

Chloroplast SecA functions as a membrane-associated component of the Sec-like protein translocase of pea chloroplasts

Sam R. HAWARD¹, Johnathan A. NAPIER² and John C. GRAY¹

¹ Department of Plant Sciences and Cambridge Centre for Molecular Recognition, University of Cambridge, Cambridge, England

² IACR-Long Ashton, Department of Agricultural Science, University of Bristol, Bristol, England

(Received 20 May/21 July 1997) – EJB 97 0699/6

Protein cross-linking studies with a thylakoid membrane translocation intermediate were used to demonstrate that chloroplast SecA functions as a membrane-associated component of the Sec-like ATP-dependent protein translocase of pea chloroplasts. In assays with isolated thylakoids, it was observed that translocation of the 33-kDa protein of the oxygen-evolving complex of photosystem II (OE33) decreased when the ATP concentration was low, and that the protein accumulated as a bound precursor. The bound precursor was able to be translocated into the lumen when the ATP concentration was raised, indicating that the precursor was bound to the translocation apparatus. Inclusion of apyrase in the import reaction prevented translocation but did not affect precursor binding to the membrane. When this translocation intermediate was treated with the cross-linking agent disuccinimidyl suberate, a single predominant cross-linked product of 120 kDa was produced. This conjugate could be immunoprecipitated with antibodies to pea chloroplast SecA, identifying the cross-linking partner as SecA. This provides direct evidence for a functional interaction between a thylakoid precursor protein and a component of the thylakoid protein-translocation apparatus.

Keywords: chloroplast; cross-linking; SecA; thylakoid membrane; translocation.

Four distinct pathways for targeting, insertion and translocation of proteins across the chloroplast thylakoid membrane have been identified, each responsible for a specific subset of thylakoid integral or lumen proteins (Cline and Henry, 1996). These pathways can be distinguished by their energetic requirements, by the involvement of different stromal protein factors and by the use of inhibitors (Cline et al., 1992; Hoffman and Franklin, 1994; Hulford et al., 1994; Kapazoglou et al., 1995; Kar-nauchov et al., 1994; Klösger et al., 1992; Li et al., 1995; Mant et al., 1994; Michl et al., 1994; Mould and Robinson, 1991; Mould et al., 1991; Nielsen et al., 1994; Pagan and Cline, 1991). In addition, competition experiments with large amounts of precursor proteins expressed in *Escherichia coli* have confirmed the distinction between these pathways (Cline et al., 1993). The four routes involve an ATP-dependent bacterial-Sec-like system, a Δ pH-dependent system, a chloroplast signal-recognition-particle-like system, and spontaneous insertion.

The chloroplast Sec-like system is responsible for the translocation of nuclear-encoded thylakoid proteins, such as the 33-kDa polypeptide of the oxygen-evolving complex of photosystem II (OE33), plastocyanin and the F subunit of photosystem I (Cline and Henry, 1996), and at least one chloroplast-encoded protein, cytochrome *f* (Mould et al., 1997; Nohara et al., 1996). Translocation of these proteins across the thylakoid membrane is absolutely dependent on the presence of ATP and

a stromal factor (Hulford et al., 1994; Kirwin et al., 1989; Mould et al., 1991). Translocation may be stimulated by a proton-motive force, the principle component of which is Δ pH (Yuan and Cline, 1994). The contribution of the protonmotive force to translocation is most critical when the ATP concentration is low (Mant et al., 1995). The energetic characteristics of this system are very similar to those of the bacterial Sec translocation system, which is responsible for the export of proteins across the cytoplasmic membrane into the periplasm (Driessen, 1992). The bacterial Sec translocase consists of six membrane proteins: SecA, a membrane ATPase; SecYEG, the membrane-embedded pore; and SecD and F, intrinsic membrane proteins with large periplasmic domains (Arkowitz and Bassilana, 1994; Wickner and Leonard, 1996). Pea cytochrome *f*, expressed in *E. coli*, was translocated across the cytoplasmic membrane in a process which was SecA-dependent (Rothstein et al., 1985).

The possibility that homologues of bacterial Sec proteins constituted the thylakoid membrane ATP-dependent translocase was supported by the identification of *secA* and *secY* homologues in algal plastid genomes (Douglas, 1992; Flachmann et al., 1993; Scaramuzzi et al., 1992a, 1992b; Valentin, 1993) and by the inhibition of translocation of OE33, plastocyanin, the F subunit of photosystem I, and cytochrome *f* across the thylakoid membrane by azide (Knott and Robinson, 1994; Mould et al., 1997), which is a specific inhibitor of bacterial SecA (Oliver et al., 1990). The involvement of a chloroplast SecA homologue was confirmed by the purification of SecA from pea leaf chloroplasts (Nakai et al., 1994; Yuan et al., 1994). Chloroplast SecA was shown to be predominantly located in the stroma, where it fulfilled the stromal requirement for translocation into isolated thylakoid membranes. The maize mutant *thal1*, deficient in the targeting and accumulation of thylakoid proteins OE33, plastocyanin and cytochrome *f*, has been positively identified as en-

Correspondence to J. C. Gray, Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, United Kingdom

Fax: +44 1223 333953.

