Journal of Bioenergetics and Biomembranes, Vol. 22, No. 6, 1990

MINI-REVIEW

Current Views on Chloroplast Protein Import and Hypotheses on the Origin of the Transport Mechanism[1]

E. Kathleen Archer[2] and Kenneth Keegstra[3]

Received March 21, 1990

Abstract

Most chloroplastic proteins are synthesized as precursors in the cytosol prior to their transport into chloroplasts. These precursors are generally synthesized in a form that is larger than the mature form found inside chloroplasts. The extra amino acids, called transit peptides, are present at the amino terminus. The transit peptide is necessary and sufficient to recognize the chloroplast and induce movement of the attached protein across the envelope membranes. In this review, we discuss the primary and secondary structure of transit peptides, describe what is known about the import process, and present some hypotheses on the evolutionary origin of the import mechanism.

Key Words: Chloroplast; import; protein; evolutionary origin; binding; translocation.

Introduction

Most chloroplastic proteins are synthesized as precursors in the cytosol prior to their transport into chloroplasts. These precursors are generally synthesized in a form that is larger than the mature form found inside

[1] Abbreviations: DHFR, dihydrofolate reductase; EPSP synthase, 5-enolpyrovylshikimate-3-phosphate synthase; hsp heat-shock protein; LHCP II, light-harvesting chlorophyll *a*/*b* binding protein; OEE 16, 23, and 33, the 16-, 23-, and 33-kDa proteins of the oxygen-evolving complex; pr, precursor; rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SS, rubisco small subunit.

[2] Department of Biology, Trinity College, Hartford, Connecticut 06106.

[3] Department of Botany, Birge Hall, 430 Lincoln Drive, University of Wisconsin, Madison, Wisconsin 53703.

0145-479X/90/1200-0789$06.00/0

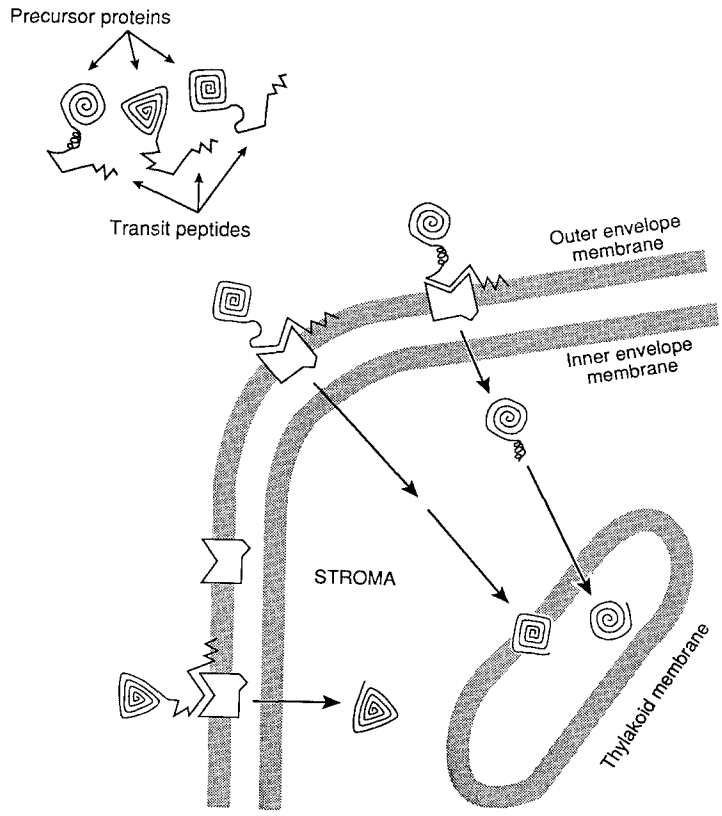

Fig. 1. General model of chloropolast protein import. After synthesis on cytosolic ribosomes, precursors bind to the chloroplast envelope. This binding may involve protein–lipid interactions and one or more envelope proteins. For convenience alone we have simply indicated a single receptor-like protein. After binding, the precursor is translocated across the envelope into the stroma, where it may be processed to mature size, or further targeted to intrachloroplastic compartments.

chloroplasts. The extra amino acids, called transit peptides, are present at the amino terminus. As described in more detail below, this transit peptide is necessary and sufficient to direct import into chloroplasts. Much of the knowledge about details of the import process derives from studies performed using an *in vitro* reconstituted assay with radiolabeled precursor proteins and isolated intact chloroplasts. For example, *in vitro* studies have revealed that import can be divided into several steps (Fig. 1). Precursor proteins first bind to the surface of chloroplasts. The bound precursors are then translocated across the two envelope membranes into the stromal space. During or immediately after translocation, a stromal processing protease removes the import domain of the transit peptide. For stromal proteins,

assembly and/or prosthetic group addition yields a functional enzyme. Thylakoid membrane proteins need to be inserted into the membrane whereas lumenal proteins need to be transported across the thylakoid membrane. The targeting information to direct these additional steps is sometimes found in part of the transit peptide, but can also be found within the mature protein. The structure of transit peptide as well as the information available about each of the steps in the import process is described in more detail below.

Transit Peptide Structure

Chloroplast transit peptides are necessary and sufficient to induce import of the attached protein into chloroplasts. Proteins lacking a transit peptide are not transported, whereas proteins with a transit peptide are successfully translocated across the envelope membranes (Mishkind *et al.*, 1985; Anderson and Smith, 1986). This is true even if the passenger protein is foreign to chloroplasts—chimeric precursor proteins containing a transit peptide at the amino terminus are imported (van den Broeck *et al.*, 1985; Lubben *et al.*, 1986). Any receptor-recognition or transport-induction domain must therefore be located within the transit peptide.

Characterization of Primary Sequences

The nucleic acid sequence of many precursor genes has been determined in recent years, leading to a great deal of information about the sequence of transit peptides (see Keegstra *et al.*, 1989 and von Heijne *et al.*, 1989 for compilations of transit sequences). In general, transit peptides from different precursors have few sequence similarities, although all chloroplast transit peptides share certain characteristics. They contain a high proportion of small aliphatic amino acids such as valine and alanine, and usually have a net positive charge. They also contain 20–35% hydroxylated amino acids and tend to be deficient in glutamate and aspartate, features shared with mitochondrial transit peptides (von Heijne *et al.*, 1989).

