JOURNAL OF BACTERIOLOGY, July 1994, p. 4197–4203
0021-9193/94/$04.00+0

Vol. 176, No. 14

In Vivo Studies of the Role of SecA during Protein Export in *Escherichia coli*

SANG-YEARN CHUN† AND LINDA L. RANDALL*

Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164-4660

Received 11 January 1994/Accepted 4 May 1994

SecA is found in *Escherichia coli* both tightly associated with the cytoplasmic membrane where it functions as a translocation ATPase during protein export and free in the cytosol (R. J. Cabelli, K. M. Dolan, L. Qian, and D. B. Oliver, J. Biol. Chem. 266:24420–24427, 1991; D. B. Oliver and J. Beckwith, Cell 30:311–319, 1982; W. Wickner, A. J. M. Driessen, and F.-U. Hartl, Annu. Rev. Biochem. 60:101–124, 1991). Here we show that SecA can be immunoprecipitated from the cytosol in complex with both fully elongated and nascent species of the precursor of maltose-binding protein, an exported, periplasmic protein. In addition, under conditions in which the distribution of SecA between the cytosolic and membrane-bound states changes from that normally observed, the distribution of precursor maltose-binding protein changes in parallel. These results support the idea that cytosolic SecA plays a role in export. With the aim of determining the roles of the multiple binding sites for ATP on SecA, we compared the export defect in a culture of *E. coli* expressing a temperature-sensitive allele of *secA* with the defect in a culture treated with sodium azide. The results indicate that the mutational change and treatment with sodium azide inhibit export by affecting different steps in the cycle of ATP binding and hydrolysis by SecA.

SecA is unique among the factors that mediate the export of protein in *Escherichia coli* in that it is found both tightly associated with the cytoplasmic membrane and free in the cytosol (5, 24). The other integral membrane components of the apparatus, i.e., SecY, SecD, SecE, SecF, band 1, and leader peptidase, are found exclusively membrane associated (3, 4, 12, 30). Work carried out in the laboratory of Wickner using an in vitro translocation system led to the conclusion that the membrane-bound form of SecA binds precursor OmpA (8, 14). However, Mizushima and his colleagues (1) have shown that free SecA forms a complex with the precursor of an OmpF-lipoprotein hybrid polypeptide in the absence of membrane. Thus, it is not clear at what stage of the cycle in vivo SecA encounters the precursor. In the work presented here, we show that SecA exists in the cytoplasm in complex both with fully elongated precursor maltose-binding protein and with nascent precursor species of maltose-binding protein. In addition, under conditions in which the distribution of SecA between the cytosolic and membrane-bound states changes, the distribution of precursor maltose-binding protein changes in parallel. These experiments lend support to the idea that cytosolic SecA participates in export by binding newly synthesized precursor and delivering it to the membrane-associated translocation apparatus.

With the aim of eventual elucidation of the role of ATP binding and hydrolysis in the export cycle, we compared the effects of treating a growing culture with sodium azide and the expression of an allele of SecA that has an altered ATP-binding site.

* Corresponding author. Mailing address: Department of Biochemistry and Biophysics, Washington State University, Pullman, WA 99164-4660. Phone: (509) 335-6398. Fax: (509) 335-9688.

† Present address: Division of Oral Biology, University of California-San Francisco, San Francisco, CA 94143-0512.

MATERIALS AND METHODS

Reagents. [^{35}S]methionine (1,000 Ci/mmol) was purchased from DuPont-New England Nuclear, and lysozyme, methionine, ampicillin, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), Tween 20, and ATP were from Sigma. ImmunoPure-immobilized proteinA was purchased from Pierce, metrizamide was from Nycomed AS Diagnostics, and sodium azide was from Merck. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories. CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) was a gift from DuPont.

Bacterial strains and growth conditions. *E. coli* K-12 strain MC4100 (F$^-$ Δ*lacU169 araD139 relA1 rpsL150 thi f1bB5301 ptsF25*) (6) is the isogenic parent for MM52 [*secA51*(Ts)] (23), IQ85 [*secY24*(Ts) *zhd*::Tn*10 rpsE*] (32), POP3234 [Φ(*lamB-lacZ*)*42-14*(Hyb)] (13), and BAR1091 (11, 29). The Δ*malE312* allele was introduced into MM52, which contains the *secA51* (Ts) mutation, by transduction and screening for the temperature sensitivity of the *secA51*(Ts) allele. Subsequently, plasmid pBAR43 (28), which is derived from pBR322 and contains the *malE* gene, was introduced to construct the strain HB1245. This strain was used without the addition of IPTG (isopropyl-β-D-thiogalactopyranoside). Induction is not required because there is insufficient LacI present to repress expression. Strains were grown at 30°C in M9 minimal salts medium (21) supplemented with 0.4% glycerol and 0.2% maltose except where otherwise indicated.

