The SecA Inhibitor, Azide, Reversibly Blocks the Translocation of a Subset of Proteins across the Chloroplast Thylakoid Membrane*

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The presence of secA and secY gene homologues in the plastid genomes of red algae and cyanophytes has raised the possibility that the products of these genes are involved in protein translocation across the thylakoid membrane. Bacterial SecA proteins are effectively inhibited by azide, and we have tested the effects of this compound on the transport of luminal proteins across the thylakoid membrane in pea chloroplasts. Recent studies have shown that luminal proteins are transported by two different mechanisms, one dependent on the thylakoidal ΔpH and the other requiring the presence of a stromal protein factor and ATP. In this report we show that azide inhibits the transport across the thylakoid membrane of the latter group of proteins, which includes plastocyanin and the luminal 33-kDa protein of photosystem II; translocation of proteins by the ΔpH-dependent pathway is unaffected. Following import into isolated chloroplasts in the presence of azide, a substantial proportion of plastocyanin and the 33-kDa protein is found as the stromal intermediate form; the proportion increases with lower ATP concentrations, suggesting that azide and ATP may compete for a single site. The presence of azide completely inhibits the import of the 33-kDa protein by isolated thylakoids, but import is restored if the azide is removed from the stromal extract or thylakoids, prior to the import incubation. The data thus indicate that azide reversibly inhibits the transport of a subset of proteins across the thylakoid membrane, consistent with the involvement of a SecA homolog. The results also indicate that azide is potentially a valuable tool for the future assignment of novel luminal proteins to one of the thylakoidal protein transport mechanisms.

The development of the photosynthetic apparatus in higher plant chloroplasts requires the assembly of numerous intrinsic and extrinsic proteins into the various protein-pigment complexes in the thylakoid membrane. A proportion of these proteins are encoded by the plastid genome, whereas the remainder are transported into the chloroplast after synthesis in the cytosol. Considerable attention has centered on the latter group of proteins, with the aim of determining how they are specifically directed either into, or in some cases across, the thylakoid membrane. Hydrophobic, integral thylakoid proteins are generally imported by means of transient, stroma-targeting presequences, after which information present in the mature proteins specifies integration into the membrane (Cline, 1986; Chitnis et al., 1987; Cai et al., 1993). In contrast, hydrophilic, extrinsic thylakoid lumen proteins, such as plastocyanin (PC),1 the 33-, 23-, and 16-kDa proteins of the evolving complex (33K, 23K, and 16K), and photosystem I subunit N (PSI-N) are imported by means of bipartite presequences which contain two signal peptides in tandem. The first domain targets the protein into the stroma, where it is usually removed by the stromal processing peptidase; the second “thylakoid transfer” signal directs translocation of the intermediate across the thylakoid membrane, after which processing to the mature size is carried out by a thylakoidal processing peptidase (Hageman et al., 1986; James et al., 1989; Ko and Cashmore, 1989).

The structures of thylakoid transfer signals are of particular interest because they resemble bacterial export or “signal” sequences in several respects, and the 33K thylakoid transfer signal is furthermore capable of functioning as an Escherichia coli export signal (Seidler and Michel, 1990; Meadows and Robinson, 1991). However, despite the fact that all thylakoid transfer signals possess signal peptide-like hydrophobic core regions and processing sites (von Heijne et al., 1989; Halpin et al., 1989), a surprising recent finding is that luminal proteins are transported across the thylakoid membrane by two very different mechanisms, and probably by two separate translocases. Translocation of a subset of proteins, including PC and 33K, requires the presence of a stromal protein factor and nucleoside triphosphates, but not the thylakoidal ΔpH (Theg et al., 1989; Hulford et al., 1994; Robinson et al., 1994); in contrast, neither stromal factors nor ATP are required for the translocation of 23K, 16K, or PSI-N, but the ΔpH is an absolute requirement (Mould and Robinson, 1991; Cline et al., 1992; Nielsen et al., 1994). In competition experiments, luminal proteins also fall into two clear groups; 23K and 16K compete with each other for translocation across the thylakoid membrane, and 33K competes with PC, but the two groups do not compete with each other (Cline et al., 1993).