Comparison of transit sequences from different plant species show that some homology can be detected when the analysis is confined to a specific protein. Keegstra *et al.* (1989) have aligned the amino acid sequences for 48 different ribulose bisphosphate carboxylase small subunit precursor proteins (prSS) from 22 different plant species. A consensus sequence developed from this analysis is shown in Table I, with a few representative sequences. Keegstra *et al.* noted two di- and tripeptide sequences that are especially conserved (see Table I, denoted by underlining). The most highly conserved

Table. I Amino Acid Sequences of prSS Transit Peptides[a]

CONSENSUS (1):	MASSMLS	SAAVATRTNP	AQASMVAPFT	GLKSAASFPV	SRKQNLDITS	IASNGGRVQC
Tobacco (2):	MASSVLS	SAAVATRSNV	AQANMVAPFT	GLKSAASFPV	SRKQNLDITS	IASNGGRVQC
Pea 3A (3):	MASMISS	SAVTTVSRAS	TVQSAAVAPF	GGLKSMTGFP	VKKVNTDITS	ITSNGGRVKC
Wheat W9 (4):		MAPAVMA	SSATTVAPFQ	GLKSTAGLPI	SGRSGSTGLS	SVSNGGRIRC
Chlamydomonas (5):		MAAVI	AKSSVSAAVA	RPARSSVRPM	AALKPAVKAA	PVAAPAQANQ

[a]The sequences are shown using the standard single-letter code for amino acids. Consensus sequence is the residue that appears at a particular position most frequently. (1) Keegstra *et al.* (1989); (2) Mazur and Chui (1985); (3) Fluhr *et al.* (1986); (4) Broglie *et al.* (1983); (5) Goldschmidt-Clermont and Rahire (1986).

Table II. Amino Acid Sequences of Transit Peptides from Spinach Precursors[a]

STROMAL PROTEINS							
Acyl Carrier Protein (1):	MASLSA	TTTVRVQPSS	SSLHKLSQGN	GRCSSIVCLD	WGKSSFPTLR	TSRRRSFISA	
Nitrite reductase (2):	MA	SLPVNKIIPS	STTLLSSSNN	NRRRNNSSIR			
Phosphoribulokinase (3):	M	AVCTVYTIPT	TTHLGSSFNQ	NNKQVFFNYK	RSSSSNNTLF	TTRPSYVITC	
Rubisco activase (4):	MATAVSTV	GAATRAPLNL	NGSSAGASVP	TSGFLGSSLK	KHTNVRFPSS	SRTTSMTVKA	
THYLAKOID MEMBRANE PROTEINS							
Ferredoxin/NADP reductase (5):	MTTAV	TAAVSFPSTK	TTSLSARSSS	VISPDKISYK	KVPLYYRNVS	ATGKMGPIRA	
LHCP II (6):	MASS	TMALSSPSLA	GKAVKLGPTA	SEIIGEGRIT			
PS II-10kDa protein (7):	M	ATSVMSSLSL	KPSSFGVDTK	SAVKGLPSLS	RSSASFTVRA		
Rieske FeS (8):	MIISIFNQ	LHLTENSSLM	ASFTLSSATP	SQLCSSKNGM	FAPSLALAKA	GRVNVLISKE	RIRGMKLTCQ
THYLAKOID LUMEN PROTEINS							
OEE33 (9): MAAS	LQASTTFLQP	TKVASRNTLQ	LRSTQNVCKA	FGVESASSGG	RLSLSLQSDL	KELANKCVDA	TKLAGLALAT SALIASGANA
OEE23 (10): M	ASTACFLHHH	AAISSPAAGR	GSAAQRYQAV	SIKPNQIVCK	AQKQDDNEAN	VLNSGVSRRL	ALTVLIGAAA VGSKVSPADA
OEE16 (10): MAQ	AMASMAGLRG	ASQAVLEGSL	QISGSNRLSG	PTTSRVAVPK	MGLNIRAQQV	SAEAETSRRA	MLGFVAAGLA SGSFVKAVLA
Plastocyanin (11):	MATVASSAA	VAVPSFTGLK	ASGSIKPTTA	KIIPTTTAVP	RLSVKASLKN	VGAAVVATAA	AGLLAGNAMA

[a]The underlined regions represent the lumenal targeting domains discussed in the text. OEE33, OEE23 and OEE16 are the 33-kDa, 23-kDa and 16-kDa proteins of the oxygen-evolving complex. (1) Scherer and Knauf (1987); (2) Back *et al.* (1988); (3) Roesler and Ogren (1988); (4) Werneke *et al.* (1988); (5) Jansen *et al.* (1988); (6) Mason (1989); (7) Lautner *et al.* (1988); (8) Steppuhn *et al.* (1987); (9) Tyagi *et al.* (1987); (10) Jansen *et al.* (1987); (11) Rother *et al.* (1986).

region is that from −10 through the processing site. This region is similar in both monocots and dicots. Curiously, the sequence for prSS from the green alga *Chamydomonas* has none of the conserved regions seen in higher plants.

Table II shows transit peptide sequences for other proteins targeted to the stroma, and for proteins targeted to the thylakoid membrane and the lumen. Although other stromal proteins are destined for the same compartment as prSS, their transit peptides show no sequence similarity with prSS. These transit peptides range in length from 32 amino acids for spinach nitrite reductase (Back *et al.*, 1988) to nearly 100 amino acids for *Arabidopsis* acetolactate synthase (not shown, Mazur *et al.*, 1987). All contain at least one positively charged residue within 11 amino acids of the processing site, but the conserved di- and tripeptides noted for prSS are not consistently present.

Once inside the chloroplast, precursor proteins may remain in the stroma or they may be directed to one of several other compartments. Two locations that have been studied are the thylakoid membrane and the thylakoid lumen. Further targeting to one of these compartments is accomplished by additional sequences located either within the mature protein itself, or at the carboxy terminus of the transit peptide.

Sequences are available for a number of thylakoid membrane and thylakoid lumen proteins. Most thylakoid lumen transit peptides have a two-domain structure, with a chloroplast targeting domain at the amino terminus followed by a lumenal targeting domain (Smeekens *et al.*, 1985, 1988). Deletion of the second domain presents translocation across the thylakoid membrane (Hageman *et al.*, personal communication). The lumenal targeting domain is very similar to bacterial signal peptides, containing a hydrophobic core flanked on the amino-terminal side by charged amino acids (von Heijne *et al.*, 1989). Like stromal targeting peptides, there is little similarity between sequences from different proteins.

Integral thylakoid membrane proteins contain only chloroplast targeting sequences at their amino termini, and do not contain a second thylakoid targeting domain within the transit peptide. Lamppa (1988) showed that the transit peptide for the chlorophyll *a*/*b* binding protein (LHCP II), an integral thylakoid membrane protein, serves only to target the precursor to the chloroplast, and can functionally be replaced by the transit peptide of SS. The SS transit-LHCP II chimeric protein was correctly targeted, imported, and inserted into the thylakoid membrane, indicating that targeting information for the thylakoid membrane must reside within the mature LHCP II. Hand *et al.* (1989) carried this approach one step further by showing that the LHCP II transit-SS mature chimeric protein remained in the stroma. Kohorn and Tobin (1989) showed that deletions at the carboxy terminus of LHCP II prevented insertion into the thylakoid membrane, suggesting that the internal targeting sequence may reside there. The PSII 10-kDa protein also contains

a chloroplast targeting sequence without a second domain (Webber *et al.*, 1989). Webber noted that the internal sequence for the PSII 10-dDa protein contains a hydrophobic region much like the hydrophobic core of the lumenal targeting domain.