Labelling. Exponentially growing cultures were labelled at a cell density of 2.5×10^8 cells per ml by the addition of 60 μCi of [^{35}S]methionine per ml and nonradioactive methionine to give a final concentration of 90 nM methionine. For the determination of the subcellular distribution of newly synthesized maltose-binding protein or SecA, the culture was rapidly frozen 15 s after addition of the isotope by transferring the culture to a dry ice-ethanol bath. For the determination of the subcellular distribution of SecA at the steady state, 15 s after the addition of isotope, nonradioactive methionine was added to a final concentration of 100 μM, and growth was continued

for 10 min, at which time the culture was transferred to a dry ice-ethanol bath. For the isolation of native complexes between SecA and precursors, 50 μM of CCCP was added 8 s after the addition of the radioisotope and the culture was immediately transferred to an ice-water bath and swirled for 1 min. After harvesting the culture, all manipulations were carried out with the temperature held below 4°C.

Flotation gradient centrifugation. When the samples were to be analyzed by flotation gradient centrifugation, cells were first converted to spheroplasts to release periplasmic proteins. Preparation of spheroplasts was as described by Thom and Randall (34). The spheroplasts were suspended in 10 mM HEPES (pH 7.6)–5 mM EDTA and disrupted by five pulses (15 s each) of sonication in a cuphorn sonicator (Tekmar) filled with an ice-water mixture. The solution containing the lysed spheroplasts was brought to a density of 1.29 g/ml with metrizamide. One hundred microliters of the sample, containing approximately 3.3×10^8 cells, was applied to the bottom of a Quick-Seal centrifuge tube, and a solution of metrizamide at a density of 1.27 g/ml in 10 mM HEPES (pH 7.6) was layered over the sample. The gradients were centrifuged for 4 h at 338,000 × *g* with a TLV100 rotor in a Beckman TL100 centrifuge. After centrifugation, 13 fractions of 0.15 ml each were withdrawn successively from the top of the gradient, and the index of refraction for each fraction was determined to allow calculation of the density. A portion of each fraction (40 μl) was analyzed by electrophoresis on sodium dodecyl sulfate (SDS)–14% polyacrylamide gels to display the pattern of cellular proteins, and the remainder of each fraction was used for immunoprecipitation of polypeptides related to maltose-binding protein or SecA as described previously (27).

Immunoprecipitation under nondenaturing conditions of a complex between maltose-binding protein and SecA. Immunoprecipitation of native proteins was performed as described previously (16) with minor modifications. Potassium acetate was added to the suspension of lysed spheroplasts to a final concentration of 150 mM, and the samples were centrifuged in a TLA100.1 rotor at 356,160 × *g* for 30 min to separate the cytosolic and membrane components. One hundred microliters of the supernatant fraction was diluted with 1 ml of 150 mM potassium acetate–0.5% Tween 20–10 mM HEPES (pH 7.6). SecA antibody bound to protein A-agarose beads was added, and after incubation for 2 h on ice with occasional mixing, the agarose beads were collected by centrifugation and washed twice with 1 ml of the buffer described above. The polypeptides were eluted from the beads by incubation with sample buffer for 5 min at 100°C and analyzed by SDS–11% polyacrylamide gel electrophoresis as described previously (27).

Purification of SecA. SecA was purified as described previously (7) by column chromatography using the phosphocellulose and Sephacryl S300 columns only.

RESULTS

Subcellular distribution of wild-type SecA and SecA51(Ts) at the steady state. Cabelli et al. (5) showed that, in exponentially growing *E. coli*, approximately half of the SecA is found free in the cytoplasm if it is the normal wild-type species and that the alteration in the export-defective SecA51(Ts) protein results in accumulation of that protein on the membrane. By using a different technique for fractionation of cellular components, we have confirmed the distribution of the two species of SecA and extended the study to correlate it with the distribution of precursor maltose-binding protein. The fractionation method we have used, flotation centrifugation, is based on the fact that, because of the large difference in density between lipids and proteins, it is possible to establish a gradient of density such that when a sample is applied at the bottom of the gradient, only those components that are associated with membranes will float; proteinaceous complexes will remain at the bottom of the gradient. An example of such a fractionation is shown in Fig. 1. Cytoplasmic proteins (in fractions 9 through 13) with densities from 1.29 to 1.43 g/ml are well separated from membrane-associated proteins that reach their equilibrium position at a density of 1.1 to 1.25 g/ml (fractions 1 to 6). The distribution of radiolabelled SecA within the gradient was determined by immunoprecipitation from each fraction in combination with SDS-polyacrylamide gel electrophoresis and autoradiography. SecA in the upper six fractions was considered to be membrane bound; that in the lower seven fractions was considered to be cytosolic. The distribution of SecA between the membrane-bound and free states was determined in several different strains under a variety of conditions. The results are summarized in Table 1 (see Fig. 2 and 4 for examples of data). Greater than 75% of the wild-type SecA expressed from the normal chromosomal

