Journal of Experimental Botany, Vol. 52, No. 354, pp. 57–66, January 2001

Journal of Experimental Botany

The effect of amino acid-modifying reagents on chloroplast protein import and the formation of early import intermediates

Paula E. Row[1] and John C. Gray[2]

Department of Plant Sciences and Cambridge Centre for Molecular Recognition, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK

Received 14 April 2000; Accepted 6 September 2000

Abstract

In order to identify functionally important amino acid residues in the chloroplast protein import machinery, chloroplasts were preincubated with amino-acid-modifying reagents and then allowed to import or form early import intermediates with precursor proteins. Incubation of chloroplasts with *N*-ethyl maleimide, diethyl pyrocarbonate, phenylglyoxal, 4,4′-di-isothiocyanatostilbene 2,2′-disulphonic acid (DIDS), dicyclohexylcarbodiimide (DCCD), and 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC) inhibited both import and formation of early import intermediates with precursor proteins by chloroplasts. This suggests that one or more of the binding components of the chloroplast protein import machinery contains functionally important solvent-exposed cysteine, histidine, arginine, and aspartate/glutamate residues, as well as functionally important lysine and aspartate/glutamate residues in a hydrophobic environment.

Key words: Chloroplast protein import, amino-acid-modifying reagents, ferredoxin NADP$^+$ reductase, Rubisco small subunit, phosphate translocator.

Introduction

Although chloroplasts are likely to contain several thousand different proteins, fewer than one hundred of these are encoded in the plastid genome. The rest are nuclear-encoded and are synthesized as precursor proteins on free cytosolic polysomes. Precursor proteins are directed to the chloroplast by a transit sequence, an *N*-terminal extension of varying length enriched in serine and threonine residues and in the small hydrophobic residues alanine and valine (Keegstra *et al*., 1989; de Boer and Weisbeek, 1991). The net positive charge of the transit sequence probably allows it to approach the negatively charged chloroplast envelope electrostatically. Once the transit sequence encounters the lipids of the outer envelope, it folds into an amphipathic helix and inserts into the membrane (van't Hof and de Kruijff, 1995). Next, the transit sequence interacts with the proteinaceous components of the chloroplast protein import machinery.

For many years it was thought that the initial interaction with the import machinery required energy in the form of ATP, since attempts to isolate chloroplasts with associated precursor after binding in the absence of ATP failed (Cline *et al*., 1985; Friedman and Keegstra, 1989). It is now known that the initial interaction of the transit sequence with the import apparatus is energy-independent and reversible (Perry and Keegstra, 1994); precursor proteins have been cross-linked specifically to several components of the import machinery in the absence of ATP or GTP, namely Toc159 (formerly Toc86; Perry and Keegstra, 1994; Schnell *et al*., 1997) in concert with Toc75 (Ma *et al*., 1996) and Toc34 (Kouranov and Schnell, 1997) which form a multi-subunit complex. If 100 μM ATP and GTP are added to the reaction, the transit sequence binds irreversibly to the

[1] Present address: Cambridge Institute for Medical Research and Department of Clinical Biochemistry, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, UK.

[2] To whom correspondence should be addressed. Fax: +44 1223 333953. E-mail: jcg2@mole.bio.cam.ac.uk

Abbreviations: DCCD, dicyclohexylcarbodiimide; DEPC, diethyl pyrocarbonate; DIDS, 4,4′-di-isothiocyanatostilbene 2,2′-disulphonic acid; EDC, 1-ethyl-3-dimethylaminopropylcarbodiimide; I_{50}, the concentration of reagent causing half-maximal inhibition of import of precursor proteins by chloroplasts; NEM, *N*-ethyl maleimide; PAR, photosynthetically active radiance; pFNR, precursor of ferredoxin-NADP$^+$ reductase; pLHCP, precursor of the light-harvesting chlorophyll *a*/*b*-binding protein; pPT, precursor of the phosphate translocator; pSSu, precursor of the small subunit of Rubisco.

import machinery (Cline *et al*., 1985; Friedman and Keegstra, 1989). At this stage, the precursor protein spans the outer envelope and is in contact with Toc159, Toc75 (thought to form a channel) as well as an Hsp70 (Com70) on the outer face of the outer envelope, an Hsp70 on the inner face of the outer envelope, and two proteins Tic20 and Tic22 of the inner envelope (Waegemann and Soll, 1991; Hirsch *et al*., 1994; Kessler *et al*., 1994; Schnell *et al*., 1994; Wu *et al*., 1994; Seedorf *et al*., 1995; Tranel *et al*., 1995; Ma *et al*., 1996; Kouranov and Schnell, 1997; Kouranov *et al*., 1998). Toc159 and Toc75 are thought to hydrolyse GTP to present precursor proteins to the import channel. This stage is now referred to as an early import intermediate.

Translocation across the inner envelope requires ATP hydrolysis in the stroma (Theg *et al*., 1989) and involves Tic110 (Schnell *et al*., 1994; Wu *et al*., 1994; Lubeck *et al*., 1996), Tic20 and Tic22 (Ma *et al*., 1996; Kouranov and Schnell, 1997; Kouranov *et al*., 1998), perhaps Tic55 (Caliebe *et al*., 1997) and two stromal chaperones which can be immunoprecipitated with Tic110, namely Cpn60 (Kessler and Blobel, 1996) and the chloroplast ClpC homologue (Nielsen *et al*., 1997). Once inside the stroma, the transit sequence is cleaved off by the stromal processing peptidase (vanderVere *et al*., 1995). Chloroplast protein import has been the subject of several excellent reviews recently (Cline, 2000; Chen and Schnell, 1999).

The aim of the work in this paper was to identify amino acid residues in the proteinaceous components of the import machinery, which are involved in the recognition of the transit sequences of chloroplast precursor proteins. This involved the use of amino-acid-modifying reagents (Eyzaguirre, 1987; Imoto and Yamada, 1989) which, under appropriate pH conditions, modify a specific type of amino acid residue by the covalent attachment of a chemical group. Only a subset of the commonly occurring amino acid residues are attacked by amino-acid-modifying reagents; these are the charged and uncharged polar amino acid residues which by virtue of their chemical reactivity play a central role in molecular recognition processes.

There are already some reports in the literature detailing the use of amino-acid-modifying reagents to implicate certain amino acids in the process of chloroplast protein import. In the first of these, pretreatment of chloroplasts with the sulphydryl reagent *N*-ethyl maleimide (NEM) reduced the ability of the chloroplasts to form an early import intermediate with the precursor of the small subunit of Rubisco, pSSu (Friedman and Keegstra, 1989). Scatchard analysis revealed that preincubation of the chloroplasts with NEM decreased the affinity of the binding site for pSSu (the K_d increased from approximately 8 nM to 19.2 nM) although the number of binding sites remained constant at approximately 2800 binding sites per chloroplast. Similarly, preincubation of chloroplasts with 4,4′-di-isothiocyanatostilbene 2,2′-disulphonic acid (DIDS, a lysine reagent) inhibited the subsequent import of pSSu into chloroplasts, with an I_{50} of 3 μM (Flügge *et al*., 1991). In addition, treatment of chloroplasts with diethylpyrocarbonate (which usually modifies histidine residues) inhibited the import of pSSu into chloroplasts, but did not affect the formation of early import intermediates (Caliebe *et al*., 1997). The DEPC probably modified the Rieske-type iron-sulphur cluster of Tic55 which copurifies with a translocation intermediate containing Toc159, Toc75, Toc34, and Tic110 (Caliebe *et al*., 1997).

