Driessen, A. J. SecA, the peripheral subunit of the Escherichia coli precursor protein translocase, is functional as a dimer. Biochemistry 32, 13190-13197

ARTICLE *in* BIOCHEMISTRY · JANUARY 1994

Impact Factor: 3.02 · DOI: 10.1021/bi00211a030 · Source: PubMed

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SecA, the Peripheral Subunit of the *Escherichia coli* Precursor Protein Translocase, Is Functional as a Dimer†

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Received June 28, 1993; Revised Manuscript Received August 30, 1993®

ABSTRACT: SecA, the peripheral ATPase domain of the *Escherichia coli* precursor protein *translocase*, was denatured in 6 M guanidine hydrochloride. Circular dichroism and intrinsic tryptophan fluorescence spectra revealed that the protein is transformed into a random-coil configuration. Upon dilution of the chaotropic agent, SecA refolds into its native, functional conformation as a homodimer. As structural criteria, the native dimeric state was assayed by size-exclusion chromatography, chemical cross-linking, tryptophan fluorescence, and circular dichroism. Functional SecA heterodimers were formed of which the individual subunits were tagged with fluorescent dyes to allow measurements of the association state of the monomers by resonance energy transfer using steady-state and time-resolved fluorescence spectroscopy. SecA retained its dimeric structure during translocation, while energy transfer was abolished only by denaturation. The "half-of-the-sites activity" was investigated by constructing heterodimers formed from native and 8-azido-ATP-inactivated SecA. Heterodimers have lost the ability to support translocation of the precursor protein proOmpA in an *in vitro* translocation system. It is concluded that the dimeric structure is maintained during translocation and required for functionality.

SecA (Schmidt et al., 1988) is a dissociable, peripheral subunit of the precursor protein *translocase* of *Escherichia coli* (Brundage et al., 1990; Hartl et al., 1990; Wickner et al., 1991). In addition to SecA, *translocase* consists of the SecY/E protein, a multisubunit integral membrane protein complex (Brundage et al., 1990, 1992; Akimura et al., 1991), and possibly SecD and SecF (Bieker-Brady & Silhavy, 1992; Matsuyama et al., 1993). SecA is an ATPase, and the concerted activities of the protonmotive force and ATP hydrolysis by SecA permit the successive progress of precursor proteins across the membrane (Tani et al., 1989, 1990; Schiebel et al., 1991; Driessen, 1992a; Arkowitz et al., 1993). At the transitional stages of translocation, the precursor protein may physically interact with the SecA and SecY subunits of the *translocase* (Joly & Wickner, 1993).

SecA is a complex protein and involved in multiple catalytic and regulatory interactions. SecA interacts with (i) the signal sequence domain and as yet unknown elements of the mature domain of precursor proteins (Lill et al., 1990; Akita et al., 1990; Joly & Wickner, 1993), (ii) the SecB protein, thereby assisting in protein targeting (Hartl et al., 1990), (iii) the SecY/E protein at the membrane surface (Hartl et al., 1990), (iv) acidic phospholipids (Lill et al., 1991), and (v) its own mRNA at a sequence around the *gene X-secA* intergenic region (Dolan & Oliver, 1991) as part of an autogenous regulation mechanism. Interactions with translocation-competent precursor proteins, the SecY/E protein, and acidic phospholipids (Lill et al., 1989, 1990; Cunningham & Wickner, 1989; Brundage et al., 1990) activate SecA for ATP hydrolysis. Binding of ATP to SecA drives the translocation of the amino terminus of the precursor protein, while ATP hydrolysis is needed to release the precursor protein from its association with SecA (Schiebel et al., 1991; Driessen, 1992b).

The number of SecA molecules present in a typical *E. coli* cell is about 10-fold higher than that of the other components of the *translocase* (Matsuyama et al., 1992). Localization studies indicate a dynamic and complex behavior of the cellular SecA. Depending on the conditions of cell fractionation, up to half of the cellular SecA can be found to be associated with the cytoplasmic membrane, while the remainder is present in a soluble fraction (Akita et al., 1991; Cabelli et al., 1991). A small amount of the cytosolic SecA appears to be present in a labile high molecular mass complex, i.e., 500–1000 kDa, with unknown composition. Size-exclusion chromatography, velocity sedimentation analysis, and chemical cross-linking experiments have shown that cytosolic SecA, and SecA purified from the cytosolic fraction, is homodimeric (Akita et al., 1991; Cabelli et al., 1991; van der Wolk et al., 1993). The dimeric state of the purified SecA is maintained over a wide range of protein concentrations (Akita et al., 1991). SecA homodimers can be functionally reconstituted from the guanidine-denatured state, and during renaturation, reducing conditions which prevented disulfide bond formation were found not to be critical for dimerization. Both *in vivo* (Cabelli et al., 1991; Kusters et al., 1992) and *in vitro* (Breukink et al., 1992; van der Wolk et al., 1993) studies suggest that the cellular location of SecA is modulated by its interaction with nucleotides. Genetic studies are consistent with the suggestion that SecA is a dissociable component of the *translocase*, arguing that the components of the *translocase* assemble and disassemble during translocation (Bieker-Brady & Silhavy, 1992). No data are available on the quaternary structure of SecA during the various stages of translocation, and the question arises as to whether the aggregation state of SecA is modulated by interactions with the various ligands. Also, the functional consequence of the dimeric organization of the soluble SecA had remained obscure.

The dimeric structure of SecA raises the question as to whether the two identical polypeptide chains constitute a single functional unit. As recently indicated (Weaver et al., 1992), genetic data are consistent with a homomultimeric association state of SecA. Dominant-negative *secA* mutations are

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® Abstract published in *Advance ACS Abstracts,* November 15, 1993.