The resemblance of thylakoid transfer signals to signal sequences, and the findings that gene homologues of secA and secY (which encode components of the bacterial protein export apparatus) are present in the plastid genomes of several species of alga (Douglas, 1992; Scaramuzzi et al., 1992; Flachmann et al., 1993; Valentin, 1993) has raised the possibility that the 33KPC translocation system may operate by a sec-type mechanism inherited from the cyanobacterial-type progenitor of higher plant chloroplasts. Both types of mechanism require the participation of soluble factors and nucleoside triphosphates; bacterial protein transport by the sec pathway involves the participation of SecB, a cytosolic “chaperone” molecule with an antifolding function, and SecA, a translocation ATPase that is found in both soluble and membrane-bound forms (reviewed by Oliver et al. (1990b) and Wickner et al. (1991)). However, there

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1 The abbreviations used are: PC, plastocyanin; 33K, 23K, and 16K, 33-, 23-, and 16-kDa proteins, respectively, of the oxygen-evolving complex of photosystem II; int-33K, intermediate form of 33K; PSI-N, photosystem I subunit N.
has been no direct evidence to date for the participation of SecA or SecY proteins in thylakoidal protein transport. In this report we show that an inhibitor of SecA, azide, effectively inhibits the translocation of 33K and PC across the thylakoid membrane.

**EXPERIMENTAL PROCEDURES**

*Plant Material—*Pea seedlings (*Pisum sativum*, var. Feltham First) were grown for 7–8 days under a 12-h photoperiod. Radioactive materials were obtained from Amersham International (Cardiff, United Kingdom).

*Import Studies—*Precursors to 33K, 23K, 16K, and PC were synthesized by transcription of CDNA clones followed by translation in a wheat germ cell-free system in the presence of [35S]methionine as described (James et al., 1989). A two-dimensional artificial intermediate form of wheat 33K (int-33K) was prepared by transcription-translation of a truncated wheat 33K CDNA as detailed by Hulford et al. (1994). Chloroplast import assays were carried out essentially as described by Hageman et al. (1986) except that 8 mM MgATP was present. Incubation mixtures (150 μl) contained intact chloroplasts (55 μg of chlorophyll), 8 mM MgATP, 30 mM methionine, and 12.5 μl of translation product. Assays for the import of proteins by isolated pea thylakoids were as described by Hulford et al. (1994) using int-33K as a substrate. In these assays, thylakoids were prepared by lysis of pea chloroplasts, pelleting of the membranes, and two washes of the thylakoid membranes in 10 mM Hepes-KOH, pH 8.0, 5 mM MgCl₂ (HM buffer), after which the thylakoids were resuspended in the stromal extract.

**RESULTS**

**Azide Inhibits the Translocation of a Subset of Proteins across the Thylakoid Membrane**—Previous work (Oliver et al., 1990a) has shown that millimolar concentrations of sodium azide effectively inhibit the transport of proteins across the bacterial plasma membrane, and that the sensitive component of the export machinery is SecA. In view of the observed similarities between thylakoid transfer signals and bacterial signal sequences, we tested whether azide is capable of inhibiting protein translocation across the thylakoid membrane by either of the translocation mechanisms known to operate. Precursors of luminal proteins were synthesized by transcription-translation and incubated with isolated chloroplasts that had been preincubated in the presence and absence of 10 mM azide, and the chloroplasts were fractionated afterwards to determine the locations of the imported proteins. In the minus-azide controls, it can be seen that the precursors of 33K, PC, and 23K are efficiently imported and localized in the thylakoid lumen as the mature-size forms; there is no evidence for the presence of the transient intermediate forms. Following incubation of the chloroplasts with azide, the precursors are also imported into the stroma efficiently (showing that azide does not affect translocation across the envelope membrane), but further translocation of a subset of proteins is severely affected. A substantial proportion of both 33K and PC is present in the chloroplasts as intermediate-size polypeptides, and fractionation tests indicate that the intermediates are found predominantly in the stroma.