Less is known about proteins targeted to the remaining chloroplast compartments. One precursor protein, the phosphate translocator, has been described which is targeted to the inner envelope membrane (Flügge *et al.*, 1989). Its transit peptide has little in common with other chloroplast targeting sequences. Work is in progress on the isolation and analysis of genes for proteins whose final destination is the outer envelope membrane or the intermembrane space. As yet, however, we have no information on sequences which target to these locations.

Secondary Structure

It is reasonable to expect transit peptides targeting to the same compartment to have some structural features in common. Since similarities in primary structure are not obvious, the next level of protein organization to examine is secondary structure. The four types of secondary structure—alpha helix, beta sheet, reverse turn and coil—can be predicted from primary sequences using several differnt approaches. Current methods suffer from an inability to predict with greater than 50–60% accuracy. As a consequence, discussions of secondary structure are necessarily constrained by a significant degree of uncertainty. Nevertheless, knowledge of predicted trends may be useful in future approaches to structure–function relationships.

For the following discussions, comparisons of secondary structure were made using the algorithms described by Garnier *et al* (1978), and by Chou and Fasman (1978) as modified by Nishikawa (1983). As much as possible, we confine our comments to structures predicted by both methods.

Comparison of the secondary structures predicted for prSS from different species gave the most consistent patterns, perhaps as a natural result of similarity at the primary sequence level. The first 10–15 amino acids at the amino terminus tend toward alpha helix potential. At the carboxy terminus, there is a region of beta sheet potential approximately 4–8 amino acids long, sometimes longer, which extends into the processing site. This region is usually associated with a hydrophobic moment; that is, there is a tendency for one side of the sheet to be hydrophobic and for the other side to be hydrophilic. The beta sheet region is generally preceeded by a reverse turn. Surprisingly, the secondary structure prediction for *Chlamydomonas* prSS, whose primary structure shows very little consensus with that for higher plants, follows the pattern of secondary structure just described.

Other stromal precursors do not exhibit the patterns observed for prSS. Analysis of the transit peptides from seven different stromal proteins including acyl carrier protein, ferredoxin, nitrate reductase, pyruvate phosphate dikinase, 5-enolpyrovylshikimate-3-phosphate (EPSP) synthase, ribulose bisphosphate carboxylase (rubisco) activase, and phosphoribulokinase shows no discernable trends in secondary structure.

The predictions for transit sequences of LHCP II protein are also variable, with some tendency for more alpha helix than beta sheet potential throughout the transit peptide. Similarities are easier to find in the transit peptides of lumenal proteins. We examined plastocyanin and the 33-kDa, the 23-kDa, and the 16-kDa proteins from the oxygen-evolving complex of photosystem II (OEE33, OEE23, and OEE16, respectively). The first domain, which targets the precursor to the chloroplast, tends to show alpha helix potential at the amino-terminal 12 amino acids, usually without hydrophobic moment. In the domain which contains the thylakoid targeting signal, alpha helix potential is favored, particularly at the carboxy terminus. This helical region is also not predicted to contain a hydrophobic moment.

The inability to detect stronger, more specific patterns at the secondary level of organization is probably due to the inaccuracy inherent in current predictive methods. The eventual purification of precursors in quantity, allowing empirical determinations of secondary and tertiary structures may be necessary before patterns can be detected.

Import

Import of precursors into chloroplasts can be divided into two steps; first, binding of the precursor to the chloroplast envelope and second, translocation across the envelope membranes. The two steps can be physically separated by keeping the import reaction cold, or by carrying out the reaction in the presence of very low levels of ATP. Those conditions permit binding, but block translocation. The ability to stop import at the binding step has proved very useful in defining specific requirements and characteristics of each stage.

Binding

As described above, binding can be studied apart from translocation by keeping the reaction mixture of precursors and isolated chloroplasts cold or by maintaining low ATP concentrations (Cline *et al.*, 1985). Warming the reaction or adding ATP causes most of the bound precursor to be imported, indicating that the binding is physiologically significant. ATP is required at

low concentrations for efficient binding, but a membrane potential is not needed (Olsen *et al.*, 1989). Binding specifically requires the presence of a transit peptide; mature proteins lacking the transit peptide bind very poorly (Friedman and Keegstra, 1989). In addition to a precursor and energy in the form of ATP, other components expected to affect binding include the envelope membrane lipids and integral membrane proteins, although their roles are not understood.

ATP Requirement for Binding. Olsen *et al.* (1989) have demonstrated that precursor binding is stimulated 5—10 fold by low levels of ATP. They found that the amount of bound precursors was maximal at approximately 75 μm ATP. Higher ATP concentrations were sufficient to allow low amounts of translocation, which decreased the apparent number of precursors bound. Other nucleotides (CTP, GTP, UTP and ADP) could substitute for ATP, but were less efficient—supporting 55–70% of the level bound in the presence of ATP. Non-hydrolyzable nucleotide analogs did not support binding.

The location of the ATP requirement was shown to be inside the outer envelope membrane, either in the intermembrane space or within the stroma. The intermembrane space and the stroma cannot yet be separated by technical means, so it is not possible to define a more exact location. More recently, Olsen has provided additional evidence that the NTP requirement for binding is located in the intermembrane space (L. Olsen and K. Keegstra, unpublished results). This location makes intuitive sense for a process believed to occur on the chloroplast surface.

The ATP requirement for precursor binding supports the hypothesis that envelope proteins are needed for binding, since it is difficult to imagine how precursor binding to lipids would require ATP. A number of proteins present in the chloroplast envelope could mediate ATP utilization in binding, including protein kinases (Soll, 1988; Soll *et al.*, 1988), ATPases (McCarty *et al.*, 1984), and hsp70 proteins (Marshall *et al.*, 1990). Identification of the ATP-requiring protein will be a key development in determining the role of ATP in binding.

Identification of Receptors. Protease-treated chloroplasts bind precursors with decreased efficiency, providing further evidence that efficient binding requires a protein, possibly a receptor, exposed on the chloroplast surface (Cline *et al.*, 1985). A second piece of evidence favoring a receptor is the observation that binding sites are limited and saturable. Friedman and Keegstra (1989) showed that binding for prSS saturated at between 1500 and 3500 sites per chloroplast. Pfisterer *et al.* (1982) reported a similar number of sites using a pool of various precursors.

Several approaches have been used to identify putative receptor proteins. Cornwell and Keegstra (1987) used a heterobifunctional cross-linker to identify a 66-kDa protein cross-linked to bound prSS. The 66-kDa

protein could represent a receptor, or possibly a component of the transport apparatus in close association with the precursor protein.