0006-2960/93/0432-13190$04.00/0

common (Jarosik & Oliver, 1991) and may arise from the formation of nonfunctional multimeric SecA proteins consisting of both defective- and wild-type subunits. Intragenic-*secA* complementation has also been found, and is best exemplified by the observation that the *secA51* (*Ts*) defect can be complemented by a 239-residue amino-terminal fragment of SecA (Cabelli et al., 1991) or similar sized amino-terminal fragments derived from the wild-type *Bacillus subtilis* SecA (Overhoff et al., 1991). When expressed as the complete polypeptide in *E. coli* in a wild-type background, an ATPase mutant of the *B. subtilis* SecA strongly interfered with protein export (Klose et al., 1993). Such interference may arise from poising of the active wild-type SecA with defective subunits of the SecA ATPase mutant protein, and competition for membrane-translocation sites (Van der Wolk et al., 1993). A particularly interesting observation is that the SecA ATP binding and precursor protein binding sites can be reconstituted from overlapping fragments of the SecA protein (Matsuyama et al., 1990; Kimura et al., 1991).

We now report on the "half-of-the-sites activity" of the SecA homodimer by constructing *in vitro* heterodimers from inactive and native SecA utilizing a spontaneous refolding technique. The results suggest that activity results from the preexisting asymmetry of the SecA dimer or multimer and that this multimer state is maintained during translocation.

EXPERIMENTAL PROCEDURES

Materials and Bacterial Strains. *E. coli* SecA (Cunningham et al., 1989) and SecB (Weiss et al., 1988) were purified from strains BL21(λDE3)/pT7-*secA* (Cabelli et al., 1988) and BL21(λDE3)/pJW25 (Weiss et al., 1988), respectively. Inverted membrane vesicles were prepared from *E. coli* strain UH203 (*lac*, *supF*, *ompA*, *recA*, *proA*, or *B*, *rpsL*/*F'*, *lacI*^{q}, *lacZ*, *M15*, *proAB*$^+$) by the procedure of Chang et al. (1978). ProOmpA was purified from *E. coli* UH203 harboring plasmid pRD87 (Freudl et al., 1985) as described (Crooke et al., 1988) and dissolved in 6 M urea, 1 mM dithiothreitol, and 20 mM Tris-HCl, pH 8.0. *E. coli* strain JM109 [*recA1*, *endA1*, *hyrA96*, *thi*$^-$, *hsdR17*, *relA1*, *supE44*, λ$^-$, Δ(*lac-proAB*), F', *traD36*, *proAB*, *lacI*^{q}2ΔM15] harboring plasmids pMKL04 and pMKL20 (Klose et al., 1993) were used for the isolation of the *B. subtilis* wild-type and K106N SecA proteins (Van der Wolk et al., 1993). SecA and proOmpA antisera were generous gifts of Dr. W. Wickner (University of California at Los Angeles). 8-Azido-ATP (N_3ATP)[1] was purchased from Sigma Chemical Co. (St. Louis, MO). 7-(ethylamino)-3-(4'-maleimidophenyl)-4-methylcoumarin maleimide (CMmal) and fluorescein-5-maleimide (Fmal) were from Molecular Probes (Molecular Probes, Eugene, OR).

Denaturation, Renaturation, and Fluorescent Labeling. SecA was denatured and renatured according to the procedure of Saxena and Wetlaufer (1970) as described by Matsuyama et al. (1990). SecA (7–15 nmol; 0.8–1.6 mg of protein) in 1 mL of denaturation buffer (6 M Gdn-HCl/50 mM Tris–acetate, pH 7.5) was diluted with 15 mL of buffer A [50 mM Tris–acetate (pH 7.5)/100 mM KOAc] supplemented with 1 mM dithiothreitol. The solution was incubated for 1 h at 4 °C and dialyzed against buffer A and 1 mM dithiothreitol. Dialyzed samples were concentrated by using a Centriprep-30 device (Amicon, Beverly, MA); the concentrated solution was supplemented with 10% (v/v) glycerol and stored at −80 °C until use.

For fluorescent labeling, SecA (2–2.5 mg of protein/mL) was incubated with 200 μM CMmal or Fmal in 50 mM Tris-HCl, pH 7.6, 30 mM KCl, 2 mM $Mg(OAc)_2$, and 2 mM MgATP for 2 h at 4 °C. ATP prevented low-level labeling of the protein at cysteine-98 located near the glycine-rich flexible loop of the ATP binding site. Reactions were terminated by the addition of 10 mM dithiothreitol. Protein samples were concentrated with an Amicon Centricon-30 concentrator, diluted in denaturation buffer, and subsequently renatured by dilution as described. Nonreacted maleimides were removed during the dialysis step.

Optical Measurements. Ultraviolet absorbance spectra were obtained on an Aminco DW2000 double-wavelength spectrophotometer (Urbana, IL) using a bandwidth of 4 nm. Fluorescence spectra and lifetimes were measured on an SLM 4800C (Urbana, IL) or a Perkin-Elmer LS-50B fluorometer (Norwalk, CT). Steady-state tryptophan and coumarin fluorescence spectra were obtained with fixed excitation wavelengths of 290 and 390 nm, respectively. Excitation and emission monochromators with a bandwidth of 2 nm and spectra were corrected for the wavelength dependence of the lamp intensity profile and detection system sensitivity. Experiments were performed by using quartz microcuvettes (200 μL) placed in a thermostated cuvette holder at 25 °C, unless indicated otherwise. All solutions were filtered through 0.22-μm HAWP Millipore filters (Bedford, MA) to remove dust particles. Excited-state lifetimes of coumarin were determined by the phase delay and demodulation technique at 6-, 18-, and 30-MHz modulation frequency as described (Spencer & Weber, 1970). Excitation was at 390 nm with a bandwidth of 0.5 nm, and emission was measured at 467 nm with a bandwidth of 8 nm. Lifetimes were referenced against a dilute solution of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene in ethanol (τ = 1.45 ns) or a glycogen scatter reference (τ = 0 ns). The phase (τ_Φ) and modulation (τ_m) lifetimes as determined at the different frequencies were fitted in a two-component heterogeneity analysis to yield the individual lifetimes according to the software package supplied by SLM Inc.

