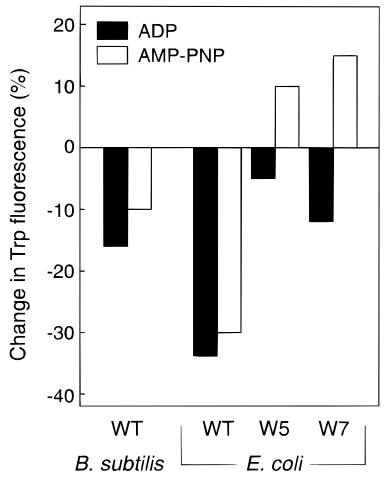

FIGURE 5: Change in tryptophan fluorescence as the percentage of total Trp fluorescence of *B. subtilis* SecA, *E. coli* SecA, and the SecAW5 and SecAW7 mutants in the presence of NBS-I saturating concentrations of Mg^{2+}/ADP (black bars) or Mg^{2+}/AMP-PNP (white bars). Negative values denote a decrease and positive values an increase in Trp fluorescence. The standard error of the mean was $\pm 4\%$ ($n \geq 3$).

332 nm, respectively. Both maxima are blue-shifted compared to the average emission maximum of 340 nm of the seven tryptophans of wild type SecA (Figure 3). The fluorescence intensities of SecAW5 and SecAW7 were 19% and 46% of the fluorescence intensity of wild type SecA, respectively (Figure 3). The fluorescence intensity of SecAW7 in combination with its high energy emission maximum suggests that this Trp is located in a relatively hydrophobic or immobilized environment with respect to the other Trps in SecA.

In order to determine if the purified SecAW5 and SecAW7 mutants were still able to assist the translocation of preproteins, the *in vitro* translocation of urea denatured ^{35}S-labeled proOmpA in the presence of SecAW5 and SecAW7 into urea-treated inner membranes was examined. Translocation of proOmpA into these vesicles is dependent on the addition of SecA and ATP (Figure 4). The efficiency of translocation by SecAW5 and SecAW7 was slightly reduced compared to that of wildtype SecA (Figure 4, compare lane 1 with lanes 3 and 4). It is concluded that both mutant SecA proteins are active.

The Trp fluorescence of the SecAW5 and the SecAW7 mutants decreased 5% and 12% upon titration with ADP in the presence of 5 mM $MgCl_2$, respectively (Figure 5). This shows that Trp7 predominantly senses the binding of ADP. The emission maximum of SecAW7 was red-shifted 2 nm, indicating that the decrease in fluorescence was not simply caused by quenching, but was due to a change in its environment. It is concluded that binding of ADP to NBS-I in the amino-terminal half of SecA changes the conformation of the carboxy-terminal domain containing Trp7.

AMP-PNP Binding to NBS-I Induces a Different Conformational Change in the Carboxyl Terminus. Titration experiments were performed with the ATP nonhydrolyzable analog AMP-PNP in a concentration range from 0.1 to 10 μM. In a NBS-I saturating AMP-PNP concentration range, again a decrease (30%) in Trp fluorescence in *E. coli* SecA was observed (Figure 5) with a K_D of approximately 0.5 μM (not shown). In *B. subtilis* SecA the decrease in Trp fluorescence was only 10%, whereas in the *E. coli* SecAW5 and SecAW7 mutants even an increase was observed in Trp fluorescence of 10% and 15%, respectively (Figure 5). This indicates that binding of AMP-PNP to NBS-I induces a conformational state of SecA which differs from that of the ADP-bound state. The fluorescence change reflects either an increase in the hydrophobicity or a decrease of the mobility of Trp7 upon AMP-PNP binding. ADP binding seems to promote the opposite changes. The observation that the Trp fluorescence of *E. coli* wild type SecA decreased, whereas it increased in both mutants, indicates that in addition to Trp5 and Trp7, the other Trps sense the conformational change in the protein and that nucleotide binding to NBS-I could cause several accumulative conformational changes throughout the protein, as for instance in adenylate kinase (Gerstein et al., 1993). It was not possible to study the conformational states of SecA in the presence of NBS-I and NBS-II saturating nucleotide concentrations due to the inner filter effects of these nucleotides.