Pain *et al.* (1988) used a unique approach to the problem of receptor identification. They raised antibodies against a synthetic peptide based on the carboxy-terminal 30 amino acids of the pea prSS transit peptide. These antibodies were used in turn as antigens to raise anti-idiotypic antibodies. The anti-idiotypic antibodies were found to block import of prSS, presumably because they resembled the three-dimensional shape of the transit peptide and bound at the receptor site. The anti-idiotypic antibodies were then used to probe Western blots of chloroplast proteins. The antibodies detected the larger subunit of rubisco for unexplained reasons, and a 30-kDa envelope protein that Pain *et al.* concluded was the prSS receptor. A 30-kDa envelope protein, present in large quantities, has been identified as a phosphate translocator (Joyard and Douce, 1988). Pain and Blobel (1988) subsequently reported, however, that the protein identified by their antibodies, although of the same molecular weight, was in fact a distinct protein.

Kaderbhai *et al.* (1988) also detected a 30-kDa protein by cross-linking with a synthetic peptide based on the 24 amino-terminal amino acids of wheat prSS. They concluded that their 30-kDa protein was actually the phosphate translocator protein. Perhaps this indicates a role for the phosphate translocator in binding, or perhaps this protein is detected because of its sheer abundance in the envelope. Clearly there is much work remaining in the elucidation of receptors and other components involved in binding.

Lipid Interactions in Binding. Lipids in the outer membrane may also be involved in precursor binding. Protease-treated chloroplasts will bind detectable amounts of precursors (Cline *et al.*, 1985); if one assumes that surface proteins have been digested, the most available alternate components to which a precursor could bind would be lipids.

Binding to lipids has been demonstrated for mitochondrial precursors. Endo and Schatz (1988) found that mitochondrial precursors bound to protein-free artificial membranes, and that this binding was lipid-specific. The precise lipid requirement implies more than simply nonspecific association. Rietveld *et al.* (1986) showed that mitochondrial precursors not only bound to protein-free membranes, but that such precursor-lipid binding can be import productive. Mitochondrial targeting sequences have some features in common with chloroplast transit peptides, suggesting that chloroplast transit peptides too might bind lipids. As yet, information on the binding of chloroplast precursors with protein-free lipid bilayers is currently lacking, but would be extremely useful in defining the role of lipids in the binding step.

General Binding Model. We propose that binding occurs in two steps. The first step is an interaction between envelope membrane lipids and a lipid-interactive domain in the transit peptide. This preliminary binding

would occur without energy input. The precursor then diffuses through the two-dimensional space of the membrane, encountering receptor proteins with a much greater efficiency than possible in the three-dimensional space of the cytosol. The receptor is recognized by a second transit peptide domain, which binds to the receptor protein in a step which requires low levels of ATP. Skerjanc *et al.* (1987) have proposed a similar model for the binding step of mitochondrial precursors.

The model predicts the existence of a transit peptide domain which interacts with lipids. In preliminary experiments, a synthetic peptide corresponding to the first 20 residues at the amino terminus of prSS was found to lyse chloroplasts, suggesting a membrane-active function for this region of the transit peptide (Perry and Keegstra, unpublished results). Edwards *et al.* reported that the first 25 amino acids of the prSS transit peptide would insert into lipid monolayers (Edwards *et al.*, 1988). Since it is expected that lipids are present in greater numbers than receptor proteins, the lipid-binding domain should saturate at higher levels than the receptor-binding domain. This test has yet to be performed. Another prediction is that a synthetic peptide corresponding to the receptor-binding domain should bind in the presence of ATP, saturating at levels similar to those found for precursor proteins. At saturating levels the receptor-binding peptide should block binding of a precursor protein by virtue of having filled all the available binding sites. Preliminary results with synthetic peptides corresponding to the middle region of the prSS transit peptide indicate that they block binding and translocation of full-length precursors (Perry and Keegstra, unpublished results). The use of synthetic peptides shows much promise as a tool for future testing of the binding model.

Translocation

The second stage of import moves the bound precursor across the membranes of the chloropolast envelope. Very little is known about the components of the transport apparatus, or the mechanism by which translocation occurs. Two areas that have been pursued are the energy requirement for translocation, and the conformation of precursors during passage across the envelope. Below we discuss the available results and compare them to findings from mitochondria.

Energy Requirement for Translocation Transport across the envelope requires energy in the form of ATP (Grossman *et al.*, 1980; Cline *et al.*, 1985). The ATP requirement is specific and cannot be substituted for by other nucleotide triphosphates such as CTP or GTP (Flugge and Hinz, 1986; Pain and Blobel, 1987; Schindler *et al.*, 1987). As in binding, the ATP requirement implies involvment of an ATP-utilizing protein; however, the ATP

concentrations necessary to support maximal translocation are at least ten-fold higher than that for maximal precursor binding (Theg *et al.*, 1989). This suggests that the ATP-utilizing activities for these two steps may be different proteins.

The site where ATP is used in translocation has been a matter of some controversy. To determine whether ATP used in import was required outside the chloroplast, Theg *et al.* (1989) controlled the external ATP concentrations with glucose-hexokinase, which rapidly depleted ATP as was demonstrated by assaying for external ATP concentrations. Under conditions where ATP was not detectable outside the chloroplasts, several different precursors were imported at high rates as long as ATP was available in the chloroplast interior. Pain and Blobel (1987), working independently, obtained similar results.

In contrast, Flugge and Hinz (1986) and Schindler *et al.* (1987) reported that protein import required ATP on the outside of the chloroplast. However, Flugge and Hinz depleted external ATP with alkaline phosphatase, an enzyme which also attacks phosphate groups on proteins. Phosphorylation of outer-membrane proteins has been linked with chloroplast protein import (Hinz and Flugge, 1988); the inhibition of import when external ATP was depeleted with this enzyme may actually be related to dephosphorylation events. Resolution of the conflicting results will be important for developing models of the transport mechanism, as ATP used within the chloroplast must be playing a different role than ATP used externally.

Mitochondrial protein transport also requires ATP; recent work indicates that the location of its utilization is internal (Hwang and Schatz, 1989). In addition, mitochondria require a protonmotive force (Chen and Douglas, 1987; Eilers *et al.*, 1987), while chloroplasts do not (Grossman *et al.*, 1980; Flugge and Hinz, 1986; Theg *et al.*, 1989). Since the chloroplast envelope membranes are not capable of generating a protonmotive force, it is not surprising that protein translocation occurs without it. Whether this signals a different translocation mechanism for the two organelles is not yet clear.