Azide has no effect on the import or localization of 23K (Fig. 1, lower panel) or 16K or PSI subunit N (not shown), consistent with the idea that these proteins are translocated by a separate pathway.

Millimolar concentrations of azide completely inhibit SecA-dependent protein transport in *E. coli* (Oliver et al., 1990a) but do not completely inhibit the translocation of 33K and PC across the thylakoid membrane in the experiment shown in Fig. 1. However, we have found that the effects of azide vary according to the incubation conditions. In the standard import assay (as used in Fig. 1), the chloroplasts are illuminated and incubation is carried out in the presence of 8 mM MgATP to drive import into the chloroplasts at maximal rates. Under these conditions we usually find that, in the presence of azide, approximately 50% of imported 33K and PC is present as the stromal intermediate form (Figs. 1 and 2, lower panel). If exogenous ATP is omitted from the incubation mixture, and import is carried out in the dark to prevent significant rates of photophosphorylation (Fig. 2, upper panel), pre-33K is still imported into chloroplasts at reasonable rates but the azide has a more dramatic effect; translocation across the thylakoid membrane is almost completely inhibited, and the stromal intermediate form now predominates. These findings are consistent with a model in which azide competes with ATP for a binding site on SecA (it is not known precisely how azide inhibits bacterial SecA).

**There Is No “Cross-talk” between the Two Pathways for Translocation of Proteins across the Thylakoid Membrane**—An alternative explanation for the data shown above is that azide does completely inhibit translocation by the 33K/PC system, but that 33K can bypass this block and enter the 23K/16K pathway.
translocation pathway. This would not be surprising since the thylakoid transfer signals of the known luminal proteins all share common features. We examined this possibility by carrying out import assays in the presence of a combination of azide and nigericin, since this uncoupler completely inhibits translocation of 23K and 16K across the thylakoid membrane (Mould and Robinson, 1991; Cline et al., 1992; Klosgen et al., 1992).

The results (Fig. 3) show that the presence of nigericin does not affect the ratio of intermediate to mature-size 33K in such assays, demonstrating that 33K does not enter the alternative ΔpH-dependent translocation pathway when its normal pathway is partially blocked. In the experiment shown in Fig. 3, control incubations were carried out using pre-23K as a substrate, and it was found that, as expected, translocation of 23K across the thylakoid membrane was completely blocked by nigericin (data not shown).

**Azide Reversibly Inhibits the Import of 33K by Isolated Thylakoids—**Further studies on the effect of azide on thylakoidal protein transport were carried out using an *in vitro* assay for the import of proteins by isolated thylakoids. Hulford et al. (1994) have analyzed the import into isolated thylakoids of a synthetic 33K intermediate form (int-33K), and have shown that import is dependent on the presence of a stromal protein factor and ATP. In the experiment shown in Fig. 4, this assay was used to examine the effects of azide on 33K transport in greater detail. Import incubations were carried out in the absence of azide, in the continuous presence of azide (incubation 2), and after treatment of either the stromal or thylakoidal fractions separately with azide; in these cases the azide was then removed before commencement of the import incubation by passing the stromal extract through a PD 10 desalting column or by washing the thylakoids (incubations 4 and 5, respectively). A final incubation (6) was carried out using both stroma and thylakoids that had been incubated with azide and then treated as above to remove the azide. The results show that import of int-33K into thylakoids is completely inhibited if azide is continuously present, whereas import proceeds normally if free azide is removed from the stromal or thylakoidal fractions prior to the import incubation. We conclude that azide does indeed have a marked effect on the import of transport of 33K across the membrane, and that the inhibition is reversible; the effect of azide on bacterial SecA has likewise been found to be reversible.2 The data are again consistent with the idea that azide and ATP compete for the same site on SecA; only low levels of ATP are present in the thylakoid import assay (0.15 μM provided by the wheat germ translation system), and it may be because of this that azide is able to completely inhibit translocation. In other tests we have found that, as expected, azide blocks the import of plastocyanin by thylakoids but has no effect on the import of 23K (not shown).