E-mail: jcg2@mole.bio.cam.ac.uk

Abbreviations. OE33, 33 kDa protein of the oxygen-evolving complex of photosystem II; iOE33, an intermediate form of OE33.

Enzymes. Apyrase (EC 3.6.1.5); thermolysin (EC 3.4.24.27).

coding chloroplast SecA (Voelker et al., 1997) and cDNAs encoding chloroplast SecA have been isolated from pea (Kapazoglou and Gray, 1995; Nohara et al., 1995) and spinach (Berghöfer et al., 1995). An *Arabidopsis* cDNA encoding a SecY homologue has been isolated and the translated protein shown to be imported by isolated chloroplasts and targeted to the thylakoid membrane, where it is assumed to function as part of the Sec-like protein-translocation system (Laidler et al., 1995).

In this study we wished to investigate directly the molecular environment of a translocating polypeptide, to identify the proteins involved in the translocation process, and to determine whether the thylakoid membrane translocase is mechanistically similar to the bacterial system. The approach used was to arrest translocation of a radiolabelled thylakoid membrane protein within the translocation complex, so that it could be conjugated to components of the translocase using cross-linking reagents. The protein used was an artificially generated intermediate form of wheat OE33 (iOE33) which represents the substrate for translocation across the thylakoid membrane by the ATP-dependent Sec-like pathway *in vivo* and is efficiently imported into isolated thylakoids *in vitro* (Hulford et al., 1994). It contains the thylakoid-targeting and mature domains and is the stromal intermediate form of the protein following cleavage of the precursor by the stromal processing peptidase.

Energetic conditions were identified in which iOE33 bound to the thylakoid protein-translocation apparatus, without being translocated into the thylakoid lumen. The translocation intermediate was exposed to the cross-linking reagent disuccinimidyl suberate, and cross-linked products were generated. The predominant cross-linking partner was identified as chloroplast SecA by immunoprecipitation with antibodies to pea chloroplast SecA. This indicated that chloroplast SecA functions as a membrane-associated component of the thylakoid protein translocase, as in the bacterial system, and that its role is not limited to that of a stromal factor.

MATERIALS AND METHODS

Materials. Pea seedlings (*Pisum sativum* cv. Feltham First) were grown in Levington compost (Fisons) in a greenhouse with supplementary lighting providing photosynthetically active radiation of 125 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 16 h/day and with peak daytime temperatures of 25–30°C.

SP6 RNA polymerase, 7-methylguanosine(5')triphospho(5')-guanosine and restriction enzymes were all from Boehringer Mannheim UK. Wheatgerm extract for translation *in vitro* and RNase inhibitor were obtained from Promega. The cross-linking reagent, disuccinimidyl suberate, was obtained from Pierce and Warriner. [³⁵S]methionine/[³⁵S]cysteine was supplied by Amersham International. MgATP, potato apyrase grade I, thermolysin, protein-A–Sepharose and *N,N,N',N'*-tetramethylethylenediamine were from Sigma. Q-tips were from Sherwood Medical.

The wheat iOE33 construct consisted of an *EcoRI*–*Bam*HI fragment encoding iOE33 ligated into pGEM4Z (Hulford et al., 1994). Anti-chloroplast SecA serum (Yuan et al., 1994) was obtained from Ken Cline (Plant Molecular and Cellular Biology Program and Horticultural Sciences Department, University of Florida, Gainesville FL, USA).

Preparation of radiolabelled precursors. Wheat iOE33 plasmid template was linearised with *Hind*III and transcribed with SP6 RNA polymerase. The reaction was made up of 2 μg linearised DNA, 2 μl 10 \times transcription buffer, 1 mM ATP/UTP/CTP, 0.2 mM GTP, 40 U RNase inhibitor, 40 U SP6 polymerase, 0.1 mM 7-methylguanosine(5')triphospho(5')guanosine in 20 μl . The mixture was incubated at 37°C for 30 min, after which the

GTP concentration was increased to 1 mM, and the reaction incubated at 37°C for 30 min. The RNA was translated in a wheatgerm translation system consisting of 5 μl wheatgerm extract, 0.2 μl RNase inhibitor (8 U), 0.8 μl 1.0 mM amino acids mixture minus methionine and cysteine, 0.5 μl [³⁵S]methionine/[³⁵S]cysteine (5 μCi , > 1000 Ci/mmol), 0.5 μl 1.0 M potassium acetate, and 0.4 μl transcription products (see above) in 10 μl . The mixture was incubated for 30 min at 25°C and the products used immediately.