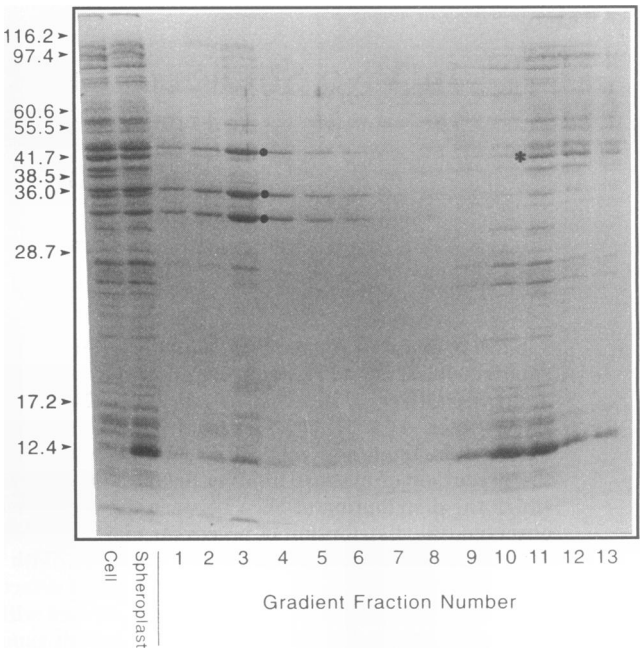

FIG. 1. Separation of membrane-associated and cytosolic proteins by flotation gradient centrifugation. An exponentially growing culture of MC4100 was labelled and prepared for analysis by flotation gradient centrifugation as described in Materials and Methods. After fractionation of the gradient, proteins from one-third of each fraction were precipitated with trichloroacetic acid (final concentration, 5%) to remove metrizamide and analyzed by SDS–14% polyacrylamide gel electrophoresis and the gel was stained with Coomassie brilliant blue. The lane labelled spheroplast contains 20% of the amount of lysate applied to the gradient. The lane labelled cell represents an equal amount of intact cells. The proteins in the gradient fractions are shown in lanes 1 (top of gradient) through 13 (bottom of gradient). The standards for molecular weight were β-galactosidase (116.2 kDa), phosphorylase *b* (97.4 kDa), α-amylase (60.6 kDa), glutamic dehydrogenase (55.5 kDa), actin (41.7 kDa), maltose-binding protein (38.5 kDa), glyceraldehyde-3-P-dehydrogenase (36 kDa), carbonic anhydrase (28.7 kDa), myoglobin (17.2 kDa), and cytochrome *c* (12.4 kDa). The most prominent membrane proteins indicated (●) are LamB, OmpF, and OmpA (from largest to smallest). EF-Tu (*) is taken as a marker for the cytosolic proteins.

TABLE 1. Distribution of SecA between membrane-bound and free states[a]

Strain	*secA* allele	Temp (°C)	At steady state		Newly synthesized	
			% Membrane bound	% Free	% Membrane bound	% Free
MC4100	Wild type	30	22	78	23	77
	Wild type	42	22	78	ND[b]	ND
MM52	*secA51*(Ts)	30	42	58	ND	ND
	secA51(Ts)	42	66	34	38	62
IQ85	Wild type	42	15	85	ND	ND
POP3234	Wild type	30	18	82	ND	ND

[a] The cellular distribution of SecA in various strains is shown. Samples were prepared and processed for fluorography as described in Materials and Methods. The bands of SecA were quantified by densitometry with a Helena Laboratories Quick Scan R&D to calculate the percentages of SecA that were membrane bound and cytoplasmic. Proteins in the upper six fractions and the lower seven fractions were considered to be membrane bound and cytosolic, respectively.