Size-Exclusion Chromatography. A Hiload 26/60 Superdex 200 size-exclusion column (10–600-kDa fractionation range) mounted to an FPLC (Pharmacia, Sweden) setup at 4 °C was used for all sizing data and purification of heterodimers, unless indicated otherwise. This resin was selected because it showed minimal interactions with SecA under the conditions employed, while it allowed preparative-scale isolation of the dimer at high elution rates. Proteins were eluted with buffer B supplemented with 1 mM dithiothreitol at an elution rate of 1 mL/min. Fractions were analyzed by SDS–PAGE and Coomassie blue staining, pooled, and concentrated.

N_3ATP Protein Labeling. Photo-cross-linking was performed with N_3ATP as described previously (van der Wolk et al., 1993). SecA (400 μg of protein/mL) was mixed with 1 mM N_3ATP in 50 mM Tris-HCl, pH 7.6, 30 mM KCl, 2 mM $Mg(OAc)_2$, and 10% (v/v) glycerol at 0 °C under reduced light. Samples of 0.5 mL were spotted on a small glass plate fixed on a lead brick placed in ice/water, and irradiated for 2 min with the 254-nm wavelength band of a Model UVG-54 UV lamp (UVP Inc., San Gabriel, CA) at 2-cm distance. Controls were subjected to the same treatment except that N_3ATP was omitted from the reactions. Reactions were quenched with 50 μL of a solution containing 50 mM

[1] Abbreviations: ATPγS, adenosine 5'-*O*-(3-thiotriphosphate); CD, circular dichroism; CMmal, 7-(ethylamino)-3-(4'-maleimidophenyl)-4-methylcoumarinmaleimide; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide; Fmal, fluorescein-5-maleimide; Gdn-HCl, guanidine hydrochloride; N_3ATP, 8-azidoadenosine 5'-triphosphate.

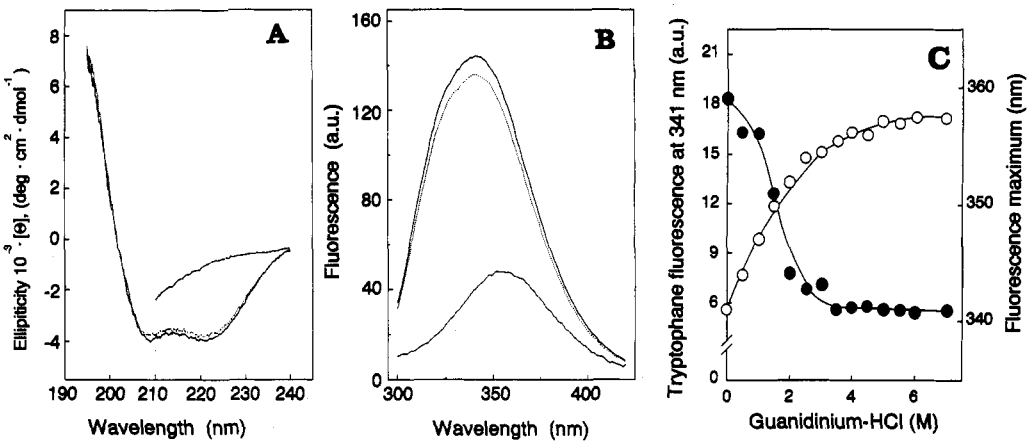

FIGURE 1: Circular dichroism and fluorescence spectra of native SecA and SecA renatured from the Gdn-HCl-denatured state. (A) Circular dichroism. Solutions were prepared at a final protein concentration of 0.4 mg/mL of native SecA in buffer A (—), native SecA denatured in 6 M Gdn-HCl and buffer A (dashed curve), or renatured SecA in buffer A (···). SecA was renatured from Gdn-HCl solution as described under Experimental Procedures. Circular dichroism spectra were obtained on a Jasco J500A spectrophotometer at 25 °C using a cell with a light path of 1 mm. Data were expressed as the total protein molecular ellipticity. In solutions of 6 M Gdn-HCl, high UV absorbance prevented data collection below 210 nm. (B) Intrinsic tryptophan fluorescence. Solutions were prepared at a final protein concentration of 40 μg/mL of native SecA in buffer A (—), native SecA denatured in 6 M Gdn-HCl and buffer A (dashed curve), or renatured SecA in buffer A (···). Tryptophan fluorescence emission spectra were recorded as described under Experimental Procedures. Five scans were averaged for each spectrum. (C) Unfolding of SecA in Gdn-HCl. SecA was denatured in buffer A supplemented with various concentrations of Gdn-HCl and incubated for 10 min at 4 °C. Tryptophan fluorescence emission spectra were recorded at 25 °C. (●) Relative fluorescence at 341 nm; (○) fluorescence emission maximum in nanometers.

dithiothreitol in 50 mM HEPES–KOH, pH 7.5. Samples were concentrated and denatured in Gdn-HCl as described above.

Translocation Assays. Translocation of proOmpA into urea-washed inner membrane vesicles of *E. coli* strain UH203 was assayed by its inaccessibility to added proteinase K (Cunningham et al., 1989). Reaction mixtures (50 μL) contained 50 mM HEPES–KOH, pH 7.3, 50 mM KCl, 0.5 mg/mL fatty acid-free bovine serum albumin, 10 mM, dithiothreitol, 2 mM $Mg(OAc)_2$, 20 μg/mL *E. coli* SecA protein, 10 μg/mL SecB protein, 2 mM MgATP, inner membranes (30 μg of protein/mL), 10 mM creatine phosphate, and 10 μg/mL creatine kinase. Reactions were initiated by the addition of 4 μg/mL proOmpA. After 30 min, samples were chilled on ice, treated with proteinase K (1 mg/mL, 15 min, 0 °C), and analyzed by SDS–PAGE and immunoblotting using the ECL Western blotting detection system (Amersham Ltd.).

Other Analytical Methods. Released orthophosphate was determined by the colorimetric assay of Lanzetta et al. (1979) as described by Lill et al. (1990). SecA was cross-linked at a concentration of 0.8 mg of protein/mL with 25 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC) (Matsuyama et al., 1990) in 50 mM potassium phosphate, pH 7.2, and 30 mM KCl. After 40-min incubation at 25 °C, reactions were terminated by the addition of 50 mM Tris-HCl, pH 7.5. Samples, precipitated with trichloroacetic acid and acetone-washed, were analyzed by SDS–PAGE and Coomassie blue staining. Protein concentrations were assayed according to Bradford (1976) using bovine serum albumin as a standard.