Preparation of isolated thylakoids. The procedure used was based on that of Kirwin et al. (1989). 30 g pea shoots, not yet fully expanded, from plants 7–8 days old, were harvested, and the tissue was homogenised with a Polytron (blade size PT20) for 10 s in 200 ml ice-cold buffer A (0.33 M sorbitol, 50 mM Hepes/KOH, pH 8.0). The homogenate was filtered through eight layers of muslin and centrifuged at 2000 \times g for 2.5 min. The supernatant was discarded and the pellet, which contained the chloroplasts, was suspended in 4 ml buffer A using a Q-tip. This suspension was divided and layered onto 4 ml 30% Percoll in buffer A and centrifuged at 2000 \times g for 7.5 min. The pellets, containing intact chloroplasts, were washed in 10 μl buffer A, recombined, and centrifuged at 2000 \times g for 2.5 min. The chloroplast pellet was suspended to 1 mg chlorophyll/ml buffer A. Chloroplasts was pelleted at 10000 \times g for 5 min, suspended in an equal volume of lysis buffer (10 mM Hepes/KOH pH 8.0, 5 mM MgCl₂, 2.5 mM CaCl₂), and lysed on ice for 10–15 min. The thylakoids were pelleted at 10000 \times g for 5 min, and the stromal supernatant was retained. The thylakoids were washed twice in lysis buffer and suspended to the original volume in stroma.

Thylakoid-protein-import assay. The basic thylakoid-import reaction consisted of 40 μl thylakoids (40 μg chlorophyll), 5 μl translation products, 5 μl 100 mM MgATP or 5 μl lysis buffer or 5 μl apyrase (0.4 U/ μl) and 1 μl 150 mM methionine/150 mM cysteine. If a reaction of larger volume was required then these amounts were scaled up. The reaction was incubated for 30 min in an illuminated waterbath at 25°C and was stopped by putting the samples on ice. The thylakoids were washed in 250 μl (5 vol.) lysis buffer and centrifuged for 5 min in a microfuge at 10000 \times g. The thylakoids were suspended in lysis buffer to the original thylakoid volume (40 μl). Thermolysin was added to samples of import reactions to 0.2 mg/ml. The samples were incubated on ice for 40 min and the treatment stopped by the addition of EDTA to 25 mM.

Cross-linking reaction. Disuccinimidyl suberate was dissolved in Me₂SO to make stock solutions that were 20-fold the final required reaction concentrations. Disuccinimidyl suberate was added to thylakoids and incubated on ice for 2 h. Reactions were terminated by quenching the cross-linker with 1 μl 1.0 M Tris/HCl pH 7.5 for 30 min on ice.

Immunoprecipitation. Products of the cross-linking reaction were solubilised in 2% SDS by the addition of 50 μl 10% SDS to 200 μl disuccinimidyl-suberate-treated thylakoids and heating at 95°C for 3 min. 800 μl buffer B (50 mM sodium phosphate pH 7.0, 0.15 M NaCl, 1% Triton X-100, 5 mM EDTA) was added and insoluble material removed by pelleting in a microfuge. Preimmune serum (40 μl) and 60 μl 12.5% (mass/vol.) protein-A–Sepharose were added to the supernatant and mixed by rotation for 1 h at room temperature. The Sepharose beads were collected by centrifugation for 20 s in a microfuge at 10000 \times g. Antiserum to pea chloroplast SecA (40 μl) and 60 μl 12.5% (mass/vol.) protein-A–Sepharose were added to the supernatant, and the suspension was mixed by rotation for 1 h at room temperature. The Sepharose beads were collected and washed five times in 400 μl buffer B and once in 400 μl 50 mM sodium phosphate pH 7.0, 0.15 M NaCl, 0.1% SDS,

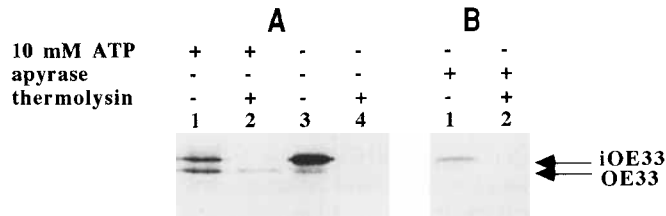


Fig. 1. The effect of ATP concentration on protein binding and translocation. Assays contained 40 μ l isolated thylakoids (40 μ g chlorophyll), 5 μ l [35 S]iOE33, 1 μ l 150 mM unlabelled methionine and cysteine, and 5 μ l 100 mM ATP, 5 μ l lysis buffer or 5 μ l apyrase (2 U). Incubations were for 30 min, in an illuminated waterbath at 25°C. Thylakoids were washed in 5 vol. lysis buffer, isolated and treated with or without thermolysin (0.2 mg/ml for 40 min on ice). (A) Effect of low ATP concentration on thylakoid import. Lanes 1 and 2, assay containing 10 mM ATP; lanes 3 and 4, assay with no added ATP. (B) Effect of apyrase on thylakoid import.

2 mM EDTA. The beads were suspended in 50 μ l 80 mM Tris/HCl, pH 8.8, 10% glycerol, 10% SDS, 0.002% bromophenol blue, 5% 2-mercaptoethanol, and heated for 3 min at 95°C prior to separation by SDS/PAGE.

Sample analysis. The products of thylakoid import and cross-linking reactions were analysed by SDS/PAGE essentially as detailed by Laemmli (1970). Up to 20 μ l protein sample/lane was separated by SDS/PAGE on a Bio-Rad protein minigel system (80 \times 80 \times 1 mm); up to 40 μ l/lane was separated on a large gel (160 \times 160 \times 1 mm). Gels were treated with boiling 5% trichloroacetic acid for 5 min, neutralised for 5 min in 1.0 M Tris, and treated for 15 min in 0.5 M sodium salicylate. The gels were dried onto Whatman 3MM paper and exposed to Fuji RX film at -70°C. The relative intensities of bands on the fluorograms were determined using a Molecular Dynamics 300S laser scanning densitometer.