Trypsin Digestion of SecA in the Presence of Nucleotides. The fluorescence intensity of Trps is very sensitive to its direct environment. The observed changes in the Trp fluorescence in the presence of nucleotides give no indication of the extent of the conformational changes in SecA. Therefore, it was investigated whether nucleotide binding to NBS-I induced a conformational change in SecA large enough to alter its susceptibility to trypsin digestion. SecA was incubated for 15 min with trypsin in the presence of increasing concentrations of AMP, ADP, or AMP-PNP. After addition of trypsin inhibitor, the tryptic digest was analyzed by SDS−PAGE and immunobloting (Figure 6A). A tryptic fragment of approximately 25 kDa, and to a minor extent of 35 kDa, was stabilized in the presence of Mg^{2+}/ADP, but not by Mg^{2+}/AMP, and only weakly by Mg^{2+}/AMP-PNP. Titration of SecA with ADP shows that the accumulation of the 25-kDa fragment saturates at ADP concentrations (<5 μM, Figure 7A) which saturate NBS-I but not NBS-II (Figure 7B). Occupation of NBS-II (>50 μM) has no further effect on the tryptic digestion pattern of SecA (Figure 7A). The same protection of the 25-kDa fragment by ADP was observed after trypsin digestion of the endogenous SecA in *E. coli* KM9 ISO vesicles (Figure 6B). This implies that the observed conformational changes are functional since they are not unique for the purified soluble SecA, but also occur in the SecA endogenously present in vesicles.

SecA Has Two Independently Unfolding Domains. The fluorescence measurements show that occupation of NBS-I at the amino-terminal part of SecA by ADP induces a change in the environment of amino acid residue 775 (Trp7) at the far end of the carboxyl terminus of SecA. Therefore, these two domains somehow seem to communicate as was found for phosphoglycerate kinase (Watson et al., 1982), or the ADP-induced conformational change is propagated up to

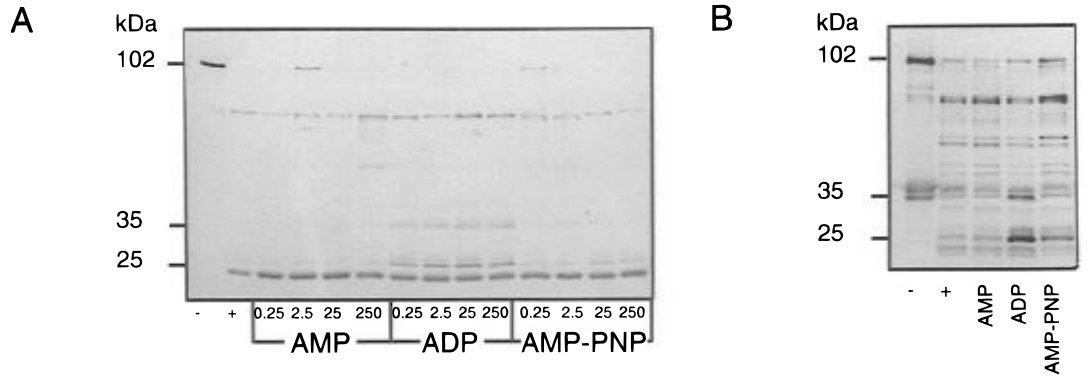

FIGURE 6: Trypsin digestion of nucleotide-bound soluble *E. coli* SecA (A) and endogenous *E. coli* SecA in vesicles (B). Undigested SecA (−), trypsin digested SecA in the absence of nucleotides (+), trypsin digested SecA in the presence of increasing concentrations of the indicated nucleotides (A), and endogenous SecA in trypsin digested vesicles with 2.5 mM AMP, ADP, and AMP-PNP (B). The incubation mixture for the samples shown in (B) contained all components used in the *in vitro* translocation apart from ATP (see Experimental Procedures). The immunoblots were analyzed with pAb against SecA and developed with BCIP/NBT (A) or with the chemiluminescence kit (B). The band below the 25-kDa fragment, visible in all lanes containing trypsin digested material, is trypsin, which at its high concentration cross-reacts with the pAb against SecA.