RESULTS

Denaturation and Renaturation of SecA. Circular dichroism (CD) spectra of native SecA (Figure 1A) showed two minima in the mean residue weight ellipticity ($[\theta]_{220} = -3950$ and $[\theta]_{209} = -4050$ deg·cm^2·dmol^{-1}) at 220 and 209 nm, characteristic of α-helical secondary structure. The spectrum of SecA in 6 M guanidine hydrochloride showed no appreciable secondary structure, indicating that the protein has adopted a random-coil configuration. The folding state of SecA was examined further using tryptophan fluorescence and UV absorption spectroscopy. The intrinsic fluorescence of the seven tryptophan residues of SecA which reside all along the polypeptide chain (residues 349, 519, 541, 622, 701, 723, and 775: total length 901 amino acid residues) was determined as a qualitative measure for tertiary structure. Native SecA exhibits a fluorescence emission maximum at 341 nm (Figure 1B). Treatment of SecA with Gdn-HCl results in a major reduction of the fluorescence quantum yield (Figure 1C, ●) and a red-shift of the emission maximum (○). Both effects indicate a more polar environment of the tryptophans after treatment with chaotropic agent.

Denatured SecA was renatured by dilution of the protein down to 40 mM Gdn-HCl in the presence of dithiothreitol to reduce disulfide bonds according to the procedure of Saxena and Wetlaufer (1970) as described by Matsuyama et al. (1990). SecA denatured in 6 M Gdn-HCl under reducing conditions regained enzymatic activity when the Gdn-HCl concentration was lowered by dilution and further removed by dialysis. On average, a recovery of 65–70% of the proOmpA-stimulated ATPase input activity (Figure 2, lanes 1 and 2) was obtained when renaturation was conducted below 10 °C. At 25 °C, most of the protein was recovered in an inactive state and eluted from a size-exclusion column at a peak size corresponding to protein aggregates. SecA renatured below 10 °C was reisolated in a dimeric state (Figure 3, c). CD (Figure 1A) and tryptophan fluorescence spectroscopy (Figure 1B) shows that the renatured protein contains nearly as much secondary and tertiary structure as the native protein. By size-exclusion chromatography, the SecA protein chromatographs as a single species in the presence of 3 M Gdn-HCl (Figure 3, b). The apparent molecular weight of the denatured protein is intermediate of that of the native dimer (a) and that expected for monomeric SecA on the basis of molecular weight calibration standards. CD spectroscopy (not shown) and intrinsic tryptophan fluorescence (Figure 1C) indicated that the protein is completely denatured in 3 M Gdn-HCl. The difference in elution volume most likely reflects the expanded hydrodynamic radius of the denatured protein. SecA, denatured in Gdn-HCl at a concentration of 3 M or

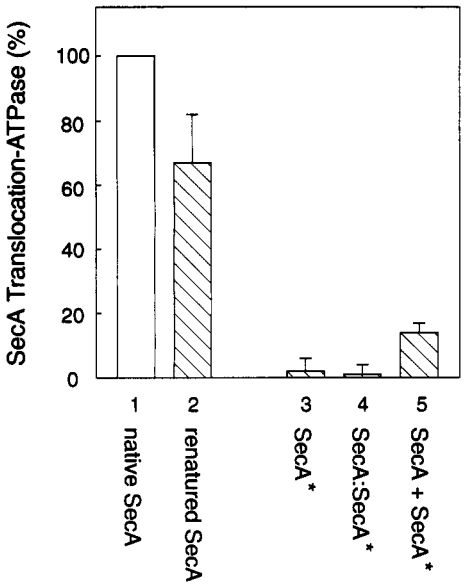

FIGURE 2: Recovery of SecA translocation ATPase activity. Reaction mixtures (50 μL) contained 50 mM HEPES–KOH, pH 7.3, 50 mM KCl, 0.5 mg/mL fatty acid-free bovine serum albumin, 2 mM Mg(OAc)$_2$, a 20 μg/mL sample of the indicated SecA protein species, 2 mM MgATP, urea-treated *E. coli* inner membranes (20 μg of protein/mL), 1 mM dithiothreitol, and 2 mM ATP. Reactions were initiated by the addition of proOmpA (40 μg/mL, final concentration), added from 6 M urea, and incubated for 40 min at 30 °C. Released inorganic phosphate was determined as described under Experimental Procedures. Lanes 1 and 2 contained 20 μg/mL native and renatured SecA, respectively. In lane 3, the SecA used was renatured from Gdn-HCl-denatured and N_3ATP-inactivated SecA (SecA*). In lane 4, a heterodimer was formed from a 1:4 mixture of Gdn-HCl-denatured native and N_3ATP-inactivated SecA (SecA:SecA*). In lane 5, a 1:4 mixture was prepared from homodimers renatured from Gdn-HCl-denatured native and N_3ATP-inactivated SecA (SecA + SecA*).

