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Genetics

Azide-resistant mutants of *Escherichia coli* alter the SecA protein, an azide-sensitive component of the protein export machinery

(protein translocation/ATPase inhibitors/sodium azide)

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ABSTRACT ***Escherichia coli azi* mutants, whose growth is resistant to millimolar concentrations of sodium azide, were among the earliest *E. coli* mutants isolated. Genetic complementation, mapping, and DNA sequence analysis now show that these mutations are alleles of the *secA* gene, which is essential for protein export across the *E. coli* plasma membrane. We have found that sodium azide is an extremely rapid and potent inhibitor of protein export *in vivo* and that *azi* mutants are more resistant to such inhibition. Furthermore, SecA-dependent *in vitro* protein translocation and ATPase activities are inhibited by sodium azide, and SecA protein prepared from an *azi* mutant strain is more resistant to such inhibition. These studies point to the utility of specific inhibitors of protein export, such as sodium azide, in facilitating the dissection of the function of individual components of the protein export machinery.**

Export of proteins synthesized in the cytoplasm of cells requires recognition of precursor proteins by soluble components that have been termed chaperones (1–3) and subsequent targeting to the appropriate membrane-bound export machinery. These membrane-localized export machineries catalyze the translocation of precursor proteins across the membrane by an as yet uncharacterized biochemical mechanism, which is driven by ATP hydrolysis (4–6). Export of proteins across the plasma membrane of *Escherichia coli* has been shown to require at least one soluble component [SecB protein (3, 7–9)], one membrane-dissociable component [SecA protein (10–12)], and four integral membrane components [SecD (13), SecE (14), SecF (Jon Beckwith, personal communication), and SecY proteins (15, 16)]. SecA protein has been shown to possess an ATPase activity that is greatly stimulated when SecA interacts with both precursor proteins and inverted plasma membrane vesicles (17). The precise function of SecA ATPase activity in protein translocation and the participation of other ATPases in the overall process need further definition. An inhibitor of SecA function, particularly its ATPase activity, would help to elucidate the role of this activity in the various steps required for precursor protein translocation across the plasma membrane.

E. coli mutants, which grow in the presence of millimolar concentrations of sodium azide, were among the first mutants isolated (18). Previous studies indicated that sodium azide resistance (*azi*) and phenethyl alcohol resistance (*pea*) are probably allelic, since these mutations are tightly linked at 2.5 min on the *E. coli* map and selection for one type of resistance often altered the phenotype of the other marker (19). Since *azi* and *pea* mutations mapped similarly to *secA*, and previous studies indicated that phenethyl alcohol addition to *E. coli* cultures results in a precursor protein processing defect (20), we have investigated the relationship between the *azi* and *secA* genes. We have found that *azi* mutations are alleles of *secA* and that sodium azide is a potent inhibitor of SecA function *in vivo* and *in vitro*.