In this study, chloroplasts were incubated with a range of amino-acid-modifying reagents, washed to remove excess reagent (to prevent modification of the precursor proteins by the reagents) and tested for their ability to import precursor proteins or form early import intermediates. Early import intermediates were formed by limiting the stromal ATP concentration below the level required for protein translocation. This was achieved by incubating the chloroplasts with nigericin to inhibit photophosphorylation (Grossman *et al*., 1980*a*; Cline *et al*., 1985) and carrying out the reactions at 4 °C (Grossman *et al*., 1980*b*; Friedman and Keegstra, 1989) which prevents the uptake of ATP into the stroma (Leheny and Theg, 1994). The energy requirement for the formation of the early import intermediate was satisfied by the addition of 100 μM ATP to the reactions. In some cases, additional assays were carried out in which chloroplasts with associated early import intermediates were incubated in the presence of 5 mM ATP in order to chase precursor proteins along the import pathway into the chloroplasts. In this way, it was possible to separate the effect of the amino acid modifying reagent on association of the precursor protein with the import machinery from any effect on non-specific binding of the precursor to other components of the chloroplast outer envelope.

Most of the experiments described in this paper were carried out using pSSu or the precursor of ferredoxin-$NADP^+$ reductase (pFNR). These precursors were chosen since their transcription and translation *in vitro* and their import into chloroplasts are very efficient.

Materials and methods

Synthesis of precursor proteins in vitro

Bacterial plasmids for the synthesis of precursor proteins in radiolabelled form have been described previously. Plasmid pSMS64 encodes pea pSSu (Anderson and Smith, 1986), plasmid pPPT.8 encodes pea pPT (Knight and Gray, 1995) and plasmid pFNR 1.4 encodes pea pFNR (Newman and Gray, 1988). Plasmids were transcribed using SP6 polymerase and the RNA was translated in a wheat germ extract in the presence of [^{35}S] methionine (Amersham) to yield the precursor protein in radiolabelled form exactly as described previously (Knight and

Gray, 1995). ATP was removed from translation mix containing radiolabelled pSSu by passing it through a Sephadex G-25 (Pharmacia) spun column, as described previously (Olsen *et al.*, 1989).

Isolation of chloroplasts and treatment with inhibitors

Chloroplasts were isolated from peas (*Pisum sativum* L. cv. Feltham First) which had been sown in Levington compost (Fisons) and grown for 7–10 d in a greenhouse with an ambient temperature of 15 °C to 25 °C with supplementary artificial lighting providing a PAR of 150 μmol photons m^{-2} s^{-1} over a 16 h photoperiod (Kirwin *et al.*, 1989). Chlorophyll determination was carried out by measuring A_{652} (Arnon, 1949).

Treatment of chloroplasts with amino-acid-modifying reagents was as follows. For import assays, chloroplasts containing 40 μg chlorophyll were incubated in the presence of various concentrations of amino-acid-modifying reagent in 330 mM sorbitol, 50 mM HEPES-KOH at either pH 7.0 or pH 8.0 (depending on the incubation pH for the reagent in question) in a total volume of 200 μl. Chloroplasts which were destined to form early import intermediates were incubated with amino-acid-modifying reagents in the presence of 400 nM nigericin and 330 mM sorbitol, 50 mM HEPES-KOH pH 7.0 or 8.0. The chloroplasts were incubated with varying concentrations of amino-acid-modifying reagent at 25 °C under room lights for 30 min with occasional mixing. Intact chloroplasts were reisolated as described previously (Knight and Gray, 1995), resuspended in 100 μl 330 mM sorbitol, 25 mM HEPES-KOH pH 8.0 and added to either an import assay or an assay for the formation of early import intermediates.

Chloroplast protein import assays

The assay for chloroplast protein import was carried out as described earlier (Knight and Gray, 1995). Chloroplasts (40 μg chlorophyll, which had been incubated with amino-acid-modifying reagent and reisolated) were incubated with 10 μl translation mix containing radiolabelled precursor protein in the presence of 5 mM ATP, 1 mM methionine, 330 mM sorbitol and 25 mM HEPES-KOH pH 8.0 in a total volume of 150 μl at 25 °C for 30 min under room lights. The chloroplasts were treated with 100 μg ml^{-1} thermolysin, 3 mM $CaCl_2$ on ice for 30 min to degrade precursor proteins which had not been imported. The thermolysin reaction was stopped with 5 mM EDTA and the chloroplasts were reisolated and subjected to SDS-PAGE.

Chloroplast early import intermediates were prepared in microfuge tubes which had been siliconized by dipping them in a 2% solution of dimethyldichlorosilane in 1,1,1-trichloroethane (BDH Ltd., Poole, Dorset) and allowing them to dry in a fume hood for approximately 1 h. Chloroplasts were incubated with 10 μl translation mix containing ^{35}S-labelled precursor protein in the presence of 100 μM ATP, 400 nM nigericin, 1 mM methionine, 330 mM sorbitol and 25 mM HEPES-KOH pH 8.0 in a total volume of 150 μl, on ice for 30 min. Intact chloroplasts were reisolated as described earlier (Knight and Gray, 1995).

To chase early import intermediates into chloroplasts, pellets of chloroplasts with precursor proteins bound as early import intermediates were resuspended in 5 mM ATP, 1 mM methionine, 330 mM sorbitol, and 25 mM HEPES-KOH pH 8.0 in a volume of 150 μl. The samples were incubated at 25 °C under room lights for 15 min and intact chloroplasts were reisolated.

Electrophoresis and quantification

Chloroplast assays were analysed on an equal chlorophyll basis by SDS-PAGE through a stacking gel containing 5% acrylamide and a resolving gel containing either 12% or 15% acrylamide (Laemmli, 1970). Following electrophoresis, the gels were soaked in boiling 5% trichloroacetic acid for 5 min to hydrolyse methionyl-tRNA. The gels were rinsed in water, stained in 0.0025% (w/v) Coomassie Brilliant Blue R-250, 10% (v/v) ethanol and 5% (v/v) acetic acid for 1 h and scanned with a Molecular Dynamics 300S laser scanning densitometer to check that equivalent amounts of each sample had been loaded. The gels were neutralized in 2 M TRIS (unbuffered) for 5 min and subjected to fluorography by soaking for 30 min in Amplify (Amersham International). The gels were dried onto filter paper and exposed against Fuji RX film at −80 °C. The bands on the fluorograms corresponding to bound or imported precursor proteins were scanned with a Molecular Dynamics 300S laser scanning densitometer and quantified using volume integration.