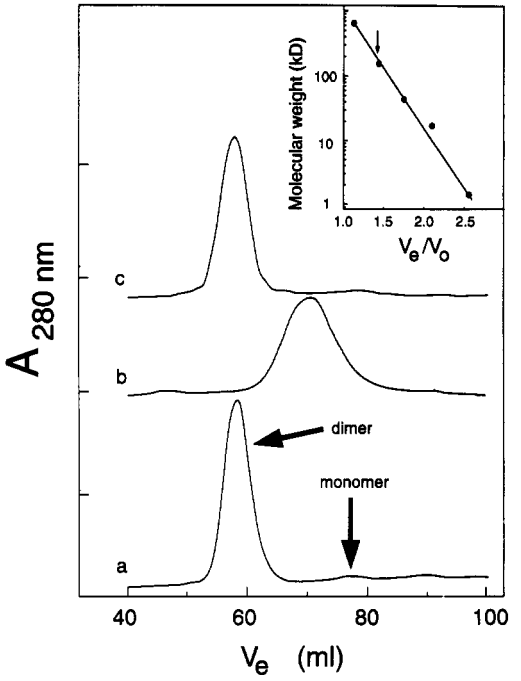

FIGURE 3: Size-exclusion chromatography of native SecA and SecA renatured from the Gdn-HCl-denatured state. Native SecA (a) and SecA renatured from the Gdn-HCl-denatured state (c) were applied on a Hiload 26/60 Superdex 200 size-exclusion column and eluted at a rate of 0.5 mL/min with buffer B supplemented with 1 mM dithiothreitol. Gdn-HCl-denatured SecA (b) was eluted with the same buffer B supplemented with 3 M Gdn-HCl. To allow a sufficiently high elution rate in 3 M Gdn-HCl solution, chromatography was performed at 18 °C. Absorbance was monitored at 280 nm, and is indicated in relative units. V_e and V_o indicate the elution and occluded volume, respectively. The expected elution volume of monomeric globular SecA is indicated by an arrow. *Inset*: calibration of the size exclusion volumes with the molecular masses of the following globular proteins (daltons): thyroglobulin, 670 000; bovine γ-globulin, 158 000; chicken ovalbumin, 44 000; equine myoglobin, 17 500; vitamin B_{12}, 1350. The elution volume (V_e/V_o) of the native and renatured SecA is indicated by an arrow.

higher, is most likely in a monomeric state. The renatured protein (Figure 3, c), however, chromatographs at the position of the native dimer (a), indicating that the protein has regained its original quaternary structure. Further structural characterization by cross-linking with EDAC (not shown) confirmed this conclusion.

SecA Retains Its Dimeric Structure during Translocation. To investigate whether SecA retains its dimeric structure during translocation, functional heterodimers were constructed consisting of two populations of fluorescently labeled SecA molecules, assuming that dimerization occurs randomly when the Gdn-HCl-denatured SecA mixture is diluted. By fluorescence energy-transfer experiments, qualitative information can be obtained on the association state of the monomers during translocation. In these experiments, the overlap in the fluorescence emission spectrum of coumarin ($\lambda_{max,em} \approx 467$ nm) and the excitation spectrum of fluorescein ($\lambda_{max,ex} \approx 502$ nm) is exploited to obtain information on the molecular proximity of each of the monomers which constitute a dimer (see Figure 5). SecA was labeled with CMmal or Fmal. At slightly alkaline pH, maleimides specifically react with cysteine residues in proteins. SecA contains four cysteinyl residues, i.e., one located near the glycine-rich flexible loop of the ATP binding site (position 98) and three in the carboxy terminus (positions 885, 887, and 896). The latter residues are not essential as this part of the protein can be truncated without noticeable loss of activity (Matsuyama et al., 1990). Reactions with the maleimides were performed in the presence of ATP to protect Cys-98, and quenched by the addition of cysteine. Fluorescently-labeled SecA proteins were denatured with Gdn-HCl, renatured by rapid dilution and dialysis, and reisolated by size-exclusion chromatography. Heterodimers were formed by mixing CMmal- and Fmal-SecA in a 1:1 ratio prior to renaturation. Labeling does not interfere with the formation of the dimeric structure as each of the proteins was found to elute at the position of the native SecA dimer. The probe stoichiometry of the derivatized protein was 2.3 ± 0.3 mol of CMmal and 2.7 ± 0.4 mol of Fmal per SecA monomer. Labeling was further evident from (i) the increase in the fluorescent quantum yield of the CMmal moiety compared to the free label which is essentially nonfluorescent in aqueous solution, (ii) a greatly reduced accessibility of Fmal to anti-fluorescein antibodies when reacted with SecA, and (iii) a comigration of the CMmal (Figure 4, lane 5) and Fmal (not shown) fluorescence with the SecA protein (lane 2) on SDS–PAGE. The derivatized cysteinyl residues are most likely located in the carboxy-terminal domain. A 97-kDa amino-terminal fragment of SecA obtained with V8 proteinase digestion in the presence of ATP (Shinkai et al., 1991) (lane 1) was not labeled with CMmal (lane 4). Similar results were obtained with a 57-kDa amino-terminal proteolytic fragment of SecA, harboring a high level of endogenous ATPase activity, purified from a SecA-overproducing *E. coli* strain (not shown). Preincubation of SecA with *N*-ethylmaleimide prior to incubation with CMmal (lane 3) or Fmal (not shown) completely prevented labeling (lane 6). Compared to identical incubations in the absence of maleimides, fluorescently-labeled and refolded SecA proteins retained their translocation ATPase activity and supported the translocation

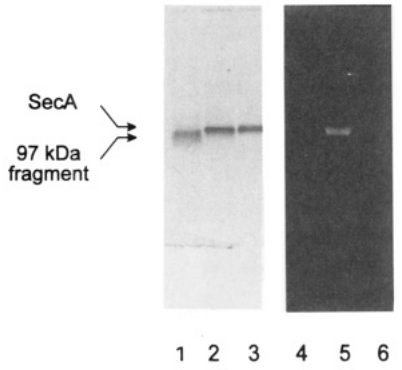

FIGURE 4: Fluorescent labeling of SecA with coumarinylmaleimide. SecA (lanes 2 and 5), a 97-kDa amino-terminal fragment of SecA obtained after V8 proteinase digestion in the presence of ATP (lanes 1 and 4), and SecA pretreated with 1 mM *N*-ethylmaleimide (lanes 3 and 6) were labeled with CMmal as described under Experimental Procedures. Samples were precipitated with trichloroacetic acid, acetone-washed, and subjected to SDS–PAGE. Western blots of the SDS–PAGE (lanes 1–3) were immunostained with polyclonal anti-SecA serum and goat anti-rabbit IgG conjugated with alkaline phosphatase. Fluorographs of the CMmal-labeled protein on SDS–PAGE are shown in lanes 4–6.

of proOmpA in an *in vitro* translocation system (not shown), demonstrating that the labeling procedure does not interfere with the functional renaturation of SecA.