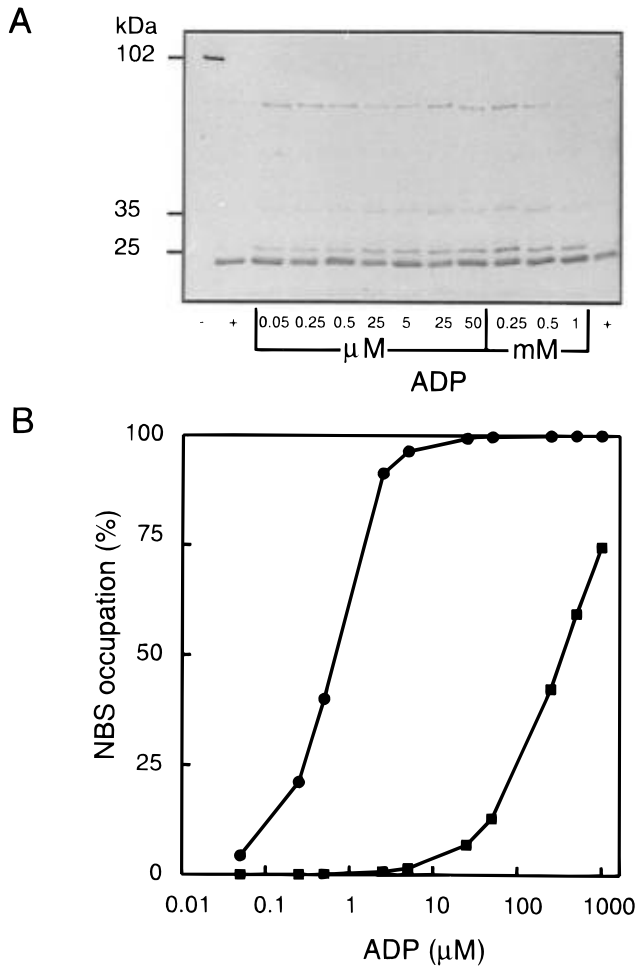

FIGURE 7: Trypsin digestion of soluble *E. coli* SecA in the presence of increasing ADP concentrations. (A) Undigested SecA (1 μM monomer, −), trypsin digested SecA (+), and in all other lanes, trypsin digested SecA with increasing ADP concentrations as indicated. The immunoblot was analyzed with pAb against SecA and developed with BCIP/NBT. (B) Nucleotide occupation of NBS-I (●) and NBS-II (■) as a function of the ADP concentration.

residue 775, as for instance in aspartate transcarbamylase or nitrogenase (Xi et al., 1994; Chen et al., 1994). To be able to discriminate between these two possibilities, and to access the conformational states of SecA in the presence of NBS-I and NBS-II saturating nucleotide concentrations, we studied the thermal denaturation of *B. subtilis* SecA in the absence and presence of ADP or AMP-PNP by differential scanning calorimetry (DSC).

The DSC profile of *B. subtilis* SecA shows two endothermic reactions with transition midpoints (T_{m1} and T_{m2}) of 40 and 49 °C, respectively (Figure 8A). This was followed by an exothermic reaction (T_{m3}) with an onset temperature at 60 °C at pH 7.5 (Figure 8B). Since aggregation of proteins is largely exothermic (Privalov & Khechnashvilli, 1974), it seems most likely that this exothermic transition corresponds to the aggregation of SecA immediate after or perhaps partly during the thermal denaturation.

The irreversibility of unfolding was evaluated by rescanning the samples after cooling at a rate of 1 °C/min and a resting period of 15 min at 25 °C. No transitions were observed during the rescan, demonstrating that the thermally induced denaturation of SecA is irreversible. To access if both endothermic transitions were irreversible, a new SecA sample was scanned from 30 to 43 °C (just passed the first transition), cooled as described, and rescanned from 25 °C up to 55 °C. The first transition appeared to be completely reversible (Figure 8A) and therefore independent from the second transition. Changing the scan rate from 1 to 1.5 °C/min upshifted the T_m of the second transition 1 °C, but not the T_m of the first transition. This indicates that unfolding of the domain of the first transition is thermally controlled whereas that of the second transition is kinetically controlled. The onset of the exothermic reaction appeared to be dependent on the buffer composition. In the buffers at pH 6.5, 7.5, and 8.0 used, the onset of the exothermic reaction was at 5, 10, and 20 °C past the second transition, respectively. At pH 8.0 where the exothermic reaction did not contribute to the heat of the second transition, this transition appeared to be also irreversible (results not shown).

The base-line problem is always a source of uncertainty in DSC, especially when the process of denaturation is irreversible. We have followed the procedure described by Takahashi et al. (1981) and carried out deconvolutions of the DSC curves in two-state transitions, characterized by the equivalence of the ΔH_{cal} (calorimetric enthalpy change) and ΔH_{vH} (van't Hoff enthalpy change). This approach is formally allowed only in the case of reversible phenomena,