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MATERIALS AND METHODS

Bacterial Strains, Phages, Plasmids, and Medium. The following *E. coli* K-12 strains resistant to sodium azide were obtained from B. Bachmann at the *E. coli* Genetics Stock Center at Yale University: W208 (F⁻ *azi-4 thr-1 leuB6 lacZ4 supE44 rpsL8 thi-1*), Hfr Hayes (Hfr *azi-7 metB1 relA1 rpsL100*), χ148 (F⁻ *azi-6 ara-14 leuB6 tonA23 lacY1 tsx-67 purE42 galK2 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1*), and SS320 [F⁻ *azi-9 pro-48 lacZ118* (Oc) *lacI22 trpA9705* (Am) *his-84* (Am) *gyrA19 rpsL171 metE70 trpR55*]. DO309 (*azi-4*), DO312 (*azi-7*), DO315 (*azi-6*), and DO318 (*azi-9*) are isogenic MC4100 (F⁻ Δ*lacU169 araD136 relA rpsL thi*) (10) derivatives constructed by P1 transduction. W208.1, χ148.1, DO309.1, and MC4100.2 are *recA1 srl*::Tn*10* derivatives of W208, χ148, DO309, and MC4100, respectively. D110.5 (*azi-7*), D110.6 (*azi-9*), D110.7 (*azi-4*), and D110.8 (*azi-6*) are isogenic derivatives of D110 (W3110 *polA1 endA thyA*) constructed by P1 transduction. BL21.10 (*azi-4 leu*::Tn*10*) is a derivative of BL21(λDE3) (21). CK1801.4 [*secA13* (Am) *supF* (Ts) *trp* (Am) *zch*::Tn*10*] is a derivative of CK1801 [MC4100 Δ(*uncB-uncC*)]; both strains are deleted for the structural genes encoding the F0F1 ATPase. Bacteriophage λ16-2 and λ16-25 (22) and λPR9 (23) have been described. M13mp18 and M13mp19 replicative form DNA was purchased from New England Biolabs. Plasmids pLG552 (24), pMF8, pAR1, pAS2 (25), and pSK6 (26) have been described. Plasmid pLG552-S1 has *secA* sequences 1019–2012 (27) replaced by the chloramphenicol acetyltransferase gene by the method of Winans *et al.* (28). Plasmids pMF8-azi4 and pMF8-azi7 were constructed by *in vivo* integration and excision of pMF8 at the *secA* locus of strains DO309 and DO312, respectively. This was accomplished by constructing derivatives of DO309/pMF8 and DO312/pMF8 that were *polA*⁻ using P1 transduction. Plasmid DNA was prepared from these transductants and used to transform χ148.1; transformants were scored for retention of sodium azide resistance. Plasmid pT7-secA-azi4 was constructed by subcloning a 1230-base-pair (bp) *Bgl* II/*Sna*BI fragment from pMF8-azi4 into pT7-secA DNA (11) lacking this fragment. TYE plates and M63 minimal medium have been described (29).

Mapping and Sequencing of the *azi* Alleles. A set of *secA* mapping cosmids was constructed by subcloning DNA fragments that contained a nested set of 5′ deletions of *secA* contained on M13 replicative form DNA (27) into the cosmid vector p343, a pBR322 derivative plasmid obtained from S. Brown (Frederick Cancer Research Center). MM294 (F⁻ *hsdR endA thi supE44*) containing the cosmids was infected with λY174 [*int6 P6* (Am) *cI857*] and these lysates were used to transduce D110.5, D110.6, D110.7, or D110.8 to ampicillin

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resistance at 30°C. Individual transductants were picked and scored for sensitivity to sodium azide by streaking on TYE plates supplemented with 100 μg of ampicillin per ml and lacking or containing 2 mM sodium azide after overnight incubation at 37°C. For DNA sequence analysis of the *azi4* mutation, three M13 clones were made and sequenced. A 612-bp *Nru* I/*Pst* I DNA fragment from pMF8-azi4 DNA was cloned into both M13mp18 and M13mp19 replicative form DNAs that had been cleaved with *Sma* I and *Pst* I. A 444-bp *Kpn* I/*Pst* I DNA fragment from pMF8-azi4 DNA was cloned into M13mp19 replicative form DNA that had been restricted similarly. For DNA sequence analysis of the *azi7* mutation, a 528-bp *Eco*RI/*Sal* I DNA fragment from pMF8-azi7 DNA was cloned into M13mp19 that had been restricted similarly. M13 clones were transfected and grown on MV1304 [Δ(*srl-recA*]306::Tn*10 thi rpsL endA sbcB15 hsdR4* Δ(*lac-proA*)/F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{Q} *lacZ*ΔM15], and single-stranded DNA was prepared and subjected to DNA sequence analysis (27) by using primer 1212 (New England Biolabs) and a Sequenase kit (United States Biochemical) according to the manufacturer's instructions.