Precursor Conformation. The conformational status of the precursor during transloction is of particular interest in the development of transport models. Many reports on the conformation of mitochondrial precursors have argued that the precursor must be unfolded for translocation to occur (for a full discussion of precursor conformation during transport into mitochondria see Verner and Schatz, 1988). In support of this contention, Eilers and Schatz (1986) showed that when a precursor was presented to mitochondria in a tighly folded conformation, translocation was blocked. They used a chimeric protein containing the transit peptide from cytochrome oxidase attached to the amino terminus of dihydrofolate reductase (DHFR). The hybrid protein was correctly targeted and imported. To test the transport

competence of DHFR in its native tertiary conformation, the substrate analog methotrexate was added. Methotrexate binds to the active site of DHFR with an extremely low dissociation constant, producing a rigid DHFR–methotrexate complex similar in shape to the native structure (Matthews *et al.*, 1977; Stammers *et al.*, 1987; Ratnam *et al.*, 1988). Import of this complex was blocked. It was suggested that methotrexate stabilized DHFR in its native tertiary conformation, preventing unfolding and thereby preventing import.

Recently, experiments were conducted with DHFR constructs containing chloroplast transit peptides (Hageman *et al.*, unpublished results). In the presence of methotrexate, the DHFR chimeric protein was imported into isolated chloroplasts with little or no inhibition. Once inside, DHFR molecules imported in the presence of methotrexate were resistant to internal proteases, whereas DHFR imported in the absence of the ligand produced several diagnostic degradation fragments. One interpretation of these results is that the DHFR–methotrexate complex can be imported into chloroplasts, and that the tight folding induced by methotrexate in the ligand binding site confers protease resistance. Since the ligand binding site is lost when DHFR is unfolded, DHFR must be transported in its native, tertiary conformation.

In a similar experiment, della-Cioppa and Kishore (1988) found that in the presence of the substrate analog glyphosate, chloroplast import of EPSP synthase was reduced about 75%, but not completely blocked. Presumably the presence of glyphosate in the active site influenced import efficiency. Della-Cioppa and Kishore suggested that precursor unfolding may be important for efficient translocation. Their results are intriguing in light of the DHFR experiment described above. It should be noted that EPSP synthase is a much larger protein (approximately 55 kDa) than DHFR (approximately 18 kDa). Perhaps molecular flexibility, often reduced in the presence of a ligand, is especially important in the import of larger proteins.

Progress towards a Translocation Model. The movement of proteins across membranes is currently a problem of considerable interest. For chloroplasts and mitochondria, translocation means passage across not one, but two, membranes. In both organelles translocation requires ATP hydrolysis, and in both it is believed to be protein-mediated. Is the mechanism of transport into both the same?

The mitochondrial requirement for a membrane potential has been clearly established and is an obvious difference in comparisons of protein import for the two organelles. Since the chloroplast envelope cannot generate a membrane potential and the mitochondrial envelope can, mitochondrial transport may have taken evolutionary advantage of what was a readily available energy source. The membrane potential may increase the efficiency of the translocation process in a way that does not affect the basic mechanism, or

it may indicate a significant difference in the way mitochondria and chloroplasts import proteins.

Models of mitochondrial protein transport generally propose precursor translocation in an unfolded conformation (Pfanner *et al.*, 1988; Verner and Schatz, 1988). Verner and Schatz have reviewed the data from several experimental approaches which support such models. As discussed above, there is some evidence that proteins may be imported into chloroplasts in a folded conformation. If these observations are substantiated, they would be good evidence in favor of different transport mechanisms.

These differences may be minor variations on a theme, or they may be symptomatic of fundamentally different mechanisms. Understanding how energy is used in transport and clarifing the nature of precursor conformation will be critical in the continued development of translocation models.

Processing

During or after translocation, precursors are processed to their mature size by a stromal processing peptidase. Robinson and Ellis (1984) partially purified a stromal protein from pea which processed prSS to its mature size. The processing activity was associated with a 180-kDa protein and was inhibited by metal chelators such as EDTA. Abad *et al.* (1989) have described a soluble processing enzyme also from pea chloroplasts which cleaved prLHCP to its mature size. The activity was partially purified on a Sephacryl S-300 gel column and was associated with a 240-kDa protein. The same fraction correctly processed prSS as well, suggesting that both stromal and thylakoid membrane precursors may be recognized by the same processing enzyme, or by a group of enzymes with similar properties.

Intrachloroplastic Transport

Insertion of Proteins into the Thylakoid Membrane

Although several thylakoid membrane proteins are synthesized in the cytosol, most transport studies have been conducted with prLHCP II. LHCP II is an integral membrane protein that spans the membrane at least three times (Karlin-Neumann *et al.*, 1985). Current evidence (Cline *et al.*, 1989) supports the hypothesis that prLHCP II is transported into chloroplasts by a process similar to that used for stromal proteins, but is subsequently inserted into the thylakoid membrane (Fig. 1). The insertion process can be reconstituted with isolated thylakoid membranes and *in vitro* synthesized

precursors (Cline, 1986; Chitnis *et al.*, 1987). Such studies have demonstrated that insertion requires energy in the form of ATP, as well as a stromal protein.

Transport to the Thylakoid Lumen

Transport from the cytoplasm to the thylakoid lumen is particularly interesting because it is one of the few situations where proteins cross three membranes. Transport to the lumen occurs in two steps. The first step, transport across the envelope membranes, is similar to the transport of stromal proteins which was discussed in detail above. The second step is transport across the thylakoid membrane. This step has many similarities with the transport of proteins across bacterial membranes. Indeed, it most probably derives from a similar step in cyanobacteria, where lumenal proteins need to be transported across the thylakoid membrane. After the endosymbiotic event, when the gene coding for a protein of the thylakoid lumen was relocated into the nucleus, the thylakoid signal peptide responsible for thylakoid transport was retained and a stromal targeting transit peptide was added on its amino terminus to ensure initial delivery into the stromal compartment. A similar model, known as the conservative sorting hypothesis, has been proposed for the targeting of several proteins to the intermembrane space of mitochondria (Hartl *et al.*, 1989).

As might be expected, a composite transit peptide with two topogenic sequences is needed to accomplish the two transport steps (see discussion above in the section on transit peptide structure). The amino terminal region of the composite transit peptide is necessary for transport into chloroplasts (Smeekens *et al.*, 1986); if it is deleted, transport is abolished. This domain is functionaly equivalent to the transit peptide of stomal precursors as demonstrated by the observation that this region can be replaced by the transit peptide of stromal precursors (Ko and Cashmore, 1989).