**DISCUSSION**

In this report we have examined the effect of a known SecA inhibitor on the transport of proteins across the thylakoid membrane, and we show that the translocation of a subset of proteins, namely 33K and PC, is markedly inhibited in intact chloroplasts and completely inhibited in a thylakoid import assay. These data represent the first biochemical evidence favoring the involvement of a SecA homolog in thylakoidal protein transport. The data do not, however, constitute proof of such an involvement; azide can not be regarded as a completely specific SecA inhibitor, having been shown to inhibit other biochemical processes, and we have been unable to identify a SecA-related protein by immunoblotting using antisera raised against bacterial SecA proteins (not shown). Nevertheless, it is now possible to probe directly for conclusive evidence of the involvement of SecA, for example by cross-linking studies, because it would be predicted that the stromal forms of 33K and PC would be bound to SecA if the latter were inactivated by azide; Hartl et al. (1990) have shown that inactivation of bacterial SecA inhibits the release of preproteins but not their binding.

As well as suggesting a role for SecA in thylakoidal protein translocation, the data also indicate that azide may be a valuable tool for the future analysis of novel luminal proteins. At present, the two types of thylakoidal protein translocation mechanism can be distinguished experimentally by the requirements for stromal factors and ATP in one pathway, and the requirement for a ΔpH in the other. However, the assignment of novel luminal proteins to one of these groups may prove to be difficult using these criteria alone. The importance of stromal factors or ATP can only be tested using the isolated thylakoid import assay, and some proteins are imported only inefficiently in this assay, and it is probable that others will not be imported at all. The role of the ΔpH can be tested by the use of uncouplers in the intact chloroplast assay, in which most proteins can be imported, but it would be dangerous to draw too many conclusions on the basis of this test alone because the response of 33K under these circumstances has been found to vary dramatically even within the same laboratory. In the presence of nigericin, the transport of 33K has variously been found to be completely blocked (Mould and Robinson, 1991), partially

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2 A. Driessen, personal communication.
inhibited (Cline et al., 1992), or completely unaffected (Nielsen et al., 1994). The importance of the ΔpH therefore seems to depend either on the precise assay conditions or on the physiological status of the chloroplasts, but the underlying reasons for the variations in results are totally unclear at present. Our data suggest that azide may well be a more suitable diagnostic reagent for discriminating between the two import pathways for luminal proteins using intact chloroplast import assays.

The data are entirely consistent with the proposed origin of the two known mechanisms for thylakoidal protein translocation. Of the five luminal proteins studied in detail to date in chloroplasts, only 33K and PC are present in the thylakoid lumen of cyanobacteria, where they are probably transported by a sec-type mechanism (Kuwahara et al., 1987; Briggs et al., 1990). Furthermore, these proteins are transported across the chloroplast thylakoid membrane by a mechanism that relies on the participation of soluble protein factors and ATP, as does bacterial protein export via the sec machinery (Hulford et al., 1994; Robinson et al., 1994). Clearly, there is now a good chance that the stromal translocation factor is either SecA or SecB (or both), and that the ATP-dependent step in the translocation of 33K and plastocyanin is SecA function. Azide has no effect on the translocation of 23K, 16K, or PSI-N across the thylakoid membrane, consistent with the idea that these proteins, which probably arose after the initial endosymbiotic event, are translocated by an entirely different mechanism, which has also arisen relatively recently.

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