Preparation of SecA and proOmpA Proteins, S300 Extracts, Membrane Vesicles, and Conditions for *in Vitro* Protein Translocation and ATPase Assays. SecA protein sensitive or resistant to sodium azide inhibition (SecA or Azi4-SecA) was purified from BL21(λDE3)/pT7secA or BL21.10/pT7secA-azi4, respectively, as described (11). ^{3}H-labeled proOmpA (^{3}H-proOmpA) was prepared from W3110/pTRC-Omp9 by a method similar to that of Crooke *et al.* (30) except that M9 medium was supplemented with 125 μg of 19 amino acids (omitting leucine) per ml, and [^{3}H]leucine (53 Ci/mmol; 1 Ci = 37 GBq; ICN) was added to a final concentration of 25 μCi/ml 5 min after induction and cells were harvested 145 min later. SecA-depleted S300 extracts were prepared from BL15.5, and inverted inner membrane vesicles were prepared from CK1801 and CK1801.4 as described (11). Urea-treated inner membrane vesicles for ATPase assays were prepared as follows: membranes derived from CK1801 were incubated for 60 min at 4°C with 5 M urea in 42 mM sodium phosphate (pH 7.2), reisolated by sedimentation for 20 min at 90,000 rpm in a TLA-100.2 rotor at 4°C, and resuspended in 10 mM Tris·OAc, pH 7.6/50 mM KCl. *In vitro* protein translocation assays were carried out by a modification of the procedure of Cabelli *et al.* (11) using purified ^{3}H-proOmpA instead of a posttranslational supernatant as a source of precursor protein. The hydrolysis of [γ-^{32}P]ATP (10 Ci/mmol; DuPont/NEN) was assayed as described by Lill *et al.* (17).

RESULTS

***azi* Mutations Are Alleles of *secA*.** To locate the gene conferring resistance to sodium azide, transducing phages and plasmids that collectively span a 10-gene region, which would include the *azi* gene, were introduced into four azide-resistant strains—W208, Hfr Hayes, χ148, and SS320 or *recA* derivatives of two of these strains, W208.1 and χ148.1—and scored for growth on medium containing sodium azide. The results shown in Fig. 1 demonstrate clearly that *azi* is recessive to *azi*$^+$ and is allelic to *secA*, since only phages or plasmids that contain a functional copy of the *secA* gene conferred an azide-sensitive phenotype. A plasmid containing a *secA51* (Ts) allele, pAS2, displayed a temperature-sensitive, azide-sensitive phenotype.

Location of the *azi* mutations within the *secA* gene by direct DNA sequence analysis would have been problematic, not only because of the large size of this gene [2703 bp (27)], but also because of the potential for additional *secA* mutations induced during the individual histories of these unrelated strains. Therefore, we devised an *in vivo* mapping strategy that utilized a set of cosmids containing a nested set of 5′ deletions in *secA* to locate the relevant mutations. These cosmids were integrated at the *secA* locus of a given *azi polA* double mutant, defective for plasmid replication, by cosmid transduction and selection for ampicillin resistance. As shown in Fig. 2, only if the crossover event was 5′ of the chromosomally located *azi* mutation (crossover 1) would the resulting transductants undergo a change to an azide-sensitive phenotype. By scoring for the loss of this 5′ crossover event with increasingly larger 5′ *secA* deletions, the four *azi* mutations were located tentatively to two regions: *azi6* and *azi7* mapped to a 528-bp region between the *Eco*RI and *Sal* I sites in *secA*, and *azi4* and *azi9* mapped to a 612-bp region between the *Nru* I and the first *Pst* I sites in *secA*. To more precisely determine the location of two representative *azi* alleles, *azi4* and *azi7*, we devised an *in vitro* mapping procedure. To this end, we constructed derivatives of a plasmid containing the gene X-*secA* operon, pMF8 (25), which carry either the *azi4* or *azi7* allele, pMF8-azi4 or pMF8-azi7, respectively (see *Materials and Methods* for details). We were able to readily map the position of *azi4* and *azi7* by simply exchanging various restriction enzyme fragments between pMF8 and the corresponding pMF8-azi plasmids and scoring the phenotype of the resulting hybrid plasmids in an *azi recA* mutant strain. The two hybrid plasmids made by exchanging 2.1-kilobase (kb) *Mlu* I fragments between pMF8 and pMF8-azi7 indicated that the *azi7* mutation was located 5′ of the *Mlu* I site in *secA*. A similar