The second step is directed by a second domain found at the carboxyl end of the transit peptide. The second domain is necessary, but not always sufficient, for transport across the thylakoid membrane. If the second domain is deleted, the truncated precursor containing only the first domain is transported into chloroplasts, but not across the thylakoid membrane (Weisbeek *et al.*, 1989). When foreign passenger proteins are imported by the prPC transit peptide, they accumulate in the stromal space as intermediate-sized molecules (Smeekens *et al.*, 1986; 1987). At present it is not understood what else is needed to transport foreign proteins across the thylakoid membrane, but one possibility is that mature PC contains additional topogenic information. Howver, if the composite transit peptide of other lumenal precursors are used, transport of some foreign proteins to the thylakoid

lumen can be observed. Both Meadows *et al.* (1989) and Ko and Cashmore (1989) reported the transport of dihydrofolate reductase to the lumen, but Ko and Cashmore reported that glycolate oxidase was not transported to the lumen. Thus, the ability to direct foreign proteins to the thylakoid lumen may depend not only upon the transit peptide employed, but also on the passenger protein.

Recently it has become possible to reconstitute the second step using isolated thylakoid membranes and *in vitro* synthesized precursors (Kirwin *et al.*, 1989; Bauerle and Keegstra, unpublished observations). The availability of this new assay should provide new insights into the details of this step. Results to date indicate that this step requires ATP and results in the accumulation of mature-sized protein within the thylakoid lumen (Kirwin *et al.*, 1989; Bauerle and Keegstra, unpublished observations). Although the two groups used different precursors (precursor to the 33-kD protein of the oxygen-evolving complex by Kirwin *et al.* and prPC by Bauerle and Keegstra), the system is currently limited in that not all lumenal precursors can be transported in this assay.

Evolutionary Origin of the Transport Mechanism

According to the endosymbiotic theory of chloroplast evolution, chloroplasts have descended from free-living photosynthetic prokaryotes. Compelling arguments in favor of the theory have been articulated by Gray and Doolittle (1982), who noted the prokaryotic nature of chloroplastic ribosomal RNA, transcription factors, DNA structure, and gene organization. Comparison of the 16S RNA sequences for cyanobacteria, cyanelles, and chloroplasts indicates that chloroplasts may be derived from a cyanobacterial lineage (Giovannoni *et al.*, 1988).

Though once independent, chloroplasts are now incapable of life on their own. This is due in part to the chloroplast genome, which is no longer complete for all the genes necessary for organelle functions. Many essential genes are now located in the nucleus, and it is thought that during the evolution of the organelle these genes were transferred from the genome of the chloroplast progenitor to their current location. Proteins coded for by these genes are translated on cytosolic ribosomes and are imported into the chloroplast where they function.

A natural question arises, how did the first nuclear-encoded protein get back inside the chloropolast? One can hardly suppose that a specific transport apparatus was in place prior to the need for it. We will consider two possibilities. First, that the original mechanism of entry was spontaneous translocation across the chloropolast envelope. This mechanism required a

precursor protein which had membrane-active properties but did not require interaction with proteins in the membrane. Second, that translocation was mediated by a proteinaceous transport apparatus that already existed for the uptake of other molecules. We hope that examining the possible sources of the modern transport mechanism will be useful in stimulating new approaches to studying chloroplast protein import.

Spontaneous Transport

Many proteins are membrane-active; that is, they can disturb the organization of a lipid bilayer, and may induce their own movement into or through the membrane (Batenburg *et al.*, 1987, 1988; Rietveld *et al.*, 1986). Once could imagine that the first chloroplast gene to migrate to the nuclear genome might have fortuitously integrated behind a genomic sequence coding for a membrane-active peptide. Once synthesized, such a protein could induce its own translocation across the envelope, perhaps by destabilzing the envelope membranes and causing a temporary lipid rearrangement. Over time, a more efficient and specific transport mechanism could have evolved, including receptor proteins and perhaps other transport-facilitating proteins.

Do features of contemporary protein transport support spontaneous translocation as the original mechanism? The key attribute is the ability of transit peptides to disturb lipid bilayers. Development of a protein-mediated transport apparatus may have reduced the need for high membrane activity, so modern transit peptides may no longer exhibit this capability to any great degree. Nevertheless, one might expect transit peptides to retain traces of their ancestral function.

One assay for chloroplast transit peptide membrane activity is the presentation of peptides to protein-free lipid bilayers, followed by analysis of membrane penetration. A preliminary report indicates that a synthetic peptide corresponding to the first 25 amino acids of the prSS transit peptide self-inserted into a protein-free lipid monolayer (Edwards *et al.*, 1987). Mitochondrial proteins, whose targeting sequences have some features in common with these for chloroplasts, are able to bind to and induce translocation through protein-free artificial membranes (Rietveld *et al.*, 1986; Endo *et al.*, 1989). That mitochondrial proteins retain the ability to transport themselves across protein-free membranes does not prove that chloropolast proteins do also, but the findings are suggestive. Chloroplast transit peptides can induce the translocation of an attached protein into yeast mitochondria (Hurt *et al.*, 1986), indicating that transport mechanisms of chloroplasts and mitochondria may be similar, and hence that the transit peptides may share similar functions.

Mediated Translocation

An alternative to spontaneous translocation is transport via an apparatus involved in the import of other molecules. It is not intuitively easy to imagine transport machinery which functions to import small molecules as capable of also transporting large proteins. Yet such transport mechanisms exist, particularly well known in the enteric bacteria.

The bacterial transport machinery for vitamin B12, nucleosides, and iron complexes involves a receptor protein in the outer membrane and at least one, probably more, protein(s) in the cytoplasmic membrane (Fischer *et al.*, 1989). The same transport apparatus has been co-opted by a bacterial defense system to induce the uptake of toxic proteins known as colicins (Sabet and Schnaitman, 1971; Pugsley and Reeves, 1975; Hantke, 1976). The colicin protein, excreted by a competing bacterial strain, induces its own uptake by a susceptible bacterium via the transport apparatus. The susceptible bacterium is then killed by the action of the toxin.

In the first step of colicin translocation, the colicin receptor recognition domain binds to the receptor protein in the outer membrane. This binding does not require a membrane potential (Kadner *et al.*, 1979). The colicin transport induction domain then moves the protein across the outer membrane via an interaction between the receptor protein and the proteins present in the cytoplasmic membrane (Fischer *et al.*, 1989). This step does require energy in the form of a membrane potential (Kadner *et al.*, 1979). Once inside the periplasmic space between the outer and cytoplasmic membranes, a number of colicins then penetrate the inner membrane and form an ion channel, producing toxic effects by the formation of the pore. Other colicins, however, cross the cytoplasmic membrane and act within the cell itself (Pugsley 1984). The mechanism of movement across the cytoplasmic membrane is not yet characterized.

The colicin transport system demonstrates that a translocation apparatus which functions in the transport of small molecules can also be induced to transport proteins. An interesting question is whether cyanobacteria, the presumed chloroplast progenitors, contain such a transport system. Cyanobacteria, like the gram-negative enteric bacteria, possess two membranes defining their exterior, and might be expected to face similar problems in transporting small molecules to the cell interior. Cyanobacteria also take up iron in the form of iron siderophere complexes, complexes which bacteria take up via the transport apparatus described above. In fact, cyanobacteria recognize and transport certain bacterial iron siderophores, suggesting that similar transport mechanisms are possible (Goldman *et al.*, 1983). Whether the cyanobacterial iron translocation apparatus resembles

that used by bacteria and whether it can be induced to take up proteins is unknown, but presents an intriguing area for future investigation.