[b] ND, not determined.

copy of the gene (Table 1, strain MC4100) was cytosolic at steady state. This distribution was the same whether the cultures were grown at 30 or 42°C (Table 1 and Fig. 2A). In contrast, as previously shown (5), over 65% of the defective SecA, produced at 42°C in a strain (MM52) expressing the temperature-sensitive allele *secA51*(Ts) was membrane bound at the steady state (Table 1 and Fig. 2B). When cells expressing *secA51*(Ts) are grown at 30°C, the SecA produced is not completely functional but promotes export at a level between that mediated by wild-type SecA and by SecA51(Ts) at 42°C (5). The subcellular distribution of SecA51(Ts) at 30°C was also intermediate between that of wild-type SecA and that of defective SecA (Table 1). The production of SecA is regulated

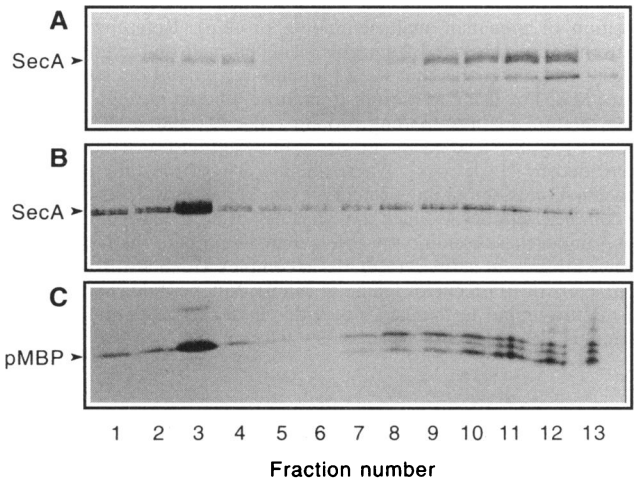

FIG. 2. Distribution of SecA and precursor maltose-binding protein. Cultures of *E. coli* were grown and radiolabelled with [^{35}S]methionine for 15 s and then incubated for 10 min in the presence of an excess of nonradioactive methionine. After incubation for 10 min to allow the distribution of radiolabelled SecA to reach the steady state, the culture was harvested and analyzed as described in Materials and Methods. The positions of SecA and precursor maltose-binding protein (pMBP) are indicated. Fractions 1 through 13 correspond to the gradient from top to bottom. (A) Steady-state distribution of radiolabelled wild-type SecA from MC4100 grown at 30°C; (B) steady-state distribution of radiolabelled SecA51(Ts) from MM52 grown at 42°C; (C) distribution of radiolabelled precursor maltose-binding protein from the same gradient as that analyzed for the distribution of SecA shown in panel B. The bands below the position of precursor in fractions 7 to 13 are degradation products of the precursor as well as a small quantity of the mature species, which is probably present as a result of incomplete release of the periplasm.

in response to the functioning of the export apparatus so that when export is impaired, the cell increases the amount of SecA made (29). Thus, the expression of the defective SecA51(Ts) results in its overproduction. The association of a high proportion of SecA51(Ts) with the membrane can be attributed to the defect in SecA and is not simply a result of its overproduction since strains that have export defects resulting from an alteration in genes other than SecA itself overproduced SecA relative to the normal level by a factor of approximately 10; however, in each strain, less than 20% of the overproduced wild-type SecA was membrane bound. The strains analyzed were those producing either defective SecY (strain IQ85) or a LamB-LacZ hybrid protein (strain POP3234) (Table 1) that blocks export. A complication in concluding that the membrane location of SecA51(Ts) is the result of a defect in SecA is the report that the strain expressing SecA51(Ts) at 42°C accumulates intracytoplasmic membranes (9). However, expression of the LamB-LacZ hybrid protein also results in the production of the abnormal membranes (9), and yet in a strain producing the hybrid protein, greater than 80% of the wild-type SecA is cytoplasmic (strain POP3234) (Table 1). Therefore, we conclude that its accumulation in a membrane-associated state is an intrinsic property of the defective SecA51(Ts) protein. Cabelli et al. (5) reached the same conclusion by using an elegant approach that allowed the simultaneous analysis of the distribution of both functional and defective SecA expressed in the same cells.

SecA and precursor maltose-binding protein accumulate on the membrane in parallel. Even though at the restrictive temperature (42°C) most of the SecA51(Ts) present at the steady state is membrane bound, when the protein is newly synthesized, it is distributed normally. A culture expressing *secA51*(Ts) at 42°C was labelled for 15 s with [^{35}S]methionine and harvested immediately. Of the newly synthesized SecA51 (Ts), only 38% was membrane bound. This is the same distribution as that seen at the steady state when the culture is grown at the permissive temperature (Table 1, 30°C). This observation indicates that the defect in SecA is expressed after it reaches the membrane. Analysis of precursor maltose-binding protein in the same cultures expressing the defective SecA reveals that the distribution of precursor maltose-binding protein parallels that of the SecA. Thirty-seven percent of the newly synthesized precursor is membrane bound, whereas after the 10-min incubation subsequent to the pulse-labelling, 64% of the precursor is found in the membrane fraction (Fig. 2C). The parallel accumulation at the membrane suggests that the precursor binds the membrane via the newly synthesized SecA before it exhibits its defect at the membrane. It is unlikely that the defective SecA51(Ts) present at steady state on the