RESULTS

Apyrase arrests translocation of iOE33. Translocation of OE33 across the thylakoid membrane is dependent on the presence of ATP (Kirwin et al., 1989). The possibility of generating a translocation intermediate by lowering the ATP concentration was investigated. Fig. 1A shows iOE33 translocation into isolated thylakoids in the presence or absence of added 10 mM ATP. After 30 min the thylakoids were isolated and treated with or without thermolysin. A comparison of the thermolysin-treated import products showed that more protease-protected mature OE33 was found in assays containing 10 mM ATP than in assays without added ATP, which was in agreement with previous work. Although some mature OE33 was observed in assays without added ATP, the non-protease-treated samples show that iOE33 mainly accumulated on the thylakoid membrane as a bound, unprocessed precursor. The amount of iOE33 associated with the membrane was greater in the absence of added ATP, than when the reaction contained 10 mM ATP. This suggests that it may be possible to accumulate a translocation intermediate by lowering the ATP concentration.

We examined whether decreasing the ATP concentration further could abolish translocation entirely. This was an important prerequisite to any cross-linking studies, because the interpretation of any observed cross-linked products might be more complicated if precursor protein was translocated across the thylakoid membrane, processed and the mature protein incorporated into its final destination, the oxygen-evolving complex of photosystem II. It might then be unclear whether cross-linked prod-

ucts were the result of cross-linking to the translocation complex or to proteins concerned with later steps.

Apyrase hydrolyses endogenous ATP and has been used previously to investigate the energetic requirement for insertion of the light-harvesting chlorophyll-*a/b*-binding protein into the thylakoid membrane (Cline et al., 1992). Fig. 1B shows the effect of added apyrase, in the absence of added ATP, on iOE33 translocation into illuminated thylakoids at 25°C for 30 min. Thermolysin treatment of the reisolated thylakoids degraded all the radiolabelled protein, showing that translocation across the membrane was stopped entirely; instead, iOE33 accumulated as a bound intermediate on the thylakoid membrane. A separate experiment showed that this bound iOE33 could not be removed simply by repeated washings of the thylakoid membranes with lysis buffer (data not shown).

Bound iOE33 is associated with the translocation apparatus. It was possible that the iOE33 that was bound to the thylakoids when the ATP concentration was low was not interacting with the translocation apparatus and was instead non-specifically associated with the membrane. To determine whether the association was productive, that is the bound precursor protein represented a true translocation intermediate, iOE33 was allowed to bind to thylakoid membranes in assays containing a low concentration of ATP, and was demonstrated to be translocated across the membrane when the ATP concentration was raised. Ideally apyrase would have been included to prevent any translocation during the first incubation; however, apyrase appeared to be associated with the thylakoid membranes after centrifugation, prior to the second incubation in the presence of ATP.

Radiolabelled iOE33 was incubated with isolated thylakoids at 25°C in the light, in the presence or absence of 10 mM ATP. After 30 min, the thylakoids were washed in 5 vol. lysis buffer, pelleted, and suspended in stroma to the original volume; this removed any radiolabelled protein not associated with the thylakoid membrane. ATP was added to both assay mixtures to 10 mM, and the thylakoids were incubated for 30 min under the above conditions. The thylakoids were isolated, washed as before, and suspended in lysis buffer. Aliquots from both incubations were treated with thermolysin. The samples were adjusted to an equal concentration of chlorophyll and separated by SDS/PAGE.

A considerable proportion of iOE33, bound to the thylakoid membrane in the absence of added ATP (Fig. 2), was translocated across the thylakoid membrane when the ATP concentration was increased to 10 mM. This result is most clearly seen when the thermolysin-treated samples are considered. The amount of mature OE33 in the protease-treated thylakoid membranes was low after the first incubation lacking exogenous ATP, compared with the control assay containing 10 mM ATP. However, the amount of protease-protected mature OE33 increased fivefold when ATP was added to the assay for the second incubation. By densitometric analysis of the fluorograms, and allowing for the different number of 35 S-labelled methionine and cysteine residues in the precursor and mature proteins (10 in iOE33, 6 in mature OE33), it can be calculated that 40–45% of the iOE33 bound to the membrane in the absence of ATP was translocated into the lumen on addition of ATP. In the control assays with 10 mM ATP present during the first and second incubations, the amount of mature OE33 did not increase markedly during the second incubation (Fig. 2).