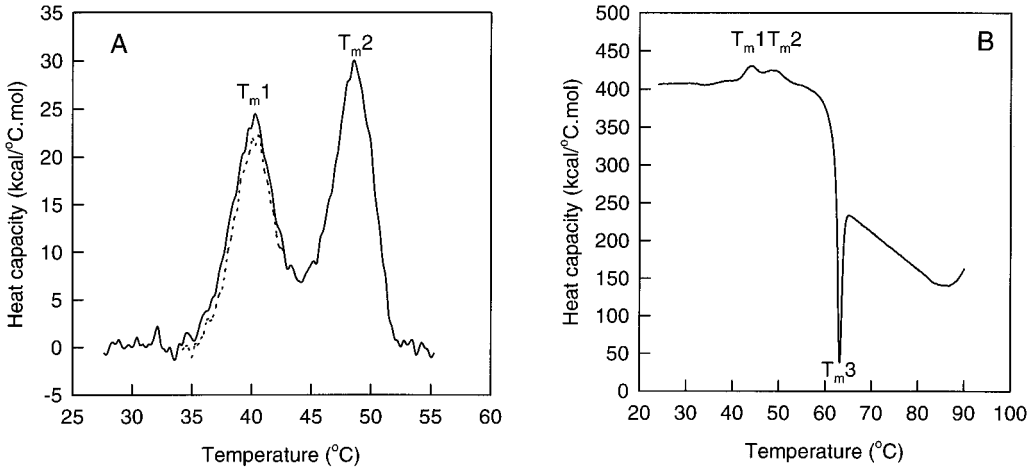

FIGURE 8: Temperature dependency of the molar heat capacity of *B. subtilis* SecA in 50 mM KP_i, pH 7.5/50 mM KCl. (A) The dotted line represents a scan from 30 to 43 °C, and the solid line represents a rescan of the same sample from 25 to 55 °C. Indicated are the transition midpoints of T_{m1} and T_{m2}. (B) Thermoscan from 20 to 95 °C; indicated are the midpoints of T_{m1}, T_{m2}, and T_{m3}.

Table 2: Thermodynamics of the Unfolding of SecA

buffer (50 mM), pH	T_{m1} (°C)	ΔH_{cal1} (kcal/mol)	ΔH_{vH1} (kcal/mol)	$\Delta H_{vH1}/\Delta H_{cal1}$	T_{m2} (°C)	ΔH_{cal2} (kcal/mol)	ΔH_{vH2} (kcal/mol)	$\Delta H_{vH2}/\Delta H_{cal2}$
KP_i, 6.5	38.3 ± 0.5	131 ± 19.5	133 ± 20	1.01	45.8 ± 1.0	107 ± 32	171 ± 26	1.60
KP_i, 7.5	40.5 ± 0.5	154 ± 23	133 ± 20	0.86	48.6 ± 0.5	175 ± 26	163 ± 24	0.93
Tris, 8.0	39.6 ± 0.5	150 ± 22.5	152 ± 20	1.01	47.3 ± 0.5	160 ± 24	161 ± 24	1.01

but shown to be admissible even on many systems having little or no reversibility at all (Engeseth & McMillin, 1986; Hu & Sturtevant, 1987). Since the first transition is reversible, the deconvolution of the DSC data of the first transition will be relatively reliable whereas the thermodynamic parameters derived from deconvolution of the second transition will be tentative. The first transition can be simulated with a two-state transition with a ΔH_{vH} approximately identical to the calorimetric enthalpy indicating the simultaneous unfolding of a subdomain in the SecA dimer (see below, Table 2). The $\Delta H_{vH}/\Delta H_{cal}$ ratio of the second transition is dependent on the onset temperature of the exothermic reaction, which in turn depends on the buffer composition in which the protein was denatured (Table 2). If the irreversible step occurs at temperatures significantly higher than the denaturation temperature, then application of equilibrium thermodynamics is valid. Therefore, the most reliable thermoscan was obtained by the sample wherein the exothermic reaction was postponed by 20 °C (see above). Using this sample, the second transition could also be simulated by a two-state transition. The ΔC_p was ca. 2000 cal/(°C·mol of dimer) for the complete unfolding of SecA in 50 mM Tris-HCl, pH 8.0.

We conclude that SecA has at least two thermally independent folding domains. Since the enthalpy of the unfolding is similar for both domains, they could possibly be of similar size.

The Interactions between the Two Domains of SecA Increase in the Presence of ADP. The thermal denaturation of *B. subtilis* SecA was studied in the presence of increasing concentration of ADP in 50 mM KP_i, pH 7.5, supplemented with 50 mM KCl and 5 mM $MgCl_2$ (Figure 9A, Table 3). The thermoscans in Figure 9A are all concentration normalized as dimer after subtraction of an instrumental base line as described (see above). Again, two unfolding transitions can be discriminated followed by the exothermic aggregation