The percentage import remaining after treatment with a given concentration of amino-acid-modifying reagent was calculated by expressing the density of the band of imported protein in that sample as a percentage of the density of the band in the control. Subtraction of this value from 100 gave the percentage inhibition of import in each sample. Values of I_{50} for the effect of an amino-acid-modifying reagent on import of a precursor protein into chloroplasts were calculated either from a graph of [amino-acid-modifying reagent]/percentage inhibition of import against [amino-acid-modifying reagent] (after Hanes, 1932), from a direct linear plot (Eisenthal and Cornish-Bowden, 1974) or from a graph of percentage inhibition of import against [amino-acid-modifying reagent].

Results

Inhibition by N*-ethyl maleimide*

Preincubation of chloroplasts with 1 mM NEM has already been shown to inhibit the formation of early import intermediates of pSSu by reducing the affinity of chloroplasts for pSSu (Friedman and Keegstra, 1989). In order to test the effect of NEM on import of pSSu under the experimental conditions used in this study, and to determine the concentration of NEM which resulted in half-maximal inhibition of import (the I_{50} for import), chloroplasts were incubated with various concentrations of NEM, reisolated and assayed for their ability to import or form early import intermediates with pSSu (Fig. 1). Preincubation of chloroplasts with increasing concentrations of NEM inhibited the import of pSSu into the chloroplasts (Fig. 1a) since the intensity of the band of mature protease-protected SSu decreased after preincubation of chloroplasts with increasing concentrations of NEM. The I_{50} for the inhibition of pSSu import by NEM was 120 μM. Preincubation of the chloroplasts with NEM also inhibited the formation of early import intermediates of pSSu (Fig. 1b). Therefore at least one component of the chloroplast protein import machinery involved in the formation of early import intermediates of pSSu contains a functionally important cysteine residue.

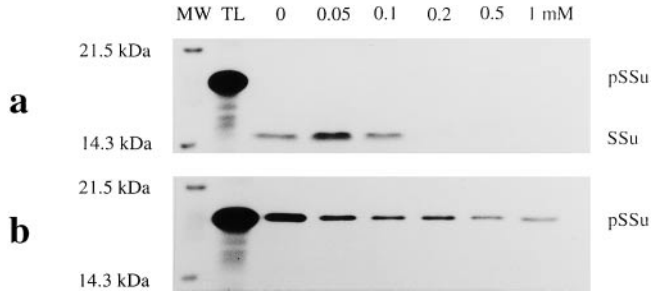

Fig. 1. The effect of NEM on import and the formation of early import intermediates with pSSu by isolated chloroplasts. Chloroplasts were incubated with 0–1 mM NEM at pH 7.0 and 25 °C for 30 min, in duplicate. Intact chloroplasts were reisolated and washed to remove excess NEM. Chloroplasts from one set of reactions were incubated with [^{35}S]-pSSu under (a) import conditions or (b) conditions which lead to the formation of early import intermediates. Intact chloroplasts were reisolated and analysed by SDS-PAGE and fluorography on an equal-chlorophyll basis. MW, molecular weight markers; TL, translation of pSSu. The concentration of NEM (mM) is given above the gel lanes. The translation lane contains 1% of the translation added to the assays and the other lanes contain approximately 22% (a) or 8% (b) of the chloroplasts recovered from the assays. I_{50} values were calculated from individual experiments. Duplicate experiments gave similar values.

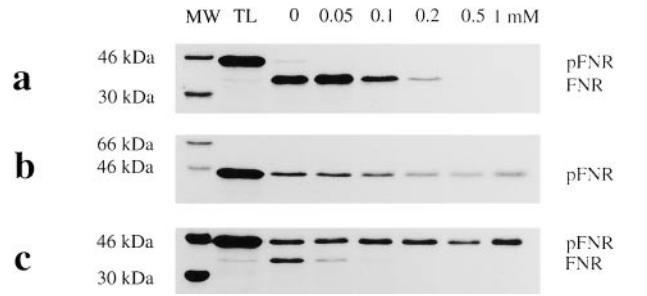

Fig. 2. The effect of NEM on import and formation of early import intermediates with pFNR by isolated chloroplasts.Chloroplasts were incubated with 0–1 mM NEM at pH 7.0 and 25 °C for 30 min, in triplicate. Intact chloroplasts were reisolated and washed to remove excess NEM. Chloroplasts were incubated with [^{35}S]-pFNR under either (a) import conditions or (b) conditions which lead to the formation of early import intermediates. Chloroplasts from the third set of reactions were incubated with [^{35}S]-pFNR under conditions which lead to the formation of early import intermediates, reisolated and incubated at 25 °C in the presence of 5 mM ATP and 1 mM methionine for 30 min in order to chase into the chloroplasts any pFNR which was bound to the import machinery. All chloroplasts were reisolated and analysed by SDS-PAGE and fluorography on an equal-chlorophyll basis. MW, molecular weight markers; TL, translation of pFNR. The concentration of NEM (mM) is given above the gel lanes. The translation lane contains 1% of the translation added to the assays and the other lanes contain approximately 9% (a and b) or 14% (c) of the chloroplasts recovered from the assays. I_{50} values were calculated from individual experiments. Replicate experiments gave similar values.

In order to provide more evidence that NEM inhibits the import and binding of precursor proteins by chloroplasts and that chloroplast precursor proteins share components of the import machinery which are involved in the formation of early import intermediates (Perry *et al.*, 1991; Schnell *et al.*, 1991), chloroplasts were incubated with various concentrations of NEM, reisolated and allowed to import or form early import intermediates with pFNR. In a third set of reactions, chloroplasts with associated pFNR early import intermediates were incubated with 5 mM ATP in order to chase pFNR which was bound to the import machinery into the chloroplasts. Any pFNR which was bound non-specifically to other components of the outer envelope should not be imported. Thus it would be possible to differentiate between the effect of NEM on association with the import machinery and the effect of NEM (if any) on non-specific binding. Preincubation of chloroplasts with NEM inhibited both import of pFNR and the formation of early import intermediates (Fig. 2a, b) without having any effect on the non-specific binding of pFNR to chloroplasts (Fig. 2c). The I_{50} for the inhibition of pFNR import by NEM, 100 μM, was similar to the I_{50} for inhibition of pSSu import into chloroplasts (120 μM).

Inhibition by DIDS

It has been shown that preincubation of chloroplasts with the lysine reagent DIDS inhibits the import of pSSu into the chloroplasts (Flügge *et al.*, 1991). To determine whether DIDS inhibits the formation of early import intermediates or the translocation of precursor proteins into the chloroplast, chloroplasts were incubated with various concentrations of DIDS and tested for their ability to import or form early import intermediates with pFNR. In addition, a third set of reactions was prepared in which chloroplasts were incubated with various concentrations of DIDS, allowed to bind pFNR and then incubated in the presence of 5 mM ATP so that any pFNR which was bound to the import machinery would be imported.