membrane can bind the precursor directly. If it could, one would expect all of the precursor to be rapidly bound since the SecA is present in at least 10-fold the amount normally on the membrane, whereas immediately after the 15-s labelling period, the precursor is predominantly cytosolic. Two possible explanations for the ability of free but not membrane-associated SecA51(Ts) to bind precursor are as follows: (i) the SecA51(Ts) can bind the precursor in the cytosol but when it associates with the membrane its conformation is changed so that it can no longer bind precursor or (ii) the SecA51(Ts) cannot release the precursor once it is bound so that it arrives at the membrane occupied and cannot exchange its ligand with the radiolabelled precursor.

Isolation of SecA from the cytosol in complex with precursor maltose-binding protein. To demonstrate directly that SecA and precursor maltose-binding protein form a complex in the cytosol, we have immunoprecipitated SecA51(Ts) under nondenaturing conditions from the cytosolic fraction of cells grown at 30°C and labelled for 15 s with [^{35}S]methionine. Analysis by SDS-gel electrophoresis of the polypeptides in the complex revealed many bands, including a candidate for precursor maltose-binding protein. To identify positively the precursor maltose-binding protein and to determine if, in addition to the fully elongated form, nascent species of the precursor were present, we used a technique that generates peptide patterns directly from proteins in a gel after they have been separated according to their size by electrophoresis (15, 17). This method of limited proteolysis generates peptides of defined size from nascent polypeptides which are heterodisperse with respect to molecular weight and thus renders them readily detectable by gel electrophoresis. The gel strip containing the polypeptides isolated in complex with SecA was laid across the top of a second gel, and limited proteolysis was carried out after the polypeptides had been electrophoresed into the stacking gel. After the period of proteolytic digestion, electrophoresis was continued to display the peptides generated. Comparison of the peptide pattern with standard patterns for fully elongated precursor and mature maltose-binding protein (Fig. 3, lanes P and M) as well as with a two-dimensional analysis of pulse-labelled maltose-binding protein isolated by immunoprecipitation via antiserum to maltose-binding protein shows that the peptide characteristic of the precursor amino terminus (Fig. 3, Np) is present not only among the peptides derived from the fully elongated precursor (Fig. 3, p) but also in the region of the gel containing peptides derived from polypeptides of lower molecular weight, i.e., the incomplete nascent chains. In addition, the peptide characteristic of the carboxyl terminus (Fig. 3, C) is also present among the peptides derived from the material migrating at the precursor position (Fig. 3, p) in the first dimension. Incubation of samples with preimmune serum followed by similar analyses showed no peptides characteristic of maltose-binding protein (data not shown). We conclude that SecA forms complexes with both fully elongated and nascent forms of maltose-binding protein in the cytosol.

Effect of sodium azide on SecA activity and subcellular distribution. Azide inhibits protein export through action on SecA (25). In vitro studies have shown that azide inhibits the ATPase activity of SecA. Three of the SecA alleles that render *E. coli* resistant to the action of azide map within the low-affinity binding site for ATP, and the fourth maps to the high-affinity site (22). The mutational changes in all of the *secA* temperature-sensitive alleles are located within the amino-terminal 170 aminoacyl residues of SecA (31). Four of the sites are within the high-affinity ATP-binding site. The change in *secA51*(Ts), the mutation studied, results in the replacement of leucine at position 43 by an alanine. This residue lies outside