This experiment confirmed that a substantial proportion of iOE33 bound to the thylakoid membrane was productively associated with the translocation machinery. When the ATP concentration was low, iOE33 was unable to be translocated across the thylakoid membrane and accumulated as a bound precursor. When the ATP concentration was subsequently raised to 10 mM,

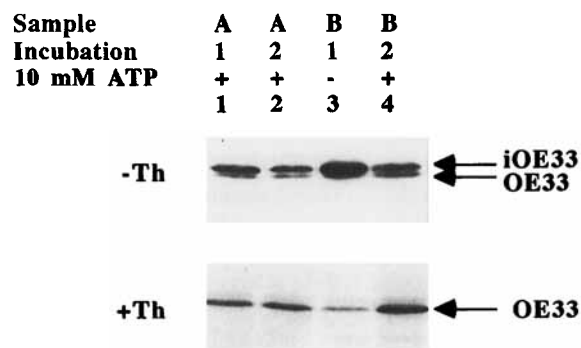


Fig. 2. Intermediate OE33 bound to the thylakoid membrane is associated with the translocation apparatus. 80 μ l isolated thylakoids (80 μ g chlorophyll) were incubated with 10 μ l [35 S]iOE33, 2 μ l 150 mM unlabelled methionine and cysteine, and either 10 μ l 100 mM ATP (sample A) or 10 μ l lysis buffer (sample B), in an illuminated waterbath at 25°C for 30 min (incubation 1). 40 μ l reisolated thylakoids (40 μ g chlorophyll) were suspended in stroma, 5 μ l 100 mM ATP added, and incubated in an illuminated waterbath at 25°C for 30 min (incubation 2); samples A and B were treated identically for the second incubation. Samples (20 μ l) from both the incubations were treated with thermolysin at 0.2 mg/ml (+Th).

this iOE33 was translocated across the membrane to the thylakoid lumen, where it was processed to mature OE33. It was theoretically possible that bound iOE33 dissociated from the thylakoid membrane when the thylakoids were suspended in fresh stromal extract, prior to engaging with the translocation machinery. However, as noted above, bound iOE33 did not dissociate from the membrane during repeated washings of the membrane with lysis buffer. The conclusion was that the bound iOE33 was interacting productively with the translocation machinery.

Cross-linking the translocation-arrested iOE33 with disuccinimidyl suberate. Apyrase was used to arrest iOE33 translocation and the resulting translocation intermediate treated with the cross-linking agent disuccinimidyl suberate, which preferentially cross-links proximal amino groups and has been widely used for conjugating radiolabelled ligands to cell-surface or organelle-surface receptors (Partis et al., 1983; Söllner et al., 1992). Using this approach it was hoped to be able to cross-link the translocation intermediate to constituents of the translocation apparatus.

The translocation intermediate was generated by incubating [35 S]iOE33 with isolated thylakoids in the presence of apyrase for 30 min in an illuminated water bath at 25°C. The thylakoids were washed with 5 vol. lysis buffer and isolated. Disuccinimidyl suberate was added from 20-fold-concentrated solutions in Me₂SO to samples of thylakoids to 0.075 mM and 0.1 mM. These concentrations had been identified previously as near optimal for cross-linking studies in this system (data not shown). The samples were incubated on ice for 2 h, and the disuccinimidyl suberate was quenched with the addition of Tris/HCl, pH 7.5, to 50 mM. Two control reactions were also included in this experiment. To eliminate the possibility that any cross-links generated were attributable to conjugation of iOE33 with components of the wheatgerm translation system, disuccinimidyl suberate was added at 0.1 mM to a 1:4 dilution of translation products in lysis buffer. To demonstrate that the thylakoids had been isolated correctly and were import competent, 35 S-labelled iOE33 was incubated with isolated thylakoids in the presence of 10 mM ATP, for 30 min, in an illuminated waterbath at 25°C, as described in previous experiments. The thylakoids were washed,

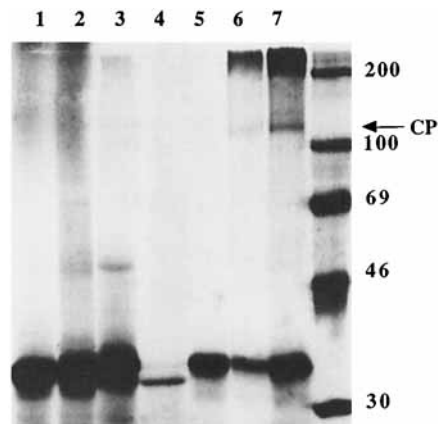


Fig. 3. Generation of cross-linked products with disuccinimidyl suberate. Translocation intermediate was generated by incubating 20 μ l [35 S]iOE33 with 80 μ l (80 μ g chlorophyll) isolated thylakoids in the presence of 10 μ l (4 U) apyrase for 30 min in an illuminated water bath at 25°C. Disuccinimidyl suberate was added from solutions in Me₂SO to 20 μ l reisolated thylakoids. Cross-linking reactions were for 2 h on ice and were stopped by quenching with 1 μ l 1.0 M Tris/HCl pH 7.5, for 30 min on ice. Lane 1, translation reaction products; lane 2, translation products diluted 1:4 with lysis buffer, and treated for 2 h with 0.1 mM disuccinimidyl suberate; lanes 3 and 4, thylakoids incubated with [35 S]iOE33 in the presence of 10 mM ATP were treated without (lane 3) or with (lane 4) thermolysin; lane 5, apyrase-generated translocation intermediate; lanes 6 and 7, cross-linked products (CP) generated by treating translocation intermediate with 0.075 mM (lane 6) or 0.1 mM disuccinimidyl suberate (lane 7).

isolated, divided in two, and one half treated with thermolysin. The products of cross-linking and the controls were separated by SDS/PAGE through a 10% resolving gel (Fig. 3).