Preincubation of chloroplasts with increasing concentrations of DIDS inhibited the import of pFNR into chloroplasts (Fig. 3a) with an I_{50} of 12 μM. This value is not dissimilar to the I_{50} of 3 μM obtained for the inhibition of pSSu import by DIDS (Flügge *et al.*, 1991). Preincubation with DIDS also inhibited the formation of early import intermediates of pFNR (Fig. 3b) with an I_{50} of 34 μM (estimated by assuming that 200 μM DIDS had reduced binding of pFNR to background levels). Since this is higher than the I_{50} value for the effect of DIDS on pFNR import, this suggests that DIDS might inhibit translocation of pFNR as well as inhibiting the formation of early import intermediates.

In order to address this possibility, chloroplasts were incubated with various concentrations of DIDS, allowed to form early import intermediates with pFNR, reisolated and then incubated with 5 mM ATP. Any pFNR which was bound to the import machinery should be chased into the chloroplasts and processed to the mature form. The amount of bound pFNR which was chased into the chloroplasts decreased with increasing concentrations of DIDS (Fig. 3c). The amount of bound pFNR which was not imported and processed in the subsequent chase

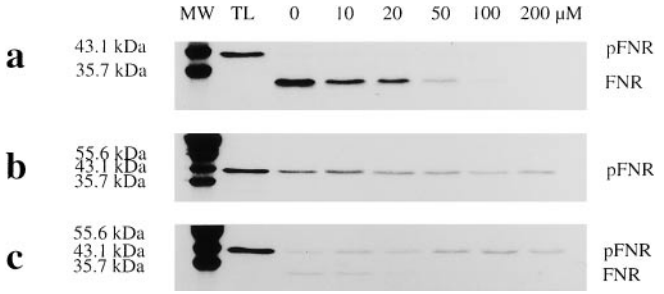

Fig. 3. The effect of DIDS on import and formation of early import intermediates with pFNR by isolated chloroplasts. Chloroplasts were incubated with 0–200 μM DIDS at pH 8.0 and 25 °C for 30 min in triplicate. Intact chloroplasts were reisolated and washed to remove excess DIDS. Chloroplasts were incubated with [^{35}S]-pFNR under either (a) import conditions or (b) conditions which lead to the formation of early import intermediates. Chloroplasts from the third set of reactions were allowed to form early import intermediates of [^{35}S]-pFNR, reisolated and incubated at 25 °C in the presence of 5 mM ATP and 1 mM methionine for 30 min in order to chase into the chloroplasts any pFNR which was bound to the import machinery. All chloroplasts were reisolated and analysed by SDS-PAGE and fluorography on an equal-chlorophyll basis. MW, molecular weight markers; TL, translation of pFNR. The concentration of DIDS (μM) is given above the gel lanes. The translation lane contains 1% of the translation added to the assays and the other lanes contain approximately 10% (a), 9% (b) or 12% (c) of the chloroplasts recovered from the assays. I_{50} values were calculated from individual experiments. Replicate experiments gave similar values.

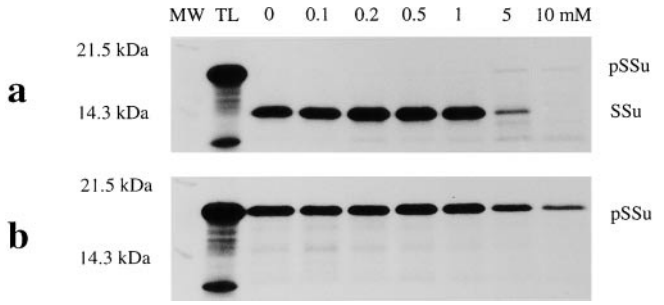

Fig. 4. The effect of phenylglyoxal on import and formation of early import intermediates with pSSu by isolated chloroplasts. Chloroplasts were incubated with 0–10 mM phenylglyoxal at pH 8.0 and 25 °C for 30 min, reisolated and allowed to (a) import or (b) form early import intermediates with [^{35}S]-pSSu. The chloroplasts were analysed by SDS-PAGE and fluorography on an equal-chlorophyll basis. MW, molecular weight markers; TL, translation of pSSu. The concentration of phenylglyoxal (mM) is given above the gel lanes. The translation lane contains 1% of the translation added to the assays and the other lanes contain approximately 13% (a) or 9% (b) of the chloroplasts recovered from the assays. I_{50} values were calculated from individual experiments. Duplicate experiments gave similar values.

reaction appeared to increase with increasing DIDS concentrations, however, suggesting that DIDS inhibited translocation as well as formation of early import intermediates with pFNR. The I_{50} for the effect of DIDS on the chased binding of pFNR to chloroplasts (5 μM) was not dissimilar to the I_{50} values of 12 μM for the effect of DIDS on pFNR import and 3 μM for the inhibition of pSSu import (Flügge *et al.*, 1991).

Inhibition by phenylglyoxal

To establish whether a component of the chloroplast protein import machinery contains a functionally important arginine residue, chloroplasts were incubated with the arginine-specific reagent phenylglyoxal, reisolated and tested for their ability to import or form early import intermediates with pSSu. Although there are several amino-acid-specific reagents which modify arginine residues, phenylglyoxal was chosen since it has a higher specificity and faster rate of reaction than the others (Takahashi, 1968). Preincubation of chloroplasts with phenylglyoxal concentrations of 5 and 10 mM inhibited both import of pSSu and the formation of early import intermediates with pSSu (Fig. 4) although no inhibition was apparent with lower phenylglyoxal concentrations. The I_{50} for the inhibition of import of pSSu was estimated to be 4 mM. These results suggest that one or more components of the import machinery involved in the formation of early import intermediates contains a functionally important arginine residue.

Inhibition by diethylpyrocarbonate

The amino acid-modifying reagent diethylpyrocarbonate (DEPC) reacts fairly specifically with histidine residues at pH 7.0 although it can also carry out slower side reactions with cysteine, lysine, arginine, tyrosine, and serine residues and α-amino groups, as well as cross-linking amine groups to the carboxyl groups of aspartate and glutamate residues (Miles, 1977; Dominici *et al.*, 1985). DEPC at 1 mM has already been shown to inhibit the import of pSSu into chloroplasts, but did not affect the formation of early import intermediates (Caliebe *et al.*, 1997). In order to determine an I_{50} for the interaction of DEPC with the import machinery, chloroplasts were preincubated with varying concentrations of DEPC at pH 7.0, reisolated and washed and allowed to import pSSu. As reported previously (Caliebe *et al.*, 1997), DEPC inhibited the import of pSSu into chloroplasts (Fig. 5). The I_{50} for the inhibition of pSSu import by DEPC was 0.8 mM which is similar to the concentration used to modify the histidine residues in purified 3,4-dihydrophenylalanine decarboxylase (Dominici *et al.*, 1985) suggesting that DEPC inhibited import by modifying at least one histidine residue, rather than by carrying out side reactions. Chloroplasts which had been pretreated with DEPC were also assayed for their ability to form early import intermediates. In this case, early import intermediates were formed by incubating chloroplasts at 25 °C with 100 μM ATP, 400 nM nigericin and [^{35}S]-pSSu translation product which had been passed through a Sephadex G-25 (Pharmacia) spun column in order to remove ATP, as described earlier (Olsen *et al.*, 1989). pSSu bound to chloroplasts as an early import intermediate if 100 μM ATP was added to the reaction, but