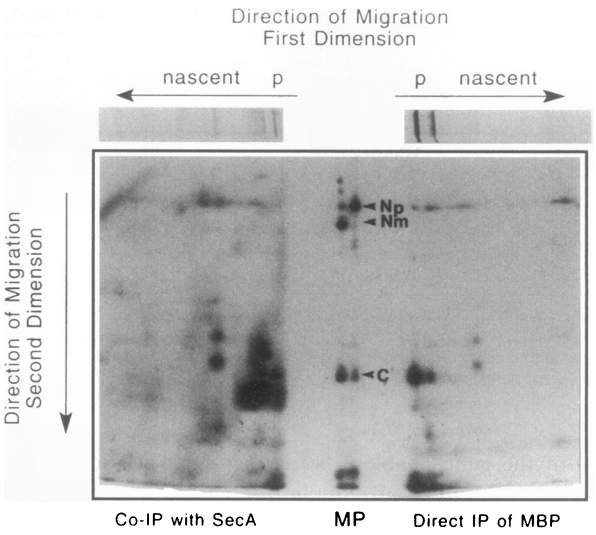

FIG. 3. Analysis of the complex of cytosolic SecA51(Ts) and precursor maltose-binding protein to detect the presence of nascent polypeptides. Strain HB1245 grown at 30°C was labelled as described in Materials and Methods. Cells were converted to spheroplasts and lysed as described for preparation of samples for flotation gradient centrifugation. Membranes were removed by sedimentation with a TLA100.1 rotor in a Beckman TL100 ultracentrifuge. Polypeptides were immunoprecipitated from the supernatant fraction with antibody to SecA under native conditions and resolved by electrophoresis in an SDS–11% polyacrylamide gel (Co-IP with SecA). The lane of the gel containing the polypeptides derived from the complex was placed lengthwise on top of a second SDS–15% polyacrylamide gel and subjected to limited proteolysis (17) and fluorography (second dimension) as described by Josefsson and Randall (15) (p indicates the position of precursor maltose-binding protein). Reference peptide patterns were generated from pulse-labelled maltose-binding protein and its nascent chains that were immunoprecipitated directly (Direct IP of MBP) by using antiserum to maltose-binding protein as well as from mature maltose-binding protein (lane M) and from precursor (lane P) synthesized in an in vitro transcription-translation system (provided by H. deCock). Approximately 3% of the fully elongated precursor maltose-binding protein present in the cytosol was recovered in complex with SecA. Since the efficiency of immunoprecipitation of SecA under these conditions was less than 5%, greater than 60% of the precursor present in the cytosol was in complex with SecA. Np, peptide characteristic of precursor amino terminus; Nm, peptide characteristic of mature amino terminus. C, peptide characteristic of the carboxyl terminus.

the ATP binding motif which comprises residues 102 through 120. Nevertheless, it is likely that azide and all of the alterations of the SecA protein resulting from the aminoacyl substitutions in the *secA* temperature-sensitive alleles exert their effects by altering the binding of ATP or its hydrolysis.

Both the presence of a *secA*(Ts) allele and treatment of the cell with azide result in derepression of the synthesis of SecA (25, 29). Since the SecA51(Ts) protein accumulates on the membrane, one might expect that the regulation of expression is modulated by the relative levels of free and membrane-bound SecA. If this were the case, then treatment with azide should also result in an accumulation of SecA at the membrane. Therefore, we examined the distribution of SecA in azide-treated cells. Both newly synthesized SecA and SecA present at the steady state were distributed normally after treatment of cells with azide (Fig. 4). The rate of processing of precursor maltose-binding protein was drastically decreased, but azide did not exert a complete blockage. The precursor

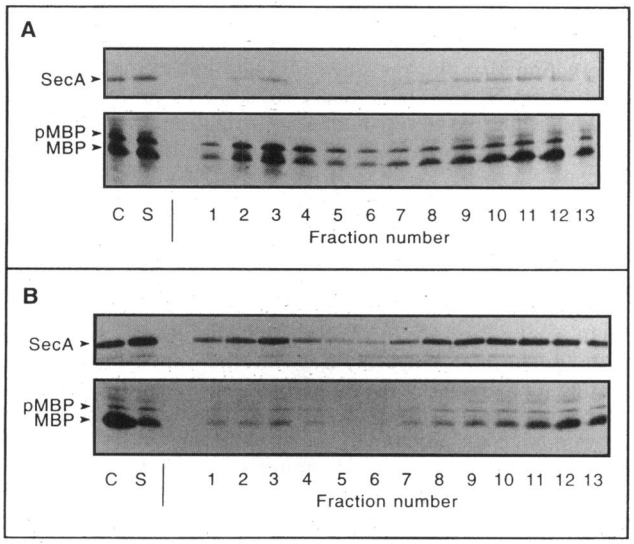

FIG. 4. Effect of sodium azide on SecA. An exponentially growing culture of MC4100 was labelled and processed for flotation gradient centrifugation as described in Materials and Methods and in the legend to Fig. 1, except that 5 min prior to the addition of the isotope, NaN_3 was added to a final concentration of 3 mM. The distribution of SecA and precursor maltose-binding protein (pMBP) is shown immediately after the 15-s labelling period (panel A) and after a further 10-min incubation after addition of an excess of nonradioactive methionine (panel B). Lane C contains a sample of the unfractionated cells, and lane S contains a sample of spheroplasts after removal of the periplasm. Each lane contains a quantity that corresponds to 20% of that applied to each gradient. The lysed spheroplast fraction was applied to the gradient. MBP, maltose-binding protein.