As before, iOE33 bound to the membrane, but was not translocated across and processed to mature OE33. In contrast, mature protease-protected OE33 was observed after thermolysin treatment when iOE33 was incubated with isolated thylakoids in the presence of 10 mM ATP, demonstrating that the thylakoids were functional. The products of the cross-linking reactions with disuccinimidyl suberate are shown. In both samples, a discrete cross-linked product of 120 kDa is clearly visible, together with some high-molecular-mass material, which just enters the resolving gel. The translation products and the products of treating the products of the translation reaction with 0.1 mM disuccinimidyl suberate are shown. In neither sample is there a band that accounts for the discrete 120-kDa cross-linked product produced by treating the translocation intermediate with disuccinimidyl suberate.

The conclusion from this experiment was that it was possible to generate a discrete 120-kDa cross-linked conjugate by treating the 35 S-labelled iOE33 thylakoid-membrane-translocation intermediate with the cross-linking agent disuccinimidyl suberate. It was possible that the cross-linking partner was a constituent of the translocation complex.

Immunoprecipitation of cross-linked products. The size of the cross-linked product suggested that the identity of the cross-link partner might be chloroplast SecA. Chloroplast SecA is an approximately 110-kDa protein (Nakai et al., 1994; Yuan et al., 1994) and if cross-linked to iOE33 would be expected to yield a product of approximately 140 kDa. This was not dissimilar to the size of the discrete cross-linked product (\approx 120 kDa) observed in Fig. 3. This cross-linked product was identified by using antibodies to pea chloroplast SecA (Yuan et al., 1994) to immunoprecipitate the cross-linked material.

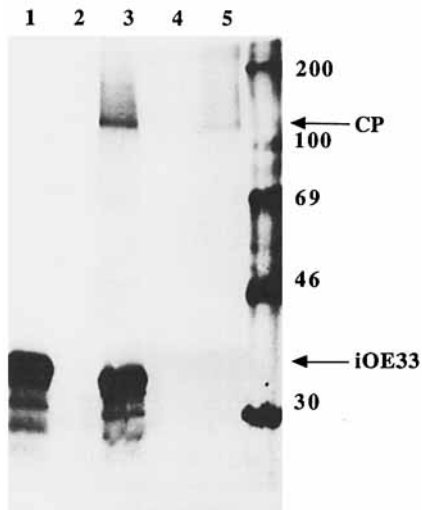


Fig. 4. Immunoprecipitation of cross-linked products with anti-SecA Ig. Translocation intermediate (lane 1) was generated by incubating 60 μ l [35 S]iOE33 with 240 μ l (240 μ g chlorophyll) isolated thylakoids in the presence of 30 μ l (12 U) apyrase, in an illuminated waterbath at 25°C for 30 min. 20 μ l of the products of this incubation were treated with thermolysin (lane 2). Cross-linked products were generated with the addition of 10 μ l 2.0 mM disuccinimidyl suberate in Me₂SO to 200 μ l washed, reisolated thylakoids containing translocation intermediate, and incubating at 4°C for 2 h. The reaction was stopped by the addition of 10 μ l 1.0 M Tris/HCl, pH 7.5. Cross-linked products (200 μ l) were immunoprecipitated sequentially with pre-immune serum (lane 4) and then anti-(chloroplast SecA) Ig (lane 5).

The result of an experiment to resolve the identity of the cross-linked material is shown in Fig. 4. The translocation intermediate was generated as described previously. The reisolated thylakoids were treated with 0.1 mM disuccinimidyl suberate, which was the concentration at which conjugation was most efficient (Fig. 3). The thylakoid membranes were solubilised with SDS and immunoprecipitated sequentially with pre-immune serum and anti-(chloroplast SecA) serum.

The translocation intermediate generated by apyrase treatment was protease sensitive. Treatment with 0.1 mM disuccinimidyl suberate generated a predominant single product of 120 kDa, as previously. Treatment of the SDS-solubilised material with preimmune serum failed to precipitate any of the cross-linked product, whereas the 120-kDa cross-linked product was precipitated by the antibodies to pea chloroplast SecA. The large excess of uncross-linked iOE33 was not immunoprecipitated, confirming the specificity of the SecA immunoprecipitation. The conclusion was that chloroplast SecA was the cross-linking partner in the 120-kDa conjugate.

Further immunoprecipitation reactions were carried out with antibodies to two internal synthetic peptides from the *Arabidopsis* chloroplast SecY protein to attempt to identify possible interactions of iOE33 with another putative component of the translocation apparatus. However, none of the cross-linked products were immunoprecipitated (data not shown). This does not necessarily exclude the presence of chloroplast SecY in these cross-linked products, since it is possible that the epitopes recognised by the antibodies were not accessible in the cross-linked complex.