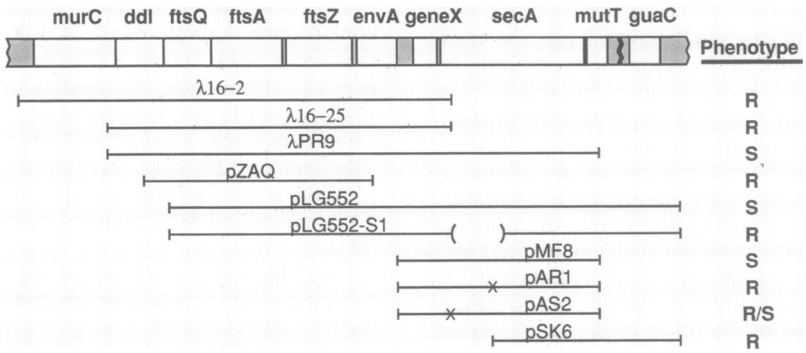

FIG. 1. Identification of the gene responsible for sodium azide resistance. The relevant genes and intergenic regions are shown at the top as open and shaded regions, respectively. The genetic content of each transducing phage or plasmid is indicated by a horizontal line and its phenotype when contained in an *azi* mutant strain is given at the right. A break in the horizontal line indicates the extent of a deletion mutation; x indicates the positions of point mutations. S or R, azide-sensitive or azide-resistant phenotype; R/S, conditional phenotype. The efficiency of plating of each plasmid contained in the W208.1 background is defined as the number of colonies on TYE plates containing 2 mM sodium azide and the appropriate selective antibiotic divided by the number of colonies on TYE plates containing the appropriate selective antibiotic and was as follows: pLG552, 2.1×10^{-5}; pLG552-S1, 0.83; pMF8, $<2 \times 10^{-7}$; pAR1, 1.4; pAS3 30°C, $<2 \times 10^{-7}$; pAS3 37°C, 0.19; pAS3 42°C, 0.75; pBR322, 1.2.

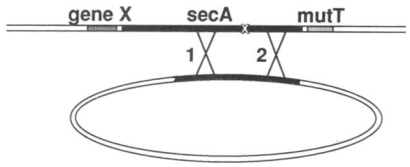

FIG. 2. Genetic mapping of the *azi* alleles within the *secA* gene. The diagram depicts the integration of a cosmid containing a 5′ *secA* deletion into the *secA* chromosomal locus. A single crossover event (1) results in an azide-sensitive phenotype, while a similar event (2) results in an azide-resistant phenotype.

exchange of a 2.5-kb *Kpn* I fragment between pMF8 and pMF8-azi4 indicated that the *azi4* mutation was located 3′ of the *Kpn* I site in *secA*.

The DNA sequence of these two *azi* mutations was determined by subcloning the appropriate restriction fragments from the pMF8-azi plasmids into bacteriophage M13mp vectors and sequencing the relevant region (see *Materials and Methods* for details). The *azi7 secA* DNA sequence between the *Eco*RI and *Mlu* I sites revealed a single alteration from the wild-type sequence, which was an A to T transversion at nucleotide position 1356 (27), corresponding to an asparagine to tyrosine change at amino acid residue 179 of SecA protein. It is noteworthy that this alteration is very near one of the predicted ATP-binding sites (31) found in SecA protein at amino acid residues 160–167 (32), as well as two *secA* (Ts) alleles mapping at amino acid residues 169 and 170 (27). The *azi4 secA* DNA sequence between the *Nru* I and *Pst* I sites revealed a single alteration from the wild-type sequence, which was a T to A transversion at nucleotide position 2755, corresponding to a leucine to glutamine change at amino acid residue 645 of SecA protein.