When prepared in a 1:1 ratio, CMmal- and Fmal-labeled heterodimers showed a significant level of energy transfer as indicated by the decrease in CMmal fluorescence and the increase in Fmal fluorescence when excited at 390 nm (Figure 5, compare traces a and c). When renatured CMmal-labeled SecA dimers were mixed with Fmal-labeled (trace b) or nonlabeled (not shown) dimers, only a small decrease in CMmal fluorescence was noted with only a minor increase in Fmal fluorescence. Fmal-labeled dimers showed only a very low level of fluorescence when excited at 390 nm (trace d). The efficiency of energy transfer increased at higher Fmal- to CMmal-labeled SecA ratios. In the experiments described below, a 1:1 ratio of heterodimer formation was used.

Phase modulation fluorometry was used to measure the lifetimes of coumarin fluorescence. The presence of fluorescein in the heterodimer decreases the fluorescence lifetime (τ) of CMmal, increases the amplitude of the shorter decay time (i.e., evident from the decrease in $\sum\tau_i\alpha_i$, which is the sum of the individual lifetimes multiplied by their fractional amplitude; Table I), and decreases the steady-state intensity (Figure 5, trace c), all indicative of resonance energy transfer between these two probes. No attempts were undertaken to calculate the molecular distance between the fluorophores as the SecA protein was derivatized at multiple positions. However, the energy-transfer efficiency observed with the heterodimers suggests that the labeled residues of the individual monomers are in close proximity.

The steady-state energy transfer between CMmal and Fmal was used to follow qualitative changes in the quaternary structure of SecA during translocation. The CMmal–Fmal SecA heterodimer prebound to *E. coli* inner membranes was incubated in the absence or presence of a 2–4-fold excess native SecA, SecB–proOmpA complex, ATP, ATPγS, ADP, and/or succinate to generate a protonmotive force across the cytoplasmic membrane (Table II). No significant changes in the steady-state level of coumarin fluorescence at 457 nm were recorded. Similar results were obtained when the fluorescent SecA heterodimer was prebound to dioleoylphosphatidylcholine/dioleoylphosphatidylglycerol (1:1, mol/mol) liposomes (not shown). Complete loss of energy transfer was

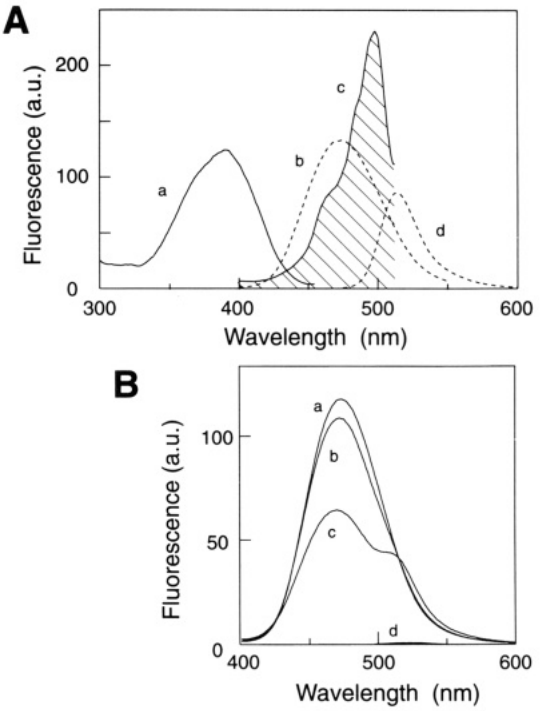

FIGURE 5: Fluorescence energy transfer of CMmal- and Fmal-labeled SecA. (A) Spectra overlap between the fluorescence emission spectrum of CMmal-labeled SecA and the fluorescence excitation spectrum of Fmal-labeled SecA. Fluorescence excitation (a) and emission (b) spectra of CMmal-labeled SecA were recorded with fixed emission and excitation wavelengths of 465 and 387 nm, respectively. Fluorescence excitation (c) and emission (d) spectra of Fmal-labeled SecA were recorded with fixed emission and excitation wavelengths of 514 and 465 nm, respectively. (B) Fluorescence emission of labeled SecA molecules in the range of 400–600 nm when excited at 387 nm. (a) 1:1 mixture of CMmal-labeled and unlabeled SecA homodimers; (b) 1:1 mixture of CMmal- and Fmal-labeled SecA homodimers; (c) heterodimers prepared from a 1:1 mixture of CMmal- and Fmal-labeled SecA; (d) 1:1 mixture of Fmal-labeled and unlabeled SecA homodimers.

Table I: Coumarin Fluorescence Lifetimes for Energy Transfer to SecA-Bound Fluorescein

sample	τ_i (ns)	$\sum\tau_i\alpha_i$ (ns)	efficiency
CMmal-SecA + Fmal-SecA	1.53 ± 0.17		
	6.85 ± 0.37	5.07	
CMmal–Fmal-SecA	0.54 ± 0.22		
	4.97 ± 0.31	3.15	0.38[a]

[a] Efficiency of energy transfer obtained from the steady-state fluorescence was 0.36 ± 0.04 (see Figure 5). $\sum\tau_i\alpha_i$ is the sum of the lifetimes for the individual species. The reduced χ^2 value which indicates the goodness-of-fit was between 1.2 and 2.1.

observed only upon digestion with proteinase K or staphylococcal V8 proteinase. These data suggest that the dimeric structure of SecA is retained during the translocation reaction in the absence and presence of the energy sources required to drive translocation. Moreover, no rapid exchange of the monomers appears to occur once the dimer is formed.