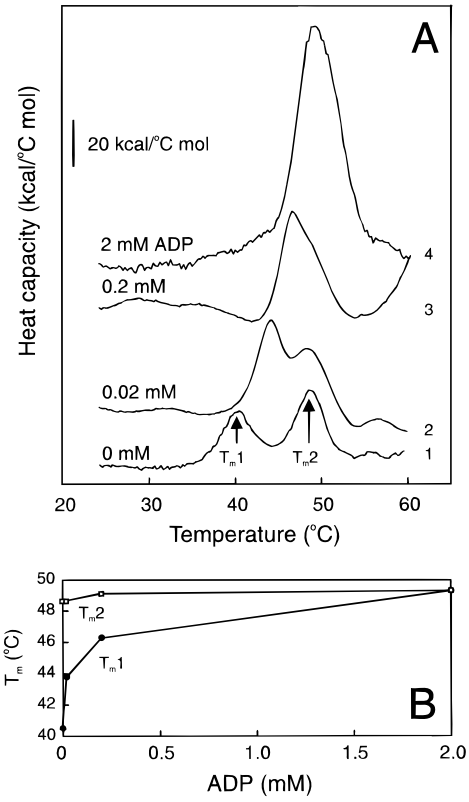

FIGURE 9: Temperature dependency of the molar heat capacity of *B. subtilis* SecA in 50 mM KP_i, pH 7.5/50 mM KCl in the absence and presence of ADP. (A) Thermal unfolding of SecA in the absence of ADP (1), and in the presence of 0.02 mM ADP (2), 0.2 mM ADP (3), and 2 mM ADP (4). Indicated are the transition midpoints of T_{m1} and T_{m2}. (B) The dependency of the midpoints of transitions 1 and 2 on the ADP concentration.

reaction onset at 60 °C. This latter reaction is partly compensated by an endothermic hydrolysis of the ADP at 80 °C. This is particularly evident in the thermoscan in the

Table 3: Thermodynamics of the Unfolding of SecA in the Presence of ADP or AMP-PNP

ligand concn	T_{m1} (°C)	ΔH_{cal1} (kcal/mol)	ΔH_{vH1} (kcal/mol)	$\Delta H_{vH1}/\Delta H_{cal1}$	T_{m2} (°C)	ΔH_{cal2} (kcal/mol)	ΔH_{vH2} (kcal/mol)	$\Delta H_{vH2}/\Delta H_{cal2}$
ADP (mM)								
0	40.5 ± 0.5	154 ± 23	133 ± 20	0.86	48.6 ± 0.5	175 ± 26	163 ± 24	0.93
0.02	43.8 ± 0.5	176 ± 26	185 ± 28	1.05	48.6 ± 0.5	172 ± 26	155 ± 23	0.90
0.2	46.3 ± 0.2	197 ± 29	235 ± 35	1.20	49.1 ± 0.2	112 ± 17	218 ± 33	1.94
2	49.3 ± 0.5	705 ± 106	129 ± 19	0.18				
AMP-PNP (mM)								
0.02	39.7 ± 0.5	142 ± 21	146 ± 22	1.03	48.5 ± 0.5	186 ± 56	145 ± 44	0.78

presence of 0.2 mM ADP (trace 3). Under all conditions, the transition profile deconvolutes approximately as two-state transitions (Table 3). At the low ADP concentrations of 0.02−0.2 mM, that saturate NBS-I but not NBS-II, an upshift in T_{m1} from 40 to 46 °C with no significant change in T_{m2} is observed. The shift in T_{m1} saturates, and T_{m2} seems to upshift slightly at higher ADP concentrations up to 2 mM (Figure 9B). This would be expected if the second transition corresponds to the binding domain (i.e., the amino-terminal part of SecA), and the first transition to the nonbinding regulatory domain (Brandts et al., 1989). At 2 mM ADP the enthalpy of unfolding of SecA increases from 350 to 700 kcal/mol of dimer (Figure 9A, Table 3). At this concentration the low-affinity ATP-binding site will be occupied by ADP for 86%, using the reported K_D at 25 °C of 340 μM (Mitchell & Oliver, 1993).

Apart from perhaps a slight destabilization of both transitions, the thermal unfolding of SecA was not altered significantly by 0.02 mM AMP-PNP compared with that of the nucleotide free protein (Table 3). The instability of AMP-PNP at high temperatures precludes us to apply higher concentrations of AMP-PNP in the DSC measurements. At the concentration used, AMP-PNP does not seem to induce changes in the interaction of the two domains of SecA.