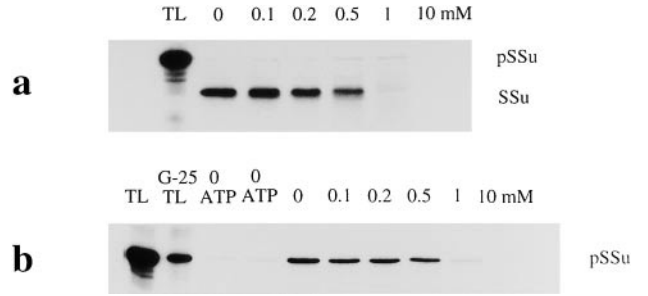

Fig. 5. The effect of DEPC on import and formation of early import intermediates with pSSu by isolated chloroplasts. Chloroplasts were incubated with 0–10 mM DEPC at pH 7.0 and 25 °C for 30 min, reisolated and allowed either to (a) import or (b) form import intermediates with [^{35}S]-pSSu. In this case, import intermediates were formed by incubating chloroplasts with 100 μM ATP, 400 nM nigericin and [^{35}S]-pSSu translation mix which had been passed through a Sephadex G-25 spun column to remove the ATP, at 25 °C for 30 min. Intact chloroplasts were reisolated and analysed by SDS-PAGE and fluorography on an equal-chlorophyll basis. MW, molecular weight markers; TL, translation of pSSu; G-25 TL, pSSu translation which had been passed through a Sephadex G-25 spun column to remove the ATP; 0 ATP, control lacking ATP. The concentration of DEPC is given above the gel lanes. The translation lane contains 1% of the translation added to the assay and the other lanes contain approximately 9% of the chloroplasts recovered from the assays. I_{50} values were calculated from individual experiments. Duplicate experiments gave similar values.

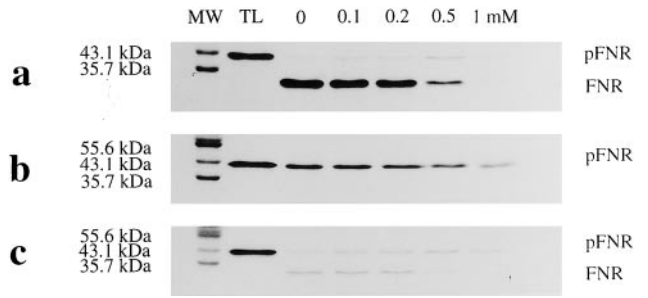

Fig. 6. The effect of DEPC on import and formation of early import intermediates with pFNR by isolated chloroplasts. Chloroplasts were incubated with 0–1 mM DEPC at pH 7.0 and 25 °C for 30 min, in triplicate. Intact chloroplasts were reisolated and washed to remove excess DEPC. Chloroplasts were incubated with [^{35}S]-pFNR under either (a) import conditions or (b) conditions which lead to the formation of early import intermediates. Chloroplasts from the third set of reactions (c) were incubated with [^{35}S]-pFNR under conditions which lead to the formation of early import intermediates, reisolated and incubated at 25 °C in the presence of 5 mM ATP and 1 mM methionine for 30 min in order to chase into the chloroplasts any pFNR which was bound to the import machinery. All chloroplasts were reisolated and analysed by SDS-PAGE and fluorography on an equal-chlorophyll basis. MW, molecular weight markers; TL, translation of pFNR. The concentration of DEPC (mM) is given above the gel lanes. The translation lane contains 1% of the translation added to the assays and the other lanes contain approximately 10% (a), 11% (b) or 16% (c) of the chloroplasts recovered from the assays. I_{50} values were calculated from individual experiments. Replicate experiments gave similar values.

not in its absence. In contrast to the findings of Caliebe *et al.* (Caliebe *et al.*, 1997), preincubation of chloroplasts with DEPC also inhibited the formation of early import intermediates of pSSu (Fig. 5b). The reason for this discrepancy is not clear. The I_{50} for the inhibition of the formation of early import intermediates of pSSu by

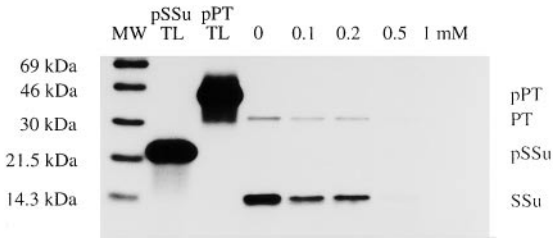

Fig. 7. The effect of DEPC on the simultaneous import of pPT and pSSu. Chloroplasts were incubated with 0–1 mM DEPC at pH 7.0 and 25 °C for 30 min before being reisolated and washed to remove excess DEPC. The chloroplasts were incubated with a mixture of [^{35}S]-pPT and [^{35}S]-pSSu translation products in a 10:1 ratio under import conditions. The chloroplasts were analysed by SDS-PAGE and fluorography on an equal-chlorophyll basis. MW, molecular weight markers; pSSu TL, translation of pSSu; pPT TL, translation of pPT. The concentration of DEPC (mM) is given above the gel lanes. The pSSu translation lane contains 1% of the translation added to the import reactions whereas the pPT translation lane contains 10% of the translation added to the import reactions. The other gel lanes contain 7% of the chloroplasts recovered from the import assays.

DEPC was 0.4 mM, which is not dissimilar to the value for the inhibition of pSSu import by DEPC.

Preincubation of chloroplasts with various concentrations of DEPC also inhibited the import of pFNR (Fig. 6a) and the formation of early import intermediates of pFNR (Fig. 6b). I_{50} values for the inhibition of pFNR import by DEPC and for inhibition of specific association of pFNR with the import machinery (obtained by chasing early import intermediates into the chloroplast, Fig. 6c) were both 0.3 mM which are similar to the values for the effect of DEPC on pSSu import and binding.

Preincubation of chloroplasts with increasing concentrations of DEPC also inhibited the simultaneous import of pSSu and the precursor of the phosphate translocator (pPT) into chloroplasts (Fig. 7) with I_{50} values of 0.25 mM and 0.22 mM, respectively. As well as corroborating the findings in the previous two sections, this also provides more evidence to suggest that different precursor proteins are imported into chloroplasts via the same import machinery. Taken together, these results all suggest that there is a functionally important histidine residue which is involved in the formation of early import intermediates.

Inhibition by carbodiimides

Preincubation of chloroplasts with dicyclohexylcarbodiimide (DCCD; Arana and Vallejos, 1981) inhibited the subsequent import of pSSu and the formation of early import intermediates of pSSu (Fig. 8), suggesting that a component of the chloroplast protein import machinery involved in the binding of precursor proteins contains a functionally important aspartate or glutamate residue in a hydrophobic environment. The I_{50} values were approximately 100 μM.