SecA Is Functional as a Dimer. In order to approach the question as to whether SecA functions as a multimer, heterodimers were constructed from the wild type and an inactivated form of the SecA protein. SecA was inactivated by N_3ATP (Lill et al., 1989), denatured with Gdn-HCl, and mixed with denatured wild-type SecA in a 1:4 ratio to increase the probability of formation of heterodimers. This ratio proved to be optimal as it still allowed detection of translocation activity with mixtures of native and inactivated SecA (see below). The renatured protein was reisolated by size-exclusion

Table II: Energy Transfer between Coumarin- and Fluorescein-Labeled Maleimides Bound to SecA during Translocation of ProOmpA

condition	energy-transfer efficiency[a]
(1) CMmal–Fmal-SecA/ *E. coli* inner membranes	0.37
(2) as (1), + unlabeled SecA	0.36
(3) as (2), + SecB–proOmpA	0.36
(4) as (3), + ATP	0.37
(5) as (3), + ADP	0.36
(6) as (3), + ATPγS	0.37
(7) as (4), + succinate	0.37

[a] The energy-transfer efficiency was calculated from the relative increase in the steady-state fluorescence emission of coumarin at 467 nm after proteinase K digestion of the sample. CMmal–Fmal-SecA (200 μg/mL) was incubated with *E. coli* membranes (0.6 mg/mL) in a final volume of 0.5 mL of buffer C [50 mM HEPES–KOH, pH 7.3, 50 mM KCl, and 2 mM $Mg(OAc)_2$]. Membranes were reisolated as described in Van der Wolk et al. (1993) and resuspended in an equal volume of buffer C, and aliquots of 30 μL were incubated in a reaction mixture (200-μL final volume) containing 10 mM creatine phosphate and 10 μg/mL creatine kinase in buffer C. As indicated, reactions were supplemented with 20 μg/mL unlabeled SecA, 10 μg/mL SecB protein, 2 mM ATP (or ADP or ATPγS), 20 mM succinate, and/or 4 μg/mL proOmpA. Proteinase K or V8 protease was added to a final concentration of 200 μg/mL. Coumarin fluorescence was recorded after 10-min incubation at 37 °C using excitation and emission wavelengths of 390 and 467 nm, respectively. Glan–Thompson cross-polarizers positioned at 68° were used in both the excitation path and the emission path to minimize the light scattering introduced by the *E. coli* inner membrane vesicles. Control incubations without Fmal–SecA protein were used for background subtraction. The standard deviation of the mean is ±0.04 with $n = 2$.

chromatography and found to elute mainly at the position of the native dimer. Fluorescent energy-transfer studies with heterodimers formed from CMmal-labeled N_3ATP-inactivated SecA and Fmal-labeled SecA indicate that the N_3ATP-inactivated SecA retains the ability to refold as a dimer (not shown). The renatured heterodimer was inactive for SecA ATPase translocation (Figure 2, lane 4), while a mixture of renatured wild type and N_3ATP-inactivated SecA (lane 2) was active to the extent expected for the amount of renatured wild-type homodimer (compare lanes 2 and 5) present. In another experiment, heterodimers were constructed from the wild-type *B. subtilis* SecA and a site-directed SecA ATPase mutant (Klose et al., 1993) which is inactive for ATPase translocation (Van der Wolk et al., 1993). Also in the latter case, the heterodimer was found to harbor a lower translocation ATPase activity than a mixture of the inactive and active homodimers (not shown). However, for measurements on the translocation activity of the heterodimers, SecA inactivated by N_3ATP was used rather than a site-directed ATPase mutant of the *B. subtilis* SecA. We have previously shown that this ATPase mutant interferes with *in vitro* translocation by occupying functional translocation sites at the membrane surface (Van der Wolk et al., 1993). In contrast, N_3ATP-inactivated SecA does not interfere with *in vitro* translocation, presumably due to its poor binding to membranes (Lill et al., 1989; not shown). We anticipated that the presence of renatured homodimers of the inactive SecA ATPase mutant would interfere with a quantitative analysis of the functionality of the renatured heterodimers. SecA-dependent translocation of proOmpA was measured in *E. coli* everted inner membrane vesicles. In these experiments, conditions were imposed such that SecA was the limiting component of the reaction. Each reaction received the same amount of SecA, *i.e.*, 20 μg of SecA/mL, while saturation required about 80–100 μg of SecA/mL (see Figure 6B). As shown in Figure 6A (compare lanes 1 and 5), most of the translocation activity of SecA was

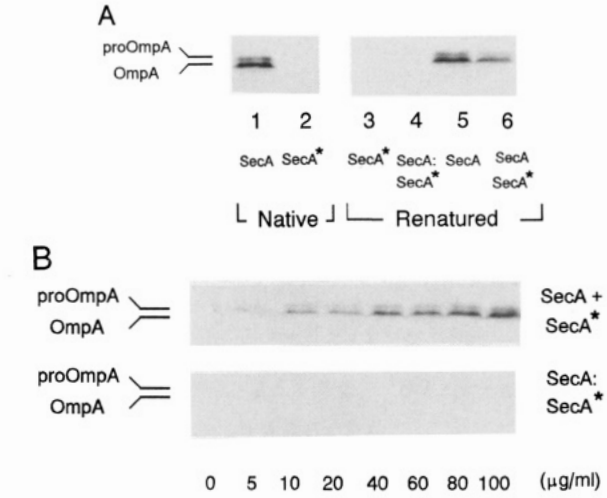

FIGURE 6: SecA-dependent translocation of proOmpA into urea-treated inner membrane vesicles. (A) Translocation reactions were assayed as described under Experimental Procedures in the presence of a 20 μg/mL aliquot of the following SecA species: lane 1, native SecA homodimer; lane 2, N_3ATP-inactivated SecA; lane 3, SecA homodimer renatured from Gdn-HCl-denatured and N_3ATP-inactivated SecA (SecA*); lane 4, SecA heterodimer formed from a 1:4 mixture of Gdn-HCl-denatured native and N_3ATP-inactivated SecA (SecA:SecA*); lane 5, renatured SecA homodimer (SecA); lane 6, a 1:4 mixture prepared from homodimers renatured from Gdn-HCl-denatured native and N_3ATP-inactivated SecA (SecA + SecA*). (B) Translocation reactions were performed with increasing amounts of the SecA heterodimer formed from a 1:4 mixture of Gdn-HCl-denatured native and N_3ATP-inactivated SecA (SecA:SecA*) or a 1:4 mixture prepared from homodimers renatured from Gdn-HCl-denatured native and N_3ATP-inactivated SecA (SecA + SecA*).