Sodium Azide Inhibits SecA-Dependent Protein Export *in Vivo*. We determined the effect of sodium azide concentration on *in vivo* protein export in isogenic wild-type and *azi* mutant strains. Bacterial cultures were pretreated with different concentrations of sodium azide for 5 min, pulse-labeled for 1 min with a mixture of [^{35}S]methionine and [^{35}S]cysteine, and the extent of precursor protein processing was monitored for the periplasmic maltose-binding protein (MBP) and the outer membrane protein OmpA. Fig. 3 shows that the wild-type strain, MC4100, displayed a severe protein export defect in the presence of 1 mM sodium azide as evidenced by the accumulation of precursors to MBP and OmpA. In contrast, the four *azi* mutant strains displayed normal protein export profiles under these conditions. Protein export in MC4100 was nearly abolished at 3 mM sodium azide, while substantial levels of protein export continued in the *azi* mutants, particularly DO309 and DO312. This pattern is identical to that observed for growth of these strains in sodium azide. MC4100 did not form colonies on TYE plates containing 1 mM sodium azide. DO315 and DO318 did not form colonies above 3 mM and 2 mM sodium azide, respectively, while DO309 and DO312 formed colonies even at 4 mM sodium azide. Fig. 3 also shows that SecA synthesis was elevated 10- to 20-fold at sodium azide concentrations that inhibited protein export, similar to the derepression of *secA* seen with other protein export blocks (33).

To determine how quickly sodium azide inhibited protein export, bacterial cultures were pulse-labeled with radioactive amino acids after growth in sodium azide for various lengths of time. Fig. 4 shows that the inhibitory effect of sodium azide on protein export was extremely rapid, occurring within the first minute after addition of this compound. Derepression of SecA synthesis was delayed, occurring 1–3 min after sodium azide addition. Nearly identical kinetic profiles that differed only in the extent of the protein export block were obtained with MC4100 and DO309, the latter strain being grown under

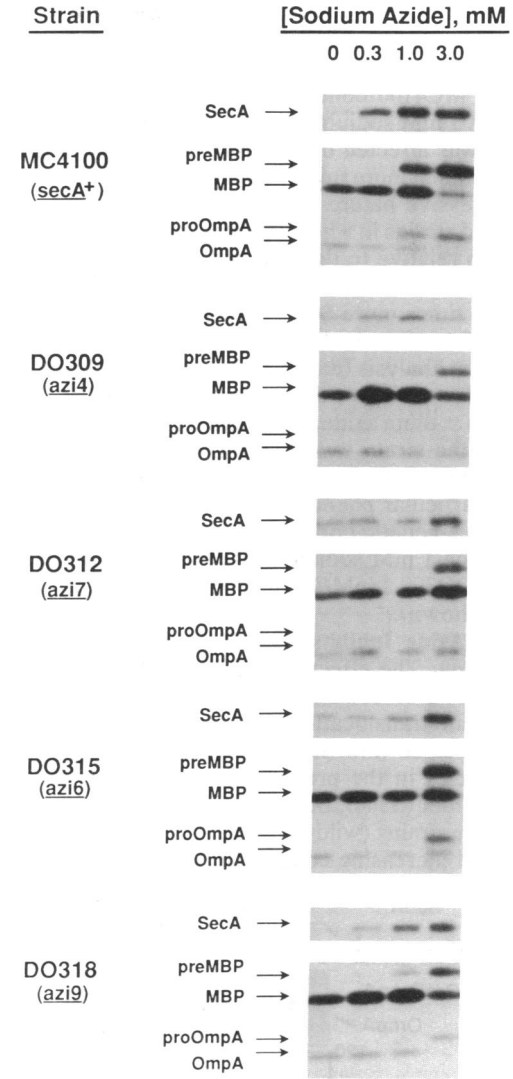

FIG. 3. Sodium azide inhibits protein export *in vivo*. Bacterial cultures were grown in M63 minimal medium containing 0.4% maltose and 25 μg of 17 amino acids per ml at 37°C to midlogarithmic phase when the culture was divided into four aliquots and sodium azide was added to the concentration indicated. Five minutes later, 1-ml aliquots of the cultures were pulse-labeled with 20 μCi of Tran^{35}S-label (1180 Ci/mmol; ICN) for 1 min, followed by the addition of an equal volume of ice-cold 10% trichloroacetic acid. Samples were prepared for immunoprecipitation and subjected to electrophoresis on polyacrylamide gels and autoradiography as described (10). The positions of SecA, MBP, and OmpA proteins, and their respective precursors (preMBP and proOmpA) are indicated.

completely viable conditions (3 mM sodium azide) during this experiment. Other essential cellular processes that were