The question at hand is how a protein which must enter the chloroplast might have taken advantage of such a transport system. Again we must invoke the fortuitous integration of the gene behind nuclear sequences which coded for receptor-recognizing peptides, and which could induce transport of the protein through the existing apparatus. Since both the protein-mediated and spontaneous transport mechanisms require nuclear sequences to facilitate translocation, it is worth noting that many random sequences from both prokaryotic and eukaryotic genomes are capable of causing protein translocation across membranes. Baker and Schatz (1987) found that more than 2.7% of clones generated from *E. coli* DNA could restore correct mitochondrial targeting to a cytochrome *c* oxidase protein that lacked a presequence. Kaiser *et al.* (1987) found that an astonishing 20% of random sequences from the human genome could function as targeting elements for the secreted protein invertase in yeast. The ability of a peptide to target and translocate a protein across a membrane is clearly not a rare one.

We have discussed two possible means for the original transport of a protein into the chloroplast. As yet, there is little direct support for either mechanism. More information on the interaction of chloroplast transit peptides and protein-free lipid bilayers would be useful in the assessment of spontaneous translocation. Identification of chloroplast proteins of the import apparatus and elucidation of their relationships with cyanobacterial and bacterial proteins will be invaluable in understanding the origin of the contemporary transport machinery. As a final note, we may find that early chloroplast protein import used a mixture of membrane perturbation and protein interaction to move across the envelope barrier; it may well be that present chloroplast protein transport employs a similar mechanism.

Acknowledgments

We would like to thank Laura Olsen for her assistance with the tables, and both Laura Olsen and Jenny Dorl for helpful comments on the manuscript.

References

Abad, M. S., Clark, S. E., and Lamppa, G. K. (1989). *Plant Physiol.* **90**, 117–124.
Anderson, S., and Smith, S. M. (1986). *Biochem. J.* **240**, 709–715.
Back, E., Burkhart, W., Moyer, M., Privalle, L. and Rothstein, S. (1988). *Mol. Gen. Genet.* **212**, 20–26.

Baker, A., and Schatz, G. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 3117–3121.
Batenburg, A. M., Hibbeln, J. C. L., and de Kruijff, B. (1987). *Biochim. Biophys. Acta* **903**, 155–165.
Batenburg, A. M., Demal, R. A., Verkleij, A. J., and de Kruijff, B. (1988) *Biochemistry* **27**, 5678–5685.
Broglie, R., Coruzzi, G., Lamppa, G., Keith, B., and Chua, N. H. (1983). *Bio/Technology* **1**, 55–61.
Chen, W. J., and Douglas, M. G. (1987). *Cell.* **49**, 651–658.
Chitnis, P. R., Nechushtai, R., and Thornber, J. P. (1987). *Plant Mol. Biol.* **10**, 3–11.
Chou, P., and Fasman, G. D. (1978). *Adv. Enzymol.* **47**, 45–148.
Cline, K. (1986). *J. Biol. Chem.* **261**, 14804–14810.
Cline, K., Werner-Washburne, M., Lubben, T. H., and Keegstra, K. (1985). *J. Biol. Chem.* **260**, 3691–3696.
Cline, K., Fulsom, D. R., and Viitanen, P. V. (1989). *J. Biol. Chem.* **264**, 14225–14232.
Cornwell, K. L., and Keegstra, K. (1987). *Plant Physiol.* **85**, 780–785.
Della-Cioppa, G., and Kishore, G. M. (1988). *EMBO J.* **7**, 1299–1305.
Edwards, J. V., Bland, J. M., Cornell, D. G., Cleveland, T. E., Landry, S., and Bartlett, S. G. (1988). *Peptides—Chemistry and Biology: Proceedings of the 10th American Peptide Symposium*, ESCOM, pp. 323–324.
Eilers, M., and Schatz, G. (1986). *Nature (London)* **322**, 228–232.
Eilers, M., Oppliger, W., and Schatz, G. (1987). *EMBO J.* **6**, 1073–1077.
Endo, T., and Schatz, G. (1988). *EMBO J.* **7**, 1153–1158.
Endo, T., Eilers, M., and Schatz, G. (1989). *J. Biol. Chem.* **264**, 2951–2956.
Fischer, E., Gunter, K., and Braun, V. (1989). *J. Bacteriol.* **171**, 5127–5134.
Flugge, U. I., and Hinz, G. (1986). *Eur. J. Biochem.* **160**, 563–570.
Flugge, U. I., Fischer, K., Gross, A., Sebald, W., Lottspeich, F. and Eckerskorn, C. (1989). *EMBO J.* **8**, 39–46.
Fluhr, R., Moses, P., Morelli, G., Coruzzi, G. and Chua, N. H. (1986). *EMBO J.* **5**, 2063–2071.
Friedman, A. L., and Keegstra, K. (1989). *Plant Physiol.* **89**, 993–999.
Garnier, J., Osguthorpe, D. J., and Robson, B. (1978). *J. Mol. Biol.* **120**, 97–120.
Giovannoni, S. J., Turner, S., Olsen, G. J., Barns, S., Lane, D. J., and Pace, N. R. (1988). *J. Bacteriol.* **170**, 3584–3592.
Goldman, S. J., Lammers, P. J., Berman, M. S., and Sanders-Loehr, J. (1983). *J. Bacteriol.* **156**, 1144–1150.
Goldschmidt-Clermont, M., and Rahire, M. (1986). *J. Mol. Biol.* **191**, 421–432.
Gray, M. W., and Doolittle, W. F. (1982). *Microbiol. Rev.* **46**, 1–42.
Grossman, A., Bartlett, S., and Chua, N. H. (1980). *Nature (London)* **285**, 625–628.
Hand, J. M., Szabo, L. J., Vasconcelos, A. C., and Cashmore, A. R. (1989). *EMBO J.* **8**, 3195–3206.
Hantke, K. (1976). *FEBS Lett.* **70**, 109–112.
Hartl, F.-U., and Neupert, W. (1989). *J. Cell. Sci. 93 Suppl.* **11**, 187–198.
Hinz, G., and Flugge, U. I. (1988). *Eur. J. Biochem.* **175**, 649–659.
Hurt, E. D., Soltanifar, N., Goldschmidt-Clermont, M., Rochaix, J.-D., and Schatz, G. (1986). *EMBO J.* **5**, 1343–1350.
Hwang, S. T., and Schatz, G. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 8432–8436.
Jansen, T., Rother, C., Steppuhn, J., Reinke, H., Bayreuther, K., Jansson, C., Andersson, B., and Herrmann, R. G. (1987). *FEBS Lett.* **216**, 234–240.
Jansen, T., Reilander, H., Steppuhn, J., and Herrmann, R. G. (1988). *Curr. Genet.* **13**, 517–522.
Joyard, J., and Douce, R. (1988). *Nature (London)* **333**, 306–307.
Kaderbhai, M. A., Pickering, T., Austen, B. M., and Kaderbhai, N. (1988). *FEBS Lett.* **232**, 313–316.
Kadner, R. J., Bassford, P. J., Jr., and Puglsey, A. P. (1979). *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg.* **244**, 90–104.
Kaiser, C. A., Preuss, D., Grisafi, P., and Botstein, D. (1987). *Science* **235**, 312–317.