The Apparent Molecular Mass Changes Dramatically in Nucleotide-Bound SecA. The enthalpy of the unfolding of a protein is directly correlated with the number of hydrophobic residues which are solvent accessible (Privalov, 1979). The doubling in the unfolding enthalpy of SecA in the presence of a NBS-I and NBS-II saturating ADP concentration suggests, therefore, either a decrease in the solvent accessibility of a number of hydrophobic residues or the dissociation of the dimer in monomers. The latter possibility could be excluded, based on gel filtration of SecA in the absence and presence of 5 mM ADP (results not shown). A decrease in the number of hydrophobic residues can be realized by increasing the compactness of the protein, for instance, by an increase in the interaction between the monomers. To further investigate the conformation of the NBS-II saturated state of SecA, the apparent size of SecA undergoing Brownian motion in solution was studied by dynamic light scattering (DLS). The fluctuations in intensity of the scattered light can be analyzed to give the translational diffusion coefficient D_T and estimates of the hydrodynamic radius of gyration and the apparent molecular weight (reviewed in Schmitz, 1990). The DLS measurements were all performed with the same batch of purified SecA to exclude any effect on the measurements of the contribution of possible different impurities of the samples. Therefore, any variation in the apparent hydrodynamic volume and the polydispersity (i.e., the variation in the hydrodynamic radius of gyration as measured by DLS) can only be attributed to variations in shape of SecA and not to variations of possible impurities in the samples. The molecular mass estimated by DLS often corresponds accurately to the calculated molecular mass of a protein or of protein complexes (Ferré-D'Amaré et al., 1994). The D_T of *B. subtilis* SecA (2 mg/mL) with 5 mM $MgCl_2$, Mg^{2+}/ADP, or Mg^{2+}/AMP-PNP in 50 mM HEPES, pH 7.5/50 mM KCl was measured at 25 °C, and the corresponding apparent molecular mass was calculated to be 190, 190, and 230 kDa, respectively (Table 4). SecA in the absence of nucleotides or $MgCl_2$ was found to have an apparent molecular mass of 164 kDa (Table 4). Saturation of NBS-I and -II with ADP or AMP-PNP increased the apparent volume of SecA, compared to nucleotide free SecA, with 21% and 51%, respectively, and the polydispersity with 25% and 54%, respectively (Table 4). If SecA would be a solid sphere with a radius R_S, the contribution of the bound water to its hydrodynamic radius R_H can be computed (eqs 2 and 3) from the average volume of a protein ($\bar{\nu}_p$) and the known molecular weight of the protein (M_p). For nucleotide free SecA the volume of the hydration shell of the protein $\delta\bar{\nu}_0 = 1.075$ g/g. However, the $\delta\bar{\nu}_0$ for ADP- or AMP-PNP-bound SecA is 1.47 and 2.02 g/g, respectively, which clearly indicates that the protein contains more water accessible surface than expected for a spherical protein. It can be concluded that shape asymmetry contributes to the R_H/R_S ratio. This indicates considerable changes in the shape and/or domain flexibility of SecA in the presence of different nucleotides.

Table 4: Determination of the Apparent Molecular Weight of *B. subtilis* SecA[a] in the Presence of Nucleotides at 25 °C by Light Scattering Measurements[b]

ligand (5 mM)	D_T[c] (10^{-13} m^2/s)	radius[d] (nm)	polydispersity (nm)	MW (kDa)
none	455 (0)	5.26 (0.02)	1.27 (0.15)	164.3 (0.5)
Mg^{2+}	433 (3)	5.60 (0.01)	1.69 (0.05)	189.8 (0.8)
Mg^{2+}/ADP	430 (1)	5.57 (0.02)	1.60 (0.04)	189.3 (1.0)
Mg^{2+}/AMP-PNP	398 (1)	6.03 (0.02)	2.24 (0.03)	229.8 (1.0)

[a] SecA concentration was 2 mg/mL in 50 mM HEPES, pH 7.5/50 mM KCl. [b] The data are based on at least ten measurements. The standard deviation is shown between brackets. [c] Translational diffusion coefficient. [d] Hydrodynamic radius of gyration.

DISCUSSION

Preprotein translocation is initiated by the ATP-dependent coinsertion of SecA and the preprotein in the integral domain of the *translocase*. Interaction of SecA with SecYEG stimulates the ATPase activity of SecA (Lill et al., 1989) and allows the deinsertion of SecA (Economou & Wickner, 1994). The Δp drives the completion of the preprotein translocation (Schiebel et al., 1991; Driessen, 1992). However, in the absence of a Δp, multiple cycles of nucleotide-modulated SecA insertion and deinsertion can also drive the

complete translocation (Schiebel et al., 1991). Both NBS-I and NBS-II are indispensable for the translocation activity of SecA (Van der Wolk et al., 1993, 1995; Mitchell & Oliver, 1993). Although they are able to bind nucleotides independently, they somehow seem to hydrolyze ATP in a cooperative manner. It has been found that ATP binding or hydrolysis can induce considerable conformational changes in proteins and over appreciable distances (Watson et al., 1982; Xi et al., 1994; Chen et al., 1994). To elucidate the mechanism underlying the nucleotide-induced SecA movements, we investigated the conformation of the ADP- and AMP-PNP-bound state of soluble SecA by intrinsic tryptophan fluorescence, tryptic digestion, differential scanning calorimetry (DSC), and dynamic light scattering (DLS).