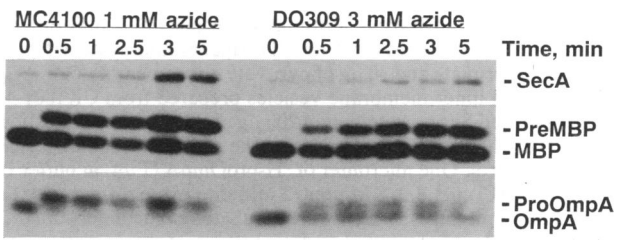

FIG. 4. Kinetics of sodium azide inhibition of protein export. Bacterial cultures were grown as described in Fig. 3 legend. Sodium azide was added to each culture at the concentration indicated and aliquots of each culture were removed at the times indicated and labeled and processed as described in Fig. 3 legend.

monitored were less affected by these treatments. For example, protein synthesis in both strains was inhibited by <50% during the first 30 min of treatment (data not shown). Growth of MC4100 continued for three generations in the presence of 1 mM sodium azide prior to arrest, while growth of DO309 was affected only slightly by this treatment.

To understand more fully the genetic and biochemical basis for the recessive nature of *azi* alleles, we constructed two merodiploid strains in which the gene dosage of *azi* was either low or high relative to the wild-type *secA*$^+$ gene. DO309.1/pMF8 (*azi4*/*azi*$^+$) and MC4100.2/pMF8-azi4 (*azi*$^+$/*azi4*) produced ≈10 times more SecA protein from the plasmid gene copy than from the chromosomal gene copy as indicated by Western blot analysis (data not shown). We found that when the *azi* allele was in low dosage, the growth of the strain was sensitive to sodium azide, and when it was in high dosage, the growth of the strain was resistant to this compound. This indicates that *azi* alleles are in fact codominant to wild type, with the particular phenotype being dependent on the gene dosage. This genetic conclusion extended to *in vivo* protein export, since 1 mM sodium azide blocked MBP and OmpA export in DO309.1/pMF8 but not in MC4100.2/pMF8-azi4 (data not shown).

Sodium Azide Inhibits SecA-Dependent *in Vitro* Protein Translocation. The effect of sodium azide on SecA protein function was assessed directly by using a SecA-dependent *in vitro* protein translocation system. Purified ^{3}H-proOmpA was translocated into SecA-depleted, inverted inner membrane vesicles in the presence of a SecA-depleted soluble protein extract (S300), SecA protein purified from *secA*$^+$ or *azi4* mutant strains (wild-type SecA and Azi4-SecA, respectively), and increasing concentrations of sodium azide. The

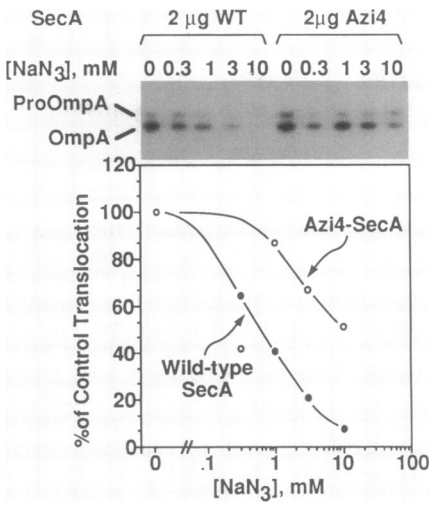

FIG. 5. Titration of SecA and Azi4-SecA-dependent translocation activities against increasing sodium azide concentrations. Reaction mixtures (100 μl) contained 50 mM Tris·OAc (pH 7.6), 21 mM NH_4Cl, 22.5 mM KCl, 5.5 mM MgOAc, 1 mM spermidine, 8 mM putrescine, 250 μg of bovine serum albumin per ml, 2 mM dithiothreitol, 1 mM ATP, 20 μM GTP, an ATP regenerating system (5 mM phospho*enol*pyruvate and 30 μg of pyruvate kinase per ml), 0.06 A_{280} unit of inverted inner membrane vesicles prepared from CK1801.4, 2.5 μg of purified SecA protein, and 200 μg of S300 extract. Reaction mixtures were held on ice for 5 min after the addition of sodium azide or a water control. One microliter of ^{3}H-proOmpA (1 μg) in buffer U (50 mM Tris·HCl, pH 8.0/8 M urea/2 mM dithiothreitol) was added to the reaction mixtures, which were incubated immediately at 40°C for 15 min. Protease treatment and processing of the samples were as described (11). % of Control Translocation = (amount of translocation obtained at a given sodium azide concentration)/(amount of translocation obtained in the absence of sodium azide) × 100 for translocation reactions done with either wild-type SecA (WT) or Azi4-SecA (Azi4) proteins.