DISCUSSION

We have demonstrated that there is a direct interaction between iOE33 and chloroplast SecA at the thylakoid membrane

during the early stages of iOE33 translocation, while the protein is still protease accessible. This suggests that chloroplast SecA acts as a component of the translocase as in bacteria (Oliver, 1993) and indicates that its role is not limited solely to that of a stromal factor. Previously, the involvement of a SecA homologue was suggested by the inhibition of OE33 and plastocyanin translocation across the thylakoid membrane by azide, a potent inhibitor of bacterial SecA (Knott and Robinson, 1994). Chloroplast SecA has been purified, and antibodies to chloroplast SecA inhibited translocation of iOE33 across the membrane (Nakai et al., 1994; Yuan et al., 1994). Approximately 80% of chloroplast SecA is located in the stroma, and chloroplast SecA was shown to replace the stromal factor requirement for translocation of iOE33 into isolated thylakoids (Nakai et al., 1994; Yuan et al., 1994). These results strongly suggested that chloroplast SecA was the stromal factor for translocation into the thylakoid lumen along the ATP-dependent pathway.

A chemical cross-linking approach was used to demonstrate a physical interaction between chloroplast SecA and iOE33. A translocation intermediate was generated by including apyrase in the import assay and this was stabilised by cross-linking with disuccinimidyl suberate. When the concentration of disuccinimidyl suberate in the cross-linking reaction was 0.075–0.1 mM, a discrete cross-linked product was observed. This was similar in size to the molecular mass of a putative SecA-iOE33 conjugate and its identity was confirmed by immunoprecipitation with anti-SecA antibodies. A similar method has been successfully used to demonstrate an interaction between mitochondrial precursor polypeptides and 19-kDa and 72-kDa mitochondrial-outer-membrane receptor proteins (Söllner et al., 1992).

The method used to generate the translocation intermediate was derived from the observation that when the ATP concentration in the import assay was low, there was only limited translocation, and the precursor protein instead accumulated as a bound intermediate. A similar result was obtained during studies on bacterial protein-translocation, in which reducing the ATP concentration did not interfere with precursor binding to the cytoplasmic membrane, but substantially reduced translocation efficiency (Swidersky et al., 1990). The similarities between the results obtained from studies on both of these translocation systems suggests that the thylakoid and bacterial translocases are similar mechanistically.

In *Escherichia coli* the precursor protein is conducted by chaperones to SecA, which recognises the signal peptide (Lill et al., 1989), and the precursor protein-SecA complex associates at the cytoplasmic membrane with the SecYEG membrane-embedded translocation pore (Lill et al., 1990). ATP-binding energy is sufficient to drive the translocation of a short segment of the preprotein into the membrane (Schiebel et al., 1991). Concomitant with this translocation event, a 30-kDa domain of SecA is inserted into the membrane so that it becomes protease inaccessible from the cytosolic side of the membrane (Economou and Wickner, 1994). The same work showed that the subsequent deinsertion of this SecA domain and release of SecA from the precursor protein required additional ATP binding and hydrolysis. If proOmpA-translocation reactions were treated with apyrase, several effects were observed. ProOmpA translocation was completely inhibited, there was a 30–40% increase in inserted SecA 30-kDa domain, and this insertion was stable and could not be reversed. The results of this study were confirmed by Rajapandi and Oliver (1996), who demonstrated that SecA proteins defective in the high-affinity ATP-binding domain were found exclusively in the integral membrane form of the protein. It is believed that preprotein-translocation is accomplished by cycles of SecA insertion and deinsertion, accompanied by SecA

disengaging and reengaging with the translocating preprotein (Economou et al., 1995).

The results obtained in this investigation suggest that a similar model may be proposed for thylakoid-membrane-protein translocation. Thylakoid luminal precursor proteins associate with chloroplast SecA, and the protein complex associates with the thylakoid membrane, initiating translocation. Apyrase hydrolyses all the ATP available to the protein-translocation apparatus in the stroma, and, without available ATP, chloroplast SecA is unable to deinsert from the membrane, stalling translocation of iOE33. When the translocation intermediate produced by this method is treated with cross-linking agents, the principle cross-linking partner is chloroplast SecA, because there is insufficient ATP available to release chloroplast SecA from the translocating precursor protein. It seems likely that the initial association of precursor protein with chloroplast SecA takes place in the stroma, since this is the predominant location of chloroplast SecA, supporting the hypothesis developed for the bacterial system that chloroplast SecA cycles on and off the membrane. However it is possible that precursor protein associates with chloroplast SecA that is already bound to the membrane.

In *E. coli*, once translocation has been initiated and SecA has deinserted from the membrane, the next phase of protein translocation begins. A proton-motive force acting across the membrane drives the translocation of the precursor protein through SecYEG preprotein translocase (Schiebel et al., 1991). SecA may reengage with precursor protein to allow multiple rounds of protein translocation. SecA is actively bound to SecYEG (Hartl et al., 1990; Douville et al., 1995) and together these four proteins are capable of reconstituting protein translocation in proteoliposomes (Hanada et al., 1994). The protein translocase has been investigated by cross-linking studies (Joly and Wickner, 1993). A translocation intermediate was generated with a proOmpA-dihydrofolate-reductase fusion protein. This could be cross-linked only to SecA and SecY, suggesting that these are the two nearest neighbours of the translocating preprotein and that the translocating protein passes through an entirely proteinaceous pathway.