recovered after renaturation of the Gdn-HCl-denatured protein, *i.e.*, 70–80%. Renatured N_3ATP-inactivated SecA was completely inactive (lane 3) as was the native SecA when inactivated with N_3ATP (lane 2). In order to determine the residual activity of the heterodimer accurately, a comparison of the translocation activity was made between the heterodimer (lane 4) and a mixture of renatured native and N_3ATP-inactivated SecA (lane 6). In each case, the same amount of native SecA was used. With the heterodimer, however, the native SecA was forced to refold into a heterodimer with the N_3ATP-inactivated SecA. A significant level of translocation was observed with the mixture of homodimers (lane 6) to about 15–20% of that of the renatured native SecA. Translocation was nearly undetectable when the heterodimer was used (lane 4). Similar observations were made when the amount of SecA was varied. Increasing levels of translocation activity could be observed when the amount of the mixture of renatured native and N_3ATP-inactivated SecA was raised (Figure 6B). On the other hand, the heterodimer proved to be completely inactive even at the higher concentrations. These results suggest that the multimeric structure of SecA is required for functionality.

DISCUSSION

The majority of the enzymes present in the cytosol of *E. coli* exist as oligomeric complexes (Goodsell, 1991). Here we determined the functional impact of the dimeric organization of the SecA protein that couples the hydrolysis of ATP to the translocation of precursor proteins across the cytoplasmic membrane. The data suggest that SecA is functional as a multimer, possibly with the dimer as the minimal functional size.

Often the catalytic site of an oligomeric protein is located at the interface between the subunits, allowing subtle motions

between subunits to be used in catalysis and regulation (Goodsell & Olson, 1993). Cooperativity may exist between the subunits, possibly mediated through complete association and dissociation of subunits. Our energy-transfer experiments suggest that such complete association/dissociation reactions do not occur with the SecA protein under conditions of translocation. Our assay does not allow for the detection of subtle motions between the subunits. The monomers appear to have a strong tendency to associate. In this respect, an electron microscopic investigation of small, nondiffracting, bipyramidal SecA crystals indicates that the individual SecA monomers are indeed organized as oligomeric particles, possibly as di- or tetrameric species (Weaver et al., 1992). In solution, the purified SecA appears to exist as a dimer rather than a higher order oligomeric structure (Akita et al., 1991; Cabelli et al., 1991; this paper).

Recently, the crystallographic structure of the nitrogenase iron protein from *Azobacter vinelandii* was solved at 2.9 Å (Georgiadis et al., 1992). This protein forms a complex with the molybdenum iron protein, and couples ATP hydrolysis and electron transfer. The Fe protein is a homodimer, and a single bound ADP molecule was located at the interface region between the two subunits. It was suggested that hydrolysis of ATP and electron transfer are coupled through a conformational change at the interfacial region of the two subunits, implying that the dimeric state is the functional unit. This structure may be highly relevant to other energy-transducing proteins which contain two nucleotide binding sites such as the ABC transporters that couple the hydrolysis of ATP to the translocation of substrates across the membrane (Higgins, 1992). Typically, these transport systems consist of two membrane-integrated domains and two peripheral nucleotide binding domains that face the cytosol. These latter domains are organized either as homodimeric or as heterodimeric species of individual subunits, or fused together to give a single polypeptide. Elimination of either ATP binding domain (Hiles et al., 1987) or inactivation by site-directed mutagenesis (Azzaria et al., 1989; Berkower & Michaelis, 1991) in representatives of this class of transport proteins dramatically reduces transport activity. Similar observations have been made with ArsA, the peripheral subunit of the *E. coli* arsenate extrusion system (Kaur & Rosen, 1991). This may also apply to the SecA homodimer, and provides a functional explanation for the dominant-negative *secA* mutations (Jarosik & Oliver, 1991). Nonfunctional SecA molecules may arise from the formation of multimers consisting of both defective- and wild-type subunits. Intragenic *secA* complementation, as found for certain amino-terminal fragments of SecA (Cabelli et al., 1991; Overhoff et al., 1991) which complement the *secA51* (*Ts*) defect, may follow a similar principle. Furthermore, it has been shown that the SecA ATP binding and precursor protein binding sites can be reconstituted from overlapping fragments of the SecA protein, although translocation activity was not recovered in these studies (Matsuyama et al., 1990; Kimura et al., 1991).

How does the dimeric structure of SecA contribute to the protein transport mechanism? The two high-affinity nucleotide binding sites of the dimer possibly allow for cooperative interactions of sites, to promote the dissociation of bound precursor proteins in a stepwise fashion, where the individual polypeptide domains are released upon the hydrolysis of ATP. We have previously shown that binding of the SecA protein to intermediates in the translocation pathway drives limited translocation (Schiebel et al., 1991). Subsequent binding of ATP to SecA promotes further limited translocation, while hydrolysis of ATP is needed to release the preprotein from its association with SecA (Schiebel et al., 1991) and the membrane (Van der Wolk et al., 1993). The preprotein may be in contact with both SecA subunits simultaneously, or be transferred from one subunit to the other with translocation progress. In the latter case, channeling of the preprotein along the SecA surface or the preprotein binding cavity/domain would allow for a stepwise translocation.

The above models require that each SecA dimer binds a single preprotein. SecA promotes the binding of SecB and the SecB–proOmpA complex to the membrane surface with 85–130 pmol of sites/mg of membrane protein (Hartl et al., 1991). Similar estimates on the number of receptor sites for SecA yield a value of 120–160 pmol/mg of membrane protein. The accuracy of these numbers, however, does not allow definite conclusions as to whether one or two molecules of proOmpA are bound per SecA dimer at the membrane surface (Hartl et al., 1991; unpublished results). Thus far, we have not been able to quantitatively immunoprecipitate preprotein–SecA complexes. Cross-linking approaches are needed to determine the binding stoichiometry. Another question is which domain of the SecA protein is responsible for dimerization. We are approaching this question through the use of chimeric SecA proteins and refolding assays. Evidence from X-ray crystallographic studies will be required to define the active centers and corroborate the proposed reaction mechanism in which residues from both subunits are thought to participate.

ACKNOWLEDGMENT

I thank Wieny Kuiper for technical assistance.

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