Karlin-Neumann, G. A., Kohorn, B. D., Thornber, J. P., and Tobin, E. M. (1985). *J. Mol. Appl. Genet.* **3**, 45–61.
Keegstra, K., Olsen, L. J., and Theg, S. M. (1989). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 471–501.
Kirwin, P. M., Meadows, J. W., Shackleton, J. B., Musgrove, J. E., Elderfield, P. D., Mould, R., Hay, N. A., and Robinson, C. (1989). *EMBO J.* **8**, 2251–2255.
Ko, K., and Cashmore, A. R., (1989). *EMBO J.* **8**, 3187–3194.
Kohorn, B. D., and Tobin, E. M. (1989). *Plant Cell.* **1**, 159–166.
Lamppa, G. K. (1988). *J. Biol. Chem.* **263**, 14996–14999.
Lautner, A., Klein, R., Ljungberg, U., Reilander, H., Bartling, D., Andersson, B., Reinke, H., and Beyreuther, K. (1988). *J. Biol. Chem.* **263**, 10077–10081.
Lubben, T. H., and Keegstra, K. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 5502–5506.
Marshall, J. S., DeRocher, A. E., Keegstra, K., and Vierling, E. (1990). *Proc. Natl. Acad. Sci. USA* **87**, 374–378.
Mason, J. G. (1989). *Nucleic Acids Res.* **17**, 5387–5387.
Matthews, D. A., Alden, R. A., Bolin, T. T., and Freer, S. T. (1977). *Science* **197**, 452–455.
Mazur, B. J., and Chui, C. F. (1985). *Nucleic Acids Res.* **13**, 2373–2386.
Mazur, B. J., Chui, C. F., and Smith, J. K. (1987). *Plant Physiol.* **85**, 1110–1117.
McCarty, D. R., Keegstra, K., and Selman, B. R. (1984). *Plant Physiol.* **76**, 584–588.
Meadows, J. W., Shackleton, J. B., Hulford, A., and Robinson, C. (1989). *FEBS Lett.* **253**, 244–246.
Mishkind, M. L., Wessler, S. R., and Schmidt, G. W. (1985). *J. Cell. Biol.* **100**, 226–234.
Nishikawa, K. (1983). *Biochim. Biophys. Acta.* **748**, 285–299.
Olsen, L. J., Theg, S. M., Selman, B. R., and Keegstra, K. (1989). *J. Biol. Chem.* **264**, 6724–6729.
Pain, D., and Blobel, G. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 3288–3292.
Pain, D., and Blobel, G. (1988). *Nature (London)* **333**, 307.
Pain, D., Kanwar, Y. S., and Blobel, G. (1988). *Nature (London)* **331**, 232–237.
Pfanner, N., Hartl, F. U., and Neupert, W. (1988). *Eur. J. Biochem.* **175**, 205–212.
Pfisterer, J., Lachmann, P., and Kloppstech, K. (1982). *Eur. J. Biochem.* **126**, 143–148.
Pugsley, A. P. (1984). *Microbiol. Sci.* **1**, 168–175.
Pugsley, A. P., and Reeves, P. (1975). *J. Bacteriol.* **127**, 218–228.
Ratnam, M., Tan, X., Prendergast, N. J., Smith, P. L., and Freisheim, J. H. (1988). *Biochemistry.* **27**, 4800–4804.
Rietveld, A., Jordi, W., and de Kruijff, B. (1986). *J. Biol. Chem.* **261**, 3846–3856.
Robinson, C., and Ellis, R. J. (1984). *Eur. J. Biochem.* **142**, 337–342.
Roesler, K. R., and Ogren, W. L. (1988). *Nucleic Acids Res.* **16**, 7192.
Rother, C., Jansen, T., Tyagi, A., Tittgen, J., and Herrmann, R. G. (1986). *Curr. Genet.* **11**, 171–176.
Sabet, S. F., and Schnaitman, C. A. (1971). *J. Bacteriol.* **108**, 422–430.
Scherer, D. E., and Knauf, V. C. (1987). *Plant Mol. Biol.* **9**, 127–134.
Schindler, C., Hracky, R., and Soll, J. (1987). *Z. Naturforsch.* **42c**, 103–108.
Skerjanc, I. S., Shore, G. C., and Silvius, J. R. (1987). *EMBO J.* **6**, 3117–3123.
Smeekens, S., and Weisbeek, P. (1988). *Photosyn. Res.* **16**, 177–186.
Smeekens, S., de Groot, M., van Binsbergen, J., and Weisbeek, P. (1985). *Nature (London)* **317**, 456–458.
Soll, J. (1988). *Plant Physiol.* **87**, 898–903.
Soll, J., Fischer, I., and Keegstra, K. (1988). *Planta* **176**, 488–496.
Stammers, D. K., Champness, J. N., Beddell, C. R., Dann, J. G., Eliopoulos, E., Geddes, A. J., Ogg, D., and North, A. C. T. (1987). *FEBS Lett.* **218**, 178–184.
Steppuhn, J., Rother, C., Hermans, J., Jansen, T., Salnikow, J., Hauska, G., and Herrmann, R. G. (1987). *Mol. Gen. Genet.* **210**, 171–177.
Theg, S. M., Bauerle, C., Olsen, L. J., Selman, B. R., and Keegstra, K. (1989). *J. Biol. Chem.* **264**, 6730–6736.

Tyagi, A., Hermans, J., Steppuhn, J., Jansson, C., Vater, F., and Herrmann, R. G. (1987). *Mol. Gen. Genet.* **207**, 288–293.
Van den Broeck, G., Timko, M. P., Kausch, A. P., Cashmore, A. R., Van Montagu, M., and Herrera-Estrella, L. (1985). *Nature (London)* **313**, 358–363.
Verner, K., and Schatz, G. (1988). *Science* **241**, 1307–1313.
von Heijne, G., Steppuhn, J., and Herrmann, R. G. (1989). *Eur. J. Biochem.* **180**, 535–545.
Webber, A. N., Packman, L. C., and Gray, J. C. (1989). *FEBS Lett.* **242**, 435.
Werneke, J. M., Zielinski, R. E., and Ogren, W. L. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 787–791.