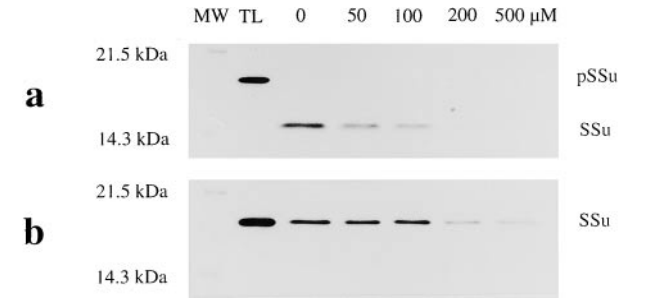

Fig. 8. The effect of DCCD on import and formation of early import intermediates with pSSu by isolated chloroplasts. Chloroplasts were incubated with 0–500 µM DCCD in the presence of 0.01% (v/v) ethanol at pH 7.0 and 25 °C for 30 min, reisolated and incubated with [^{35}S]-pSSu under either (a) import conditions or (b) conditions which lead to the formation of early import intermediates. The chloroplasts were analysed by SDS-PAGE and fluorography on an equal-chlorophyll basis. MW, molecular weight markers; TL, translation of pSSu. The concentration of DCCD (µM) is given above the gel lanes. The translation lane contains 1% of the translation added to the assays and the other lanes contain approximately 18% (a) or 12% (b) of the chloroplasts recovered from the assays. I_{50} values were calculated from individual experiments. Duplicate experiments gave similar values.

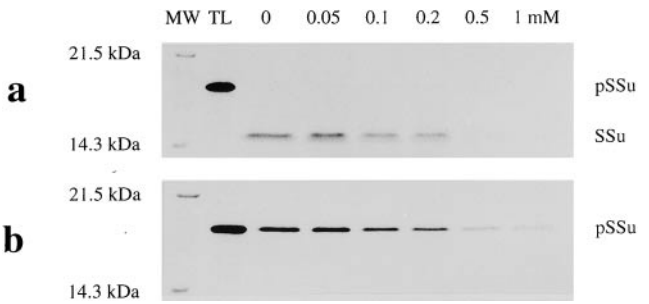

Fig. 9. The effect of EDC on import and formation of early import intermediates with pSSu by isolated chloroplasts. Chloroplasts were incubated with 0–1 mM EDC at pH 7.0 and 25 °C for 30 min, reisolated and incubated with [^{35}S]-pSSu under either (a) import conditions or (b) conditions which lead to the formation of early import intermediates. The chloroplasts were analysed by SDS-PAGE and fluorography on an equal-chlorophyll basis. MW, molecular weight markers; TL, translation of pSSu. The concentration of EDC (mM) is given above the gel lanes. The translation lane contains 1% of the translation added to the assays and the other lanes contain approximately 20% (a) or 8% (b) of the chloroplasts recovered from the assays. I_{50} values were calculated from individual experiments. Duplicate experiments gave similar values.

In order to determine whether the chloroplast protein import machinery also contains a functionally-important aspartate or glutamate residue in a hydrophilic environment, chloroplasts were incubated with the water-soluble carbodiimide 1-ethyl-3-dimethylaminopropyl-carbodiimide (EDC) before being included in assays to study the import or formation of early import intermediates of pSSu. Modification reactions involving EDC are usually carried out by incubating the protein to be modified at 25 °C with approximately 0.1 M EDC in the presence of 1 M amine (for instance glycine methyl ester) at pH 4.75–5.0 (Carraway and Koshland, 1972). The carbodiimide activates accessible carboxyl groups and the activated carboxyl groups then react with the amine to yield amidated carboxyl groups and the urea derivative of the carbodiimide. In the absence of an amine, the activated carboxyl groups can be hydrolysed to regenerate free carboxyl groups. This reaction cannot be carried out with chloroplasts, however, since a pH of 5.0 would result in lysis of the chloroplasts. Instead the reaction was carried out at pH 7.0. In addition, it was not possible to add an amine to carry out the second part of the reaction since 0.1 M glycine methyl ester lysed the chloroplasts (data not shown).

Preincubation of chloroplasts with increasing concentrations of EDC inhibited the import of pSSu into the chloroplasts (Fig. 9a) with an I_{50} of 0.2 mM. EDC also inhibited the formation of early import intermediates of pSSu (Fig. 9b). The I_{50} value of 0.13 mM which was estimated by assuming that 1 mM EDC inhibited the binding of pSSu to background levels was similar to the I_{50} value for the effect of EDC on import.

Identification of the amino acid residue which was modified by EDC was not straightforward because the reaction conditions were not those which are normally used for modification of protein with EDC, and might be expected to result in no net modification of the import machinery, due to hydrolysis of the carboxyl group. However, reaction of proteins with EDC at pH values near 8.0 favours the formation of intramolecular cross-links; the activated carboxyl formed by the EDC can react with a basic residue nearby in the protein to form a cross-link. The basic residue might be the lysine, arginine or histidine residues which were implicated in the earlier sections. Another possibility is that the EDC reacted with the cysteine residue which was shown to be functionally important for the import and binding of pSSu and pFNR by studies with NEM, since EDC carries out side reactions with cysteine residues in the absence of added nucleophile (Carraway and Koshland, 1972).

Discussion

The results presented in this paper have shown that both the import of precursor proteins and the formation of early import intermediates were inhibited by pretreatment of the chloroplasts with the cysteine reagent NEM, the histidine reagent DEPC, the arginine reagent phenylglyoxal, DIDS (which modifies lysine residues in a hydrophobic environment) and DCCD and EDC which react with aspartate and glutamate residues in a hydrophobic and hydrophilic environment, respectively.

The results with NEM confirm the previous finding that NEM inhibits the formation of early import intermediates with pSSu (Friedman and Keegstra, 1989). These results have been extended to include pFNR. This suggests that one or more functionally important

cysteine residue(s) is involved in the formation of early import intermediates of chloroplast precursor proteins and consequently, in import. A functionally important cysteine residue has been implicated by other experiments which have been reported in the literature. Treatment with dithiothreitol (DTT) and glutathione stimulated import (Pilon *et al*., 1992). As reducing agents, DTT and glutathione break disulphide bonds and reduce mercaptides (e.g. S-Cu) which are often formed when intracellular organelles are purified away from the reducing environment of the cytosol. Conversely, the oxidizing agent copper chloride inhibited the formation of an early import intermediate and subsequent import of pSSu (Seedorf and Soll, 1995). The inhibition was relieved by DTT. The fact that NEM lowered the affinity of chloroplasts for pSSu without totally destroying the pSSu binding site (Friedman and Keegstra, 1989) suggests that addition of an ethyl group to a free sulphydryl of a cysteine residue might cause steric hindrance of precursor protein binding. Since treatment of chloroplasts with copper chloride led to the formation of a cross-linked complex consisting of Toc159, Toc34 and Toc75 (Seedorf and Soll, 1995), this suggests that the functionally important cysteine residues are present in one or more of these components, which are known to constitute the initial receptor and outer envelope insertion site for chloroplast precursor proteins (Perry and Keegstra, 1994; Ma *et al*., 1996; Kouranov and Schnell, 1997).