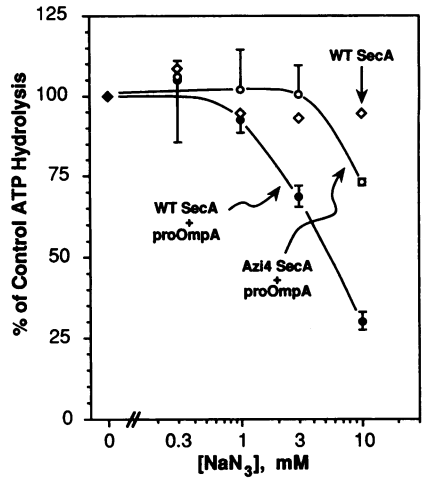

FIG. 6. Titration of SecA and Azi4-SecA-dependent translocation ATPase activities against increasing sodium azide concentrations. All reaction mixtures (25 μl) contained 50 mM Tris·OAc (pH 7.6), 21 mM NH_4Cl, 22.5 mM KCl, 2.5 mM MgOAc, 1 mM spermidine, 8 mM putrescine, 250 μg of bovine serum albumin per ml, 2 mM dithiothreitol, 2.5 mM ATP, ≈300,000 cpm of [γ-^{32}P]ATP (10 Ci/mmol), 90 μg of urea-treated inverted inner membrane vesicles per ml prepared from CK1801, 40 μg of SecB protein per ml, 60 μg of SecA protein per ml, and 40 μg of proOmpA per ml as indicated. Reaction mixtures were held on ice for 5 min after addition of sodium azide, followed by incubation at 38°C for 6 min. Precursor-independent SecA ATPase activity (◇) = ATPase activity for SecA and membrane vesicles − ATPase activity for membrane vesicles alone. Translocation ATPase activities for wild-type SecA protein (●) and Azi4-SecA protein (○) = (ATPase activity for SecA, proOmpA, and membrane vesicles − ATPase activity for membrane vesicles alone) − [(ATPase activity for SecA and membrane vesicles − ATPase activity for membrane vesicles alone) + (ATPase activity for proOmpA and membrane vesicles − ATPase activity for membrane vesicles alone)]. Data are expressed as percentage of ATPase activity obtained in the absence of sodium azide and are an average of two experiments.

results shown in Fig. 5 are completely consistent with sodium azide directly inhibiting SecA-dependent protein translocation activity, since the Azi4-SecA-dependent translocation activity was much more resistant to inhibition by sodium azide than that of wild-type SecA. We note that the sodium azide concentrations required to inhibit SecA-dependent proOmpA translocation activity by 50% (between 0.3 and 1 mM) and Azi4-SecA-dependent proOmpA translocation activity by 33% (3 mM) are similar to what was observed *in vivo*.

Sodium Azide Inhibits SecA ATPase Activity. SecA is known to possess an ATPase activity that is stimulated upon binding to precursor proteins and inverted inner membrane vesicles and has been termed translocation ATPase (17). Since sodium azide is known to inhibit certain classes of ATPases, we have tested whether it inhibits translocation ATPase activity by titrating this activity for wild-type and Azi4-SecA proteins against increasing concentrations of sodium azide. As shown in Fig. 6, translocation ATPase activity catalyzed by wild-type SecA protein was more sensitive to inhibition by sodium azide concentrations above 1 mM than the comparable activity catalyzed by Azi4-SecA protein: 50% inhibition occurred at 5 mM sodium azide for the former activity, while only 27% inhibition occurred at 10 mM sodium azide for the latter activity. The precursor-independent SecA ATPase activity was unaffected by sodium azide at all concentrations tested.