Titration of *E. coli* or *B. subtilis* SecA with ADP caused a decrease in the Trp fluorescence in a concentration range which corresponds to saturation of the NBS-I but not of NBS-II. Since *B. subtilis* SecA has only two carboxy-terminal Trps, it was concluded that binding of ADP to the amino-terminal located NBS-I induces a change in a carboxy-terminal domain of SecA. This was confirmed by the decrease in the tryptophan fluorescence of the *E. coli* SecA mutant SecAW7 where all Trps apart from the most carboxy-terminal Trp (amino acid residue 775) were replaced by phenylalanine. The increase in Trp fluorescence in this mutant in the presence of the nonhydrolyzable ATP analog AMP-PNP suggests a different conformational change of the carboxyl terminus in ATP-bound SecA. During trypsin digestion, a 25-kDa fragment of SecA accumulated in the presence of NBS-I saturating ADP concentrations, but hardly in the presence of AMP-PNP and not with AMP. This phenomenon is observed with both soluble SecA and membrane-bound SecA, and independent of the presence of preprotein and SecB. Therefore, it seems that at least the conformation of the soluble ADP-bound state of SecA is similar to that of the membrane associated ADP-bound state of SecA.

The DSC analysis of the unfolding of SecA shows that *B. subtilis* SecA has two thermally discernible domains of approximately equal size. *E. coli* SecA differs in this respect, since only one transition at 42 °C was observed upon thermal unfolding directly measured by DSC (1030 kcal/mol) (Ulbrandt et al., 1992). ADP binding to NBS-I stabilizes the SecA domain of the first transition, but not that of the second transition. Such a pattern is frequently observed in two domain proteins, which will bind a ligand at the more stable domain (T_{m2}), whereas the ligand interaction with this domain is influenced by ligand dependent changes in the interactions between the binding domain (T_{m2}) and the regulatory domain (T_{m1}; Brandts et al., 1990). In such a case, T_{m2} is expected to upshift in temperature at an excess ligand concentration. Unfortunately, the thermodynamic effects of nucleotide binding to NBS-II conceal potential upshifts of T_{m2} in SecA. Cross-linking of [α^{32}-P]ATP to amino-terminal peptides containing NBS-I of *E. coli* SecA is only possible in the presence of the carboxy-terminal counterpart (Matsuyama et al., 1990). These data seem to support our model in which the interaction between the two domains of SecA increases upon ADP binding to NBS-I.

We assume that the nucleotide free form of SecA is nonexistent *in vivo* and that the soluble form of SecA will be predominantly in the ADP-bound state. For SecYEG membrane association of SecA, either ATP and preprotein or AMP-PNP is needed (Economou et al., 1995). AMP-PNP, in a concentration which seems to occupy at least NBS-II partly, locks SecA in an insertion competent conformation which is unable to translocate preproteins. Mutants of *E. coli* and *B. subtilis* SecA which are defective in ATP hydrolysis but not in ATP binding at NBS-I are also able to insert into the membrane but are not able to deinsert or to translocate preproteins (Van der Wolk et al., 1993, Economou et al., 1995). We find that ADP binding to NBS-I in the amino-terminal half of SecA results in a conformational change in at least the carboxyl terminus, the protection of a 25-kDa fragment against trypsin digestion, and an increased interaction in the two thermodynamically discernible domains which are assumed to be the carboxy- and amino-terminal halves of SecA. AMP-PNP binding to NBS-I results in a different conformational change of the carboxyl-terminal domain and seems not to induce a change in the interaction of both domains. This could be interpreted as movements that conceal and reveal the membrane insertion site of SecA, respectively.

In the presence of 2 mM ADP, NBS-I is fully occupied whereas NBS-II is occupied for about 86% under the experimental conditions employed (see eqs 4 and 5 in the Experimental Procedures). The doubling in enthalpy, as measured by DSC, of the unfolding reaction in the presence of 2 mM ADP cannot be attributed to the enthalpy of the dissociation of the bound ADP since this has never been reported to be more than ca. 30 kcal/mol ADP (e.g., Hu & Sturtevant, 1987). Therefore, the increase in enthalpy must be caused by the partial occupation of NBS-II. Since the occupation of the NBS-II does not result in a substantial increase in T_{m2}, it seems unlikely that the low-affinity domain is located in the more stable domain which was assigned as the amino-terminal domain (see above). Therefore, the domain of the first transition is tentatively assigned to contain the low-affinity binding site or to be the carboxy-terminal half, and the domain of the second transition to be the amino-terminal half of the protein. The overall unfolding enthalpy of SecA in the absence of ADP or in the presence of NBS-I saturating ADP concentration is unusually small, 329 $\pm$ 49 kcal/mol of dimer or 1.71 cal (g of protein^{-1} but not unprecedented (Blandamer et al., 1994). In the presence of 2 mM ADP, the specific enthalpy of unfolding increases to 3.67 cal g^{-1}, which is in the range normally found for globular proteins (Hu & Sturtevant, 1987; Takahashi et al., 1981). The enthalpy of unfolding is largely dependent on the amount of amino acid residues which are inaccessible to the solvent (Privalov, 1979). The increase in enthalpy upon ADP binding to NBS-I and NBS-II suggests an increase in the amount of solvent shielded residues or a more compact conformation. Alternatively, a considerable increase in the amount of shared protein surface of the monomers in the dimer could result in an increase in the enthalpy of unfolding. For instance, if NBS-II would be located at the interface of the monomers. In the presence of NBS-II saturating concentrations of the slowly hydrolyzable ATP analog ATP-γ-S, or ADP, the proteolytic site at Glu443 of *B. subtilis* SecA is protected against V8 proteolysis (Van der Wolk et al., 1995). The *B. subtilis* SecAD215N mutant defective in NBS-I is permanently resistant against V8 proteolysis even in the absence of nucleotides (Van der Wolk et al., 1995).