DIDS has previously been shown to inhibit the import of pSSu into chloroplasts suggesting that the import machinery contains a functionally important lysine residue. This study has been extended to show that DIDS inhibits the formation of an early import intermediate suggesting that the functionally important lysine residue is in the initial receptor and or envelope insertion site. Experiments with pFNR confirmed these findings.

DEPC has previously been shown to inhibit the import of pSSu into chloroplasts (Caliebe *et al*., 1997). This suggested that a component of the import machinery contained a functionally important histidine residue. These findings have been extended to include pFNR and pPT, providing more evidence that different precursors are imported via the same import machinery. In these experiments, DEPC also inhibited the formation of early import intermediates of pSSu and pFNR, although this contradicts other findings (Caliebe *et al*., 1997) which showed that treatment of chloroplasts with DEPC did not affect the formation of early import intermediates of pSSu. The reason for this discrepancy is not clear. It may result from the different reaction conditions used to treat the chloroplasts with DEPC: the chloroplasts were incubated with varying concentrations of DEPC at pH 7.0 and 25 °C for 30 min whereas Caliebe *et al*. treated their chloroplasts with DEPC on ice for 10 min at pH 7.6. Perhaps our experiments exhaustive DEPC reaction conditions modified a histidine residue involved in the formation of early import intermediates which was not modified by the gentler reaction conditions of Caliebe *et al*. Since the formation of early import intermediates precedes translocation across the inner envelope, an inhibition of the formation of early import intermediates necessarily causes an inhibition of import. Caliebe *et al*. saw a separate inhibition of translocation across the inner envelope by forming early import intermediates of pSSu, treating with DEPC, reisolating the chloroplasts and chasing the pSSu into the chloroplasts. These findings, and those of Caliebe *et al*. (Caliebe *et al*., 1997), suggest that the protein import machinery contains at least one functionally important histidine residue.

The finding that EDC and DCCD inhibited import and the formation of an early import intermediate of pSSu suggests that a component of the initial receptor and envelope insertion site contains functionally important aspartate or glutamate residue(s) in both hydrophilic and hydrophobic environments. These carboxyl groups might be involved in recognizing positively charged residues in transit sequences. It has been shown that the synthesis of pSSu (which normally contains two arginine and two lysine residues in the transit sequence) with an uncharged arginine analogue inhibited import by 60%, whereas incorporation of an uncharged lysine analogue inhibited import by 50% (Robinson and Ellis, 1985). Similarly, synthesis of pea pLHCP (whose transit sequence contains two lysine residues and an arginine residue) with an uncharged lysine analogue completely blocked import. Perhaps the arginine and lysine residues in transit sequences interact with aspartate and glutamate residues in the import machinery. An obvious candidate for the component containing the functionally important aspartate and glutamate residues is the import receptor Toc159 and the newly discovered related import receptors Toc132 and Toc120, which contain an extrachloroplastic acidic domain with numerous aspartate and glutamate residues (Bauer *et al*., 2000). Site-directed mutagenesis will be needed to investigate this further.

Experiments with diethylpyrocarbonate and phenylglyoxal suggest that the import machinery also contains functionally important histidine and arginine residues involved in the formation of early import intermediates. These, together with the lysine residue implicated by the DIDS studies, might complex with the aspartate and glutamate residues when the import machinery is in the resting state, as well as interacting with phosphorylated serine and threonine residues of the transit sequence (Waegemann and Soll, 1996). Again, site-directed mutagenesis would further pinpoint these residues.

To summarize, the results in this paper suggest that components of the chloroplast protein import machinery involved in the formation of early import intermediates

contain at least one solvent exposed cysteine, histidine, arginine, and aspartate/glutamate residue and a lysine and aspartate/glutamate residue in a hydrophobic environment. It cannot be excluded, however, that these results are a result of amino-acid modification of an outer envelope protein which is required only indirectly for import, perhaps for uptake of ATP into chloroplasts. This is less likely for studies involving the formation of early import intermediates than for chloroplast protein import studies, however, since the energy which drives the formation of early import intermediates is required in the intermembrane space, and the outer envelope is freely permeable to small molecules (Olsen and Keegstra, 1992).

The finding that import and formation of early import intermediates of several different precursor proteins was inhibited by the amino acid-modifying reagents used provides more evidence that there is only one import pathway into the chloroplast.

Acknowledgements

PER thanks Jacqui Knight, Paco Madueño and Simon Barnes for teaching her to prepare chloroplasts which imported proteins and carry out SDS-PAGE, as well as for helpful advice and discussions. PER also thanks Renuka Sornarajah, Aliki Kapazoglou, Ruth Mould, and Sam Haward for helpful advice and discussions and Rainer Duden for help in preparing the figures. PER thanks the Science and Engineering Research Council and St John's College, Cambridge for funding.

References

Anderson S, Smith SM. 1986. Synthesis of the small subunit of ribulose-bisphosphate carboxylase from genes cloned into plasmids containing the SP6 promoter. *Biochimical Journal* **240,** 709–715.

Arana JL, Vallejos RH. 1981. Two different types of essential carboxyl groups in chloroplast coupling factor. *FEBS Letters* **123,** 103–106.

Arnon DI. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* **24,** 1–15.

Bauer J, Chen K, Hiltbunner A. Wehrli E, Eugster M, Schnell D, Kessler F. 2000. The major protein import receptor of plastids is essential for chloroplast biogenesis. *Nature* **403,** 203–207.

Caliebe A, Grimm R, Kaiser G, Lübeck J, Soll J, Heins L. 1997. The chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding protein. *EMBO Journal* **16,** 7342–7350.

Carraway KL, Koshland DE. 1972. Carbodiimide modification of proteins. *Methods in Enzymology* **25,** 616–623.

Chen X, Schnell DJ. 1999. Protein import into chloroplasts. *Trends in Cell Biology* **9,** 222–227.

Cline K. 2000. Gateway to the chloroplast. *Nature* **403,** 148–149.

Cline K, Werner-Washburne M, Lubben TH, Keegstra K. 1985. Precursors to two nuclear-encoded chloroplast proteins bind to the outer envelope membrane before being imported into chloroplasts. *Journal of Biological Chemistry* **260,** 3691–3696.

de Boer AD, Weisbeek PJ. 1991. Chloroplast protein topogenesis: import, sorting and assembly. *Biochemica et Biophysica Acta* **1071,** 221–253.

Dominici P, Tancini B, Voltattorni CB. 1985. Chemical modification of pig kidney 3,4-dihydrophenylalanine decarboxylase with diethyl pyrocarbonate. Evidence for an essential histidyl residue. *Journal of Biological Chemistry* **260,** 10583–10589.

Eisenthal R, Cornish-Bowden A. 1974. The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. *Biochemical Journal* **139,** 715–720.

Eyzaguirre J. (ed.) 1987. *Chemical modification of enzymes; active site studies*. Chichester, England: Ellis Horwood.