DISCUSSION

A classical genetic approach has led us to the discovery that *azi* mutations, which confer resistance of growth to sodium

azide and were among the first mutations isolated in *E. coli* (18), are alleles of the *secA* gene, whose product catalyzes protein export (10, 11). This conclusion was based on the complementation, genetic mapping, and DNA sequencing results presented. Furthermore, we showed that concentrations of sodium azide that inhibited cell growth also inhibited protein export. Although the proton ATPase will be inhibited under these conditions, the fact that protein export in *azi* mutant strains was substantially more resistant to inhibition by sodium azide than the isogenic wild-type strain argues that sodium azide blocks protein export by direct inhibition of the SecA protein. This conclusion was confirmed more directly by showing that SecA-dependent *in vitro* protein translocation into inner membrane vesicles lacking the proton ATPase was inhibited by sodium azide at similar concentrations to the *in vivo* experiments, and that the Azi4-SecA protein displayed a higher level of resistance to such inhibition. Taken together, these findings demonstrate clearly that SecA protein is the major essential, cellular target inhibited by sodium azide at the concentrations used in these studies. Although other cellular processes are clearly affected by sodium azide treatment, they must be either essential components of redundant biochemical pathways or nonessential for cell growth and viability.

Antibiotic and small molecule inhibitors of the protein export apparatus could prove to be very valuable in elucidating the mechanistic details of the protein export process. In this regard, sodium azide should prove to be valuable in the study of both SecA function and regulation *in vivo* and *in vitro*. Since the available conditional-lethal *secA* mutants show substantial delays in the appearance of protein export defects (10, 32), the very rapid inhibition of SecA function *in vivo* produced by sodium azide should allow a better assessment of the SecA dependence of export of different noncytoplasmic proteins, as well as the *in vivo* ordering of the protein export pathway by established genetic methodologies (34). Furthermore, the relatively short delay observed between sodium azide inhibition of protein export and derepression of SecA synthesis places constraints on the potential models of *secA* regulation by protein export proficiency. The inhibition of SecA-dependent *in vitro* protein translocation activity and translocation ATPase activity by sodium azide should allow a further biochemical dissection of the role of SecA protein and its ATPase function in catalyzing precursor protein translocation across the plasma membrane. The existence of *azi* alleles of *secA* and Azi-SecA protein provide ideal controls for the validity of all of these different experimental approaches.

We note that translocation ATPase activity was significantly less sensitive to inhibition by sodium azide than *in vivo* growth and protein export and *in vitro* protein translocation, whose concentration dependencies for inhibition by sodium azide were similar. This discrepancy may be due to the nature of the coupling between ATP hydrolysis and protein translocation or, alternatively, to a lack of azide sensitivity (and coupling to protein translocation) of a significant portion of the translocation ATPase activity described here. In this regard, it has been reported that the quantity of ATP hydrolyzed is far in excess of what might reasonably be expected for the amount of precursor protein translocated in the *in vitro* system described here and elsewhere (35).

The inhibition of translocation ATPase activity but not precursor-independent SecA ATPase activity by sodium azide is of interest, since the well-characterized F1 ATPase displays a somewhat similar phenomenon. Sodium azide does not inhibit the low activity state of F1 ATPase but rather the high activity state, which requires an allosteric activation of the enzyme by ATP binding (a phenomenon known as multisite activation) (36). With this paradigm, it is not clear whether the *azi* mutations result in a loss of the sodium azide-binding site on SecA protein or whether they might result in a conformationally altered form of SecA protein that is more easily activated by interactions with precursor proteins and membrane-associated export machinery components (35). We have yet to measure the binding of sodium azide to SecA protein and to determine whether this binding results in a covalent modification. Since SecA protein also possesses multiple ATP-binding sites (17), has states corresponding to low and high levels of ATPase activity (17, 35), and appears to associate with integral membrane components to couple its ATPase activity to molecular movement through membranes, future studies should reveal whether these two enzymes share certain mechanistic features.

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