These data support our observation that binding of ADP to both NBSs increases the interaction between both domains and promotes a more compact conformation of SecA. Possibly, the monomers in *B. subtilis* SecA dimer could be loosely associated in the absence of ADP, whereas under these conditions the monomers in *E. coli* SecA are already tightly associated in a dimer. This would be in agreement with the specific enthalpy of *E. coli* SecA unfolding of 5 cal (g of protein)$^{-1}$ (Ulbrandt, et al., 1992).

DLS measurement showed an increase of 16% and 40% in apparent mass upon saturation of NBS-I and -II with ADP or AMP-PNP, respectively. The increment of $\delta\bar{\nu}_0$ in the presence of these nucleotides suggests an increase in the amount of solvent accessible protein surface and indicates an asymmetry in the shape of the protein. Based on the DSC measurements, the ADP-bound conformation seemed to be relatively compact. In combination with the DLS results, the ADP-bound conformation of SecA could possibly be visualized as irregular spherical, whereas in the AMP-PNP-bound conformation the protein could have a more elongated shape. However, to determine the actual shape of SecA in the presence of these nucleotides, the rotational diffusion coefficient as well as the translational diffusion coefficient are needed.

Mutants in NBS-II which still have 4% of the wild type activity in ATP binding and hydrolysis (Mitchel & Oliver, 1993) are able to insert and deinsert in the membrane, but cannot translocate preproteins (Economou et al., 1995). It is possible that ATP binding and hydrolysis at NBS-II are required for a more stable insertion, since AMP-PNP binding to both sites protects a membrane-bound 30-kDa fragment against protease K digestion (Economou et al., 1995). The NBSs cannot hydrolyze ATP independently (Mitchel & Oliver, 1993), and after ATP-induced insertion, additional ATP binding and hydrolysis seem to be required for the deinsertion of SecA (Economou & Wickner, 1994). This indicates that ATP hydrolysis at both sites might be required for deinsertion. Signal sequence repressing mutations are also found in both NBS regions, indicating that both sites are involved in the recognition and presentation of the signal sequence to the *translocase* (Huie & Silhavy, 1995). The observation that ADP and AMP-PNP to NBS-I as well as to NBS-II cause considerable changes in conformation and shape of SecA shows that both sites are functionally involved in the process of preprotein translocation. We propose that the initiation of preprotein translocation is a two-step process in which ATP binding to NBS-I exposes a membrane inserted domain of SecA to the integral subunits of the *translocase* and that ATP binding to NBS-II causes a second conformational change in SecA enabling the presentation of the preprotein to SecYEG. ATP hydrolysis at NBS-II could promote a more compact conformation, which excludes the preprotein, and hydrolysis at NBS-I could subsequently result in the deinsertion of SecA.

ADDED IN PROOF

Based on the tentative assignment of the T_{m1} and T_{m2} domains of SecA, an amino-terminal fragment (N-SecA, amino acids 1−443) was expressed separately and purified. The DSC profile of N-SecA in Tris-HCl, pH 8.0, 50 mM KCl, and for stabilization 5 mM $MgCl_2$, shows only one endothermic transition at 50 °C and subsequently an exothermic transition indicating aggregation similar to the second transition of intact SecA. This confirms the hypothesis that SecA consists of an independently folding amino- and carboxy-terminal half and that binding of ADP to the amino-terminal domain increases its interaction with the carboxy-terminal domain. The transition of N-SecA could also be simulated with a two state transition assuming the protein to be a dimer of 100 kDa. Consequently, the amino-terminal domain must be at least partly responsible for the dimerization of SecA.

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