Flügge U-I, Weber A, Fischer K, Lottspeich F, Eckerskorn C, Waegemann K, Soll J. 1991. The major chloroplast envelope polypeptide is the phosphate translocator and not the protein import receptor. *Nature* **353,** 364–367.

Friedman AL, Keegstra K. 1989. Chloroplast protein import. Quantitative analysis of precursor binding. *Plant Physiology* **89,** 993–999.

Grossman A, Bartlett S, Chua N-H. 1980*a*. Energy-dependent uptake of cytoplasmically synthesized polypeptides by chloroplasts. *Nature* **285,** 625–628.

Grossman AR, Bartlett SG, Schmidt GW, Chua N-H. 1980*b*. Post-translational uptake of cytoplasmically synthesized proteins by intact chloroplasts *in vitro*. *Annals of the New York Academy of Science* **343,** 266–274.

Hanes CS. 1932. Studies on plant amylases. 1. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *Biochemical Journal* **26,** 1406–1421.

Hirsch S, Muckel E, Heemeyer F, von Heijne G, Soll J. 1994. A receptor component of the chloroplast protein translocation machinery. *Science* **266,** 1989–1992.

Imoto T, Yamada H. 1989. In: Creighton TE, ed. *Protein function. A practical approach*. Oxford: IRL Press, 247–277.

Keegstra K, Olsen LJ, Theg SM. 1989. Chloroplast precursors and their transport across the envelope membranes. *Annual Reviews of Plant Physiology and Plant Molecular Biology* **40,** 471–501.

Kessler F, Blobel G. 1996. Interaction of the protein import and folding machineries in the chloroplast. *Proceedings of the National Academy of Sciences, USA* **93,** 7684–7689.

Kessler F, Blobel G, Patel HA, Schnell DJ. 1994. Identification of two GTP-binding proteins in the chloroplast protein import machinery. *Science* **266,** 1035–1039.

Kirwin PM, Meadows JW, Shackleton JB, Musgrove JE, Elderfield PD, Mould RM, Hay NA, Robinson C. 1989. ATP-dependent import of a lumenal protein by isolated thylakoid vesicles. *EMBO Journal* **8,** 2251–2255.

Knight JS, Gray JC. 1995. The N-terminal hydrophobic region of the mature phosphate translocator is sufficient for targeting to the chloroplast inner envelope membrane. *The Plant Cell* **7,** 1421–1432.

Kouranov A, Chen X, Fuks B, Schnell D. 1998. Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane. *Journal of Cell Biology* **143,** 991–1002.

Kouranov A, Schnell DJ. 1997. Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. *Journal of Cell Biology* **139,** 1677–1685.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227,** 680–685.

Leheny EA, Theg SM. 1994. Apparent inhibition of chloroplast protein import by cold temperatures is due to energetic considerations not membrane fluidity. *The Plant Cell* **6,** 427–437.

Lubeck J, Soll J, Akita M, Nielsen E, Keegstra K. 1996. Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. *EMBO Journal* **15,** 4230–4238.

Ma Y, Kouranov A, LaSala SE, Schnell DJ. 1996. Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope. *Journal of Cell Biology* **134,** 315–327.

Miles EW. 1977. Modification of histidyl residues in proteins by diethylpyrocarbonate. *Methods in Enzymology* **47,** 431–422.

Newman BJ, Gray JC. 1988. Characterisation of a full-length cDNA clone for pea ferredoxin-$NADP^+$ reductase. *Plant Molecular Biology* **10,** 511–520.

Nielsen E, Akita M, Davila-Aponte J, Keegstra K. 1997. Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. *EMBO Journal* **16,** 935–946.

Olsen LJ, Theg SM, Selman BR, Keegstra K. 1989. ATP is required for the binding of precursor proteins to chloroplasts. *Journal of Biological Chemistry* **264,** 6724–6729.

Olsen LJ, Keegstra K. 1992. The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. *Journal of Biological Chemistry* **267,** 433–439.

Perry SE, Buvinger WE, Bennett J, Keegstra K. 1991. Synthetic analogues of a transit peptide inhibit binding or translocation of chloroplastic precursor proteins. *Journal of Biological Chemistry* **266,** 11882–11889.

Perry SE, Keegstra K. 1994. Envelope membrane proteins that interact with chloroplastic precursor proteins. *The Plant Cell* **6,** 93–105.

Pilon M, de Kruijff B, Weisbeek PJ. 1992. New insights into the import mechanism of the ferredoxin precursor into chloroplasts. *Journal of Biological Chemistry* **267,** 2548–2556.

Robinson C, Ellis RJ. 1985. Transport of proteins into chloroplasts. The effect of incorporation of amino acid analogues on the import and processing of chloroplast polypeptides. *European Journal of Biochemistry* **152,** 67–73.

Schnell DJ, Blobel G, Pain D. 1991. Signal peptide analogs derived from two chloroplast precursors interact with the signal recognition system of the chloroplast envelope. *Journal of Biological Chemistry* **266,** 3335–3342.

Schnell DJ, Kessler F, Blobel G. 1994. Isolation of components of the chloroplast protein import machinery. *Science* **266,** 1007–1012.

Schnell DJ, Blobel G, Keegstra K, Kessler F, Ko K, Soll J. 1997. A consensus nomenclature for the protein import components of the chloroplast envelope. *Trends in Cell Biology* **7,** 179–190.

Seedorf M, Soll J. 1995. Copper chloride, an inhibitor of protein import into chloroplasts. *FEBS Letters* **367,** 19–22.

Seedorf M, Waegemann K, Soll J. 1995. A constituent of the chloroplast import complex represents a new type of GTP-binding protein. *The Plant Journal* **7,** 401-411.

Takahashi K. 1968. The reaction of phenylglyoxal with arginine residues in proteins. *Journal of Biological Chemistry* **243,** 6171–6179.

Theg SM, Bauerle C, Olsen LJ, Selman BR, Keegstra K. 1989. Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. *Journal of Biological Chemistry* **264,** 6730–6736.

Tranel PJ, Froehlich J, Goyal A, Keegstra K. 1995. A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. *EMBO Journal* **14,** 2436–2446.

vanderVere P, Bennett R, Oblong JE, Lamppa GK. 1995. A chloroplast processing enzyme involved in precursor maturation shares a zinc-binding motif with a recently recognized family of metalloendoproteases. *Proceedings of the National Academy of Sciences, USA* **92,** 7177–7181.

van't Hof R, de Kruijff B. 1995. Transit sequence-dependent binding of the chloroplast precursor protein ferredoxin to lipid vesicles and its implications for membrane stability. *FEBS Letters* **361,** 35–40.

Waegemann K, Soll J. 1991. Characterisation of the protein import apparatus in isolated outer envelopes of chloroplasts. *The Plant Journal* **1,** 149–158.

Waegemann K, Soll J. 1996. Phosphorylation of the transit sequence of chloroplast precursor proteins. *Journal of Biological Chemistry* **271,** 6545–6554.

Wu C, Seibert FS, Ko K. 1994. Identification of chloroplast envelope proteins in close physical proximity to a partially translocated chimeric precursor protein. *Journal of Biological Chemistry* **269,** 32264–32271.