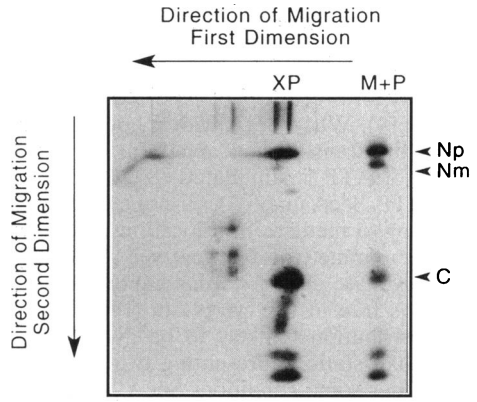

FIG. 5. Analysis of species of maltose-binding protein that are membrane bound after azide treatment. Exponentially growing cultures of MC4100 were treated with sodium azide (final concentration, 3 mM) and radiolabelled with [^{35}S]methionine for 1 min as described previously (25). Lysates were prepared and membrane separated from cytosol by flotation gradient centrifugation. Polypeptides related to maltose-binding protein were immunoprecipitated from the membrane-containing fraction and separated in a first dimension of electrophoresis in an SDS–11% polyacrylamide gel. The lane containing the polypeptides was excised and placed lengthwise on top of a second gel and subjected to limited proteolysis (15). The position of precursor maltose-binding protein (P) and a broad band at the position of mature maltose-binding protein (X) are indicated. The horizontal streak representing the amino-terminal peptide from fully elongated precursor and nascent polypeptides is indicated (Np). Nm, peptide characteristic of the mature amino terminus; C, peptide characteristic of the carboxyl terminus; M + P, reference peptide patterns generated from mature and precursor maltose-binding proteins loaded into the same lane.

labelled in a 15-s pulse (Fig. 4A, lanes C and S) was almost completely processed during a subsequent 10-min incubation (Fig. 4B, lanes C and S). It should be noted that, in the sample taken at 15 s, the material in the position of mature maltose-binding protein is not the authentic mature protein (Fig. 4A). It is a nascent species that carries the leader and is missing approximately 25 amino acids from the carboxyl terminus as demonstrated by the two-dimensional analysis of limited proteolysis of the polypeptides (Fig. 5). The protein that appears in that position during the subsequent 10-min incubation was demonstrated to be the authentic mature protein by the same technique (data not shown). In addition, comparison of the protein pattern from intact cells (Fig. 4B, lane C) with that from spheroplasts (Fig. 4B, lane S) indicates that the majority of the protein is released from spheroplasts as expected for the authentic mature protein. This was not the case with the species present in that position in the pulse-labelled sample (Fig. 4A, compare C and S). A small quantity of mature protein remains with the spheroplasts and is recovered in the soluble fraction. This probably occurs because release of periplasmic protein is not complete. In contrast to these results of azide treatment, the mutational change in SecA51(Ts) effects an almost complete blockage of processing and the precursor remains membrane bound (Fig. 2C).

Binding of ATP to SecA in solution can be demonstrated by monitoring the sensitivity of SecA to degradation by *S. aureus* V8 protease as described by Shinkai et al. (33). When ATP binds to SecA, it renders the amino-terminal, 95-kDa fragment of SecA resistant to the protease. By using this assay, it was shown that the addition of 3 mM sodium azide did not interfere with binding of ATP to the site that results in resistance of SecA to proteolysis (Fig. 6).

DISCUSSION

SecA is unusual among the components of the export apparatus of *E. coli* in that it exists both free in the cytoplasm

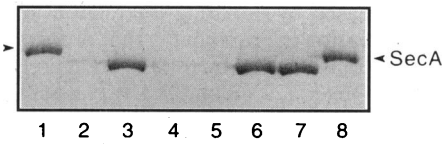

FIG. 6. Effect of azide on the binding of ATP by SecA. Proteolytic digestion of SecA was carried out as described by Shinkai et al. (33). Purified SecA at 4 μM monomer was incubated with no addition (lanes 1, 2, and 8), 2 mM ATP–5 mM $MgSO_4$ (lane 3), 3 mM sodium azide (lane 4), 20 mM sodium azide (lane 5), 2 mM ATP–5 mM $MgSO_4$–3 mM sodium azide (lane 6), or 2 mM ATP–5 mM $MgSO_4$–20 mM sodium azide (lane 7). Five hundred nanograms of protease V8 was added to each of the reaction mixtures shown in lanes 2 to 7, and samples were incubated for 30 min at 37°C. The proteolytic digestion was terminated on ice by the addition of sample buffer containing phenylmethylsulfonyl fluoride prepared as described by Shinkai et al. (33). A portion of each reaction mixture containing 2 μg of SecA was analyzed by SDS–11% polyacrylamide gel electrophoresis and then stained with Coomassie brilliant blue. The positions of SecA and the 95-kDa fragment are indicated. The activity of the purified SecA was confirmed by assessing stimulation of protein translocation in vitro by using urea-treated inner membrane vesicles and precursor maltose-binding protein as described previously (7).

and stably integrated into the membrane (5, 24). The cytoplasmic form is a homodimer with a subunit molecular weight of 102 kDa (2, 31) and has multiple binding sites for ATP (19, 22). SecA has affinity for precursors of exported proteins, for SecB, and for SecY (14). When SecA with a bound precursor is in complex with the translocation apparatus at the membrane, the hydrolysis of ATP is stimulated (8, 20). Coupled to this hydrolysis of ATP, SecA undergoes a cyclic binding and release of the precursor to mediate translocation of the polypeptide through the membrane (for a review, see reference 35).

