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*.

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 rpsL108 thi-1), Hfr Hayes (Hfr azi-7 metB1 relA1 rpsL100), χ148 (F− azi-6 ara-14 leuB6 tonA23 lacY1 tsx-67 purE42 galK2 trpE38 rfd11 rpsL109 ydx-5 mut-1 thi-1), and SS320 (F− azi-9 pro-48 lacZ118 (Oc) lacY122 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− ΔlacU169 araD136 relA1 rpsL1 thi-1) (10) derivatives constructed by P1 transduction. W208.1, χ148.1, DO309.1, and MC4100.2 are recA1 srl::Tn10 derivatives of W208, χ148, DO309, and MC4100, respectively. D110.6 (azi-7), D110.7 (azi-6), and D110.8 (azi-5) are isogenic derivatives of D110 (W3110 polA1 endA thyA) constructed by P1 transduction. BL21.10 (azi-4 leuc::Tn10) is a derivative of BL21(ΔADE3) (21). CK1801.4 (secA13 (Am) supF (Ts) trp (Am) och::Tn10) is a derivative of CK1801 [MC4100 Δ(uncB-uncC)] (22); both strains are deleted for the structural genes encoding the F0F1 ATPase. Bacteriophage A16-2 and A16-25 (22) and APR9 (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 cloning the 1230-base-pair (bp) Bgl II/SnaB1 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 [infH P6 (Am) cl857] and these lysates were used to transduce D110.5, D110.6, D110.7, or D110.8 to ampicillin

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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, x148, and SS320 or recA derivatives of two of these strains, W208.1 and x148.1—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 secA (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: azi and azi7 mapped to a 528-bp region between the EcoRI and Sal I sites in secA, and azi6 and azi7 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 vivo mapping procedure. To this end, we constructed derivatives of a plasmid containing the gene X-secA operon, pMF8 (25), which carry either the azi 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 x 10^-2; pLG552-S1, 0.83; pMF8, <2 x 10^-2; pAR1, 1.4; pAS3 30°C, <2 x 10^-2; pAS3 37°C, 0.19; pAS3 42°C, 0.75; pBR322, 1.2.
exchange of a 2.5-kb \textit{pkn} I fragment between \textit{pMF8} and \textit{pMF8-azi4} indicated that the \textit{azi} mutation was located 3' of the \textit{pkn} I site in \textit{secA}.

The DNA sequence of these two \textit{azi} mutations was determined by subcloning the appropriate restriction fragments from the \textit{pMF8-azi} plasmids into bacteriophage M13mp vectors and sequencing the relevant region (see Materials and Methods for details). The \textit{azi7 secA} DNA sequence between the \textit{EcoRI} and \textit{MluI} 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 \textit{secA} (T's) alleles mapping at amino acid residues 169 and 170 (27). The \textit{azi4 secA} DNA sequence between the \textit{NruI} and \textit{PstI} 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 \textit{in vivo} protein export in isogenic wild-type and \textit{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 \textit{[^{35}S]}methionine and \textit{[^{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 \textit{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 \textit{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 \textit{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 completely viable conditions (3 mM sodium azide) during this experiment. Other essential cellular processes that were
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, whereas 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 Vivo 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 [3H]-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 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 Azi-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 *vitro* and *in vivo*. 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 *vitro* 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 interaction with precursor proteins and membranes, and possibly related 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 covariant 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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