At the steady state during normal export, between 50 and 75% of SecA is free in the cytoplasm (reference 5 and this work). The distribution is likely to be dynamic, reflecting a cycle, rather than static, representing two different forms of SecA, since under some conditions SecA accumulates on the membrane. These observations make it reasonable to think that the free cytoplasmic SecA plays a role in export. However, it must be remembered, as pointed out by Cabelli et al. (5), that in addition to its function in export, SecA binds its own mRNA to regulate translation (10, 26). In addition, SecA has also been shown to be associated with ribosomes (18), and while this interaction might indicate a role in export of nascent chains, it might also reflect a regulatory role. Studies by Wickner and his colleagues (7, 14) have demonstrated, using an in vitro translocation system, that membrane-bound SecA is capable of carrying out all necessary functions to mediate binding and translocation of the precursor of OmpA. However, this does not necessarily mean that, in vivo, the cytoplasmic form of SecA is not involved. In support of a role for cytoplasmic SecA is the work of Mizushima et al. (1) that demonstrates the formation of a complex between free SecA and precursor. Thus, free SecA can bind precursors; the question of whether this is a function of SecA in vivo remains.

The direct demonstration by coimmunoprecipitation of cytosolic SecA in complex with both fully elongated and nascent forms of precursor maltose-binding protein and the observed parallel accumulation of precursor maltose-binding protein and the defective SecA51(Ts) at the membrane lend strong support to the idea that SecA can interact with precursors in the cytosol and subsequently bind to the membrane translocation site through its affinity for SecY. However, it is known that wild-type SecA is able to bind precursors after it has reached the membrane; thus, it seems that it is not a requirement that the interaction occurs in the cytosol, but perhaps the efficiency of export is increased by initiating interaction in the cytosol and thereby decreasing the probability that precursors will lose competence for translocation by folding or aggregation.

Mitchell and Oliver (22) identified two essential sites on SecA that bind ATP: one site has a high affinity (K_d, 0.13 μM), and the other has a low affinity (K_d, 340 μM). The mutational change in the temperature-sensitive allele, *secA51*(Ts), analyzed in these studies is located in the amino-terminal portion of SecA but outside of the high-affinity ATP-binding site (22). When cultures carrying this allele are grown at the restrictive temperature, there is a tight blockage in the processing of precursor maltose-binding proteins and SecA accumulates on the membrane. Mitchell and Oliver (22) showed that species of SecA having ATP-binding sites altered by site-directed mutagenesis accumulate in a membrane-bound state. In contrast, after treatment of cells with sodium azide, the distribution of SecA between the cytosolic and membrane-bound states remained normal and the rate of processing of precursor maltose-binding protein was drastically decreased but a complete blockage did not occur. The difference between the effects of azide treatment and the mutational change could be explained if sodium azide had its primary effect on the low-affinity binding site and the mutational change affected the high-affinity site. Support for this interpretation comes from the observation that the presence of azide had no effect on the ability of ATP to render SecA resistant to proteolysis. The binding event that results in this conformational change occurs at the high-affinity site since it can be induced by ATP at a concentration of 5 μM (33). However, allelic changes that result in resistance to the action of azide have been mapped to both the low- and high-affinity sites. Thus, it is also possible that the mutational change in *secA51*(Ts) and azide both affect the high-affinity site but do so differently. For example, the mutational change might inhibit the binding of ATP and azide might inhibit hydrolysis, or both the mutational change and azide might affect hydrolysis, with the mutational change causing a complete blockage and azide affecting the rate. It is interesting to note that both the mutational change and azide treatment result in a derepression of synthesis of SecA, whereas only the mutational change causes SecA to accumulate on the membrane. Thus, it is unlikely that the mechanism of regulation of synthesis of SecA involves sensing the subcellular distribution of SecA.

ACKNOWLEDGMENTS

We thank Traci B. Topping and Vijaya J. Khisty for critically reading the manuscript. We are grateful to Traci B. Topping for the purification of SecA and to William Wickner and Hans de Cock for providing antisera to SecA.

This research was supported by Public Health Service grant GM 29798 to L.L.R. from the National Institutes of Health.

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