A cDNA encoding chloroplast SecY has been identified and the translated protein demonstrated to be imported into isolated chloroplasts (Laidler et al., 1995). It is therefore reasonable to assume that chloroplast SecY operates as an essential part of the thylakoid Sec translocation apparatus. However, while cross-linking to chloroplast SecA was demonstrated in this study, no discrete cross-linked products that corresponded to the size of an iOE33-chloroplast-SecY conjugate (80–90 kDa) were observed in the experiments described (Figs 3 and 4) and antibodies to peptides from chloroplast SecY failed to immunoprecipitate any of the cross-linked complexes (data not shown). This may be because the precursor protein does not insert sufficiently far into the translocation pore for a successful chloroplast SecY cross-link to be generated. Only about 20 residues of proOmpA are translocated into the membrane during the initial SecA-insertion event (Schiebel et al., 1991; Economou and Wickner, 1994). Cross-links to chloroplast SecY or to other constituents of the thylakoid Sec apparatus may be generated either by use of alternative cross-linking agents or by developing strategies for arresting precursor translocation at a later stage of the translocation process.

We thank Colin Robinson, for the wheat iOE33 plasmid construct and antibodies to chloroplast SecY peptides, and Ken Cline, for antibodies to chloroplast SecA. Thanks to Steve High, Martin Poole, Ruth Mould, Paula Row and Alik Kapazoglou, for useful discussions on experiments and results. This work was supported by a Research Studentship funded by the Biotechnology and Biological Sciences Research Council, UK and IACR-Long Ashton, University of Bristol.

REFERENCES

- Arkowitz, R. A. & Bassilana, M. (1994) Protein translocation in *Escherichia coli*. *Biochim. Biophys. Acta* **1197**, 311–343.
- Berghöfer, J., Karnauchov, I., Herrmann, R. G. & Klösgen, R. B. (1995) Isolation and characterisation of a cDNA encoding the SecA protein from spinach chloroplasts: evidence for azide resistance of Sec-dependent protein translocation across thylakoid membranes in spinach. *J. Biol. Chem.* **270**, 18341–18346.
- Cline, K., Etinger, W. F. & Theg, S. M. (1992) Protein-specific energy requirements for protein transport across or into thylakoid membranes: two luminal proteins are transported in the absence of ATP. *J. Biol. Chem.* **267**, 2688–2696.
- Cline, K., Henry, R., Li, C. & Yuan, J. (1993) Multiple pathways for protein transport into or across the thylakoid membrane. *EMBO J.* **12**, 4105–4114.
- Cline, K. & Henry, R. (1996) Import and routing of nucleus-encoded chloroplast proteins. *Annu. Rev. Cell Dev. Biol.* **12**, 1–26.
- Douglas, S. E. (1992) A *secY* homologue is found in the plastid genome of *Cryptomonas* Ø. *FEBS Lett.* **298**, 93–96.
- Douville, K., Price, A., Eichler, J., Economou, A. & Wickner, W. (1995) SecYEG and SecA are the stoichiometric components of preprotein translocase. *J. Biol. Chem.* **270**, 20106–20111.
- Driessen, A. J. M. (1992) Bacterial protein translocation: kinetic and thermodynamic role of ATP and the protonmotive force. *Trends Biochem. Sci.* **17**, 219–223.
- Economou, A. & Wickner, W. (1994) SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell* **78**, 835–843.
- Economou, A., Pogliano, J. A., Beckwith, J., Oliver, D. B. & Wickner, W. (1995) SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecE. *Cell* **83**, 1171–1181.
- Flachmann, R., Michlalsowski, C. B., Löffelhardt, W. & Bohnert, H. J. (1993) SecY, an integral subunit of the bacterial preprotein translocase, is encoded by a plastid genome. *J. Biol. Chem.* **268**, 7514–7519.
- Hanada, M., Nishiyama, K., Mizushima, S. & Tokuda, H. (1994) Reconstitution of an efficient protein translocation machinery comprising SecA and the three membrane proteins, SecY, SecE, and SecG (p12). *J. Biol. Chem.* **269**, 23625–23631.
- Hartl, F.-U., Lecker, S., Schiebel, E., Hendrick, J. P. & Wickner, W. (1990) The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. *Cell* **63**, 269–279.
- Hoffman, N. E. & Franklin, A. E. (1994) Evidence for a stromal GTP requirement for the integration of a chlorophyll *a/b*-binding polypeptide into thylakoid membranes. *Plant Physiol. (Rockv.)* **105**, 295–304.
- Hulford, A., Hazell, L., Mould, R. M. & Robinson, C. (1994) Two distinct mechanisms for the translocation of proteins across the thylakoid membrane, one requiring the presence of stromal factor and nucleotide triphosphates. *J. Biol. Chem.* **269**, 3251–3256.
- Joly, J. C. & Wickner, W. (1993) The SecA and SecY subunits of translocase are the nearest neighbours of a translocating preprotein, shielding it from phospholipids. *EMBO J.* **12**, 255–263.
- Kapazoglou, A. & Gray, J. C. (1995) Characterization of a cDNA for pea chloroplast SecA, in *Photosynthesis: from light to biosphere* (Mathis, P., ed.) vol. III, pp. 751–754, Kluwer Academic Publishers, Dordrecht.
- Kapazoglou, A., Sagliocco, F. & Dure III, L. (1995) PSII-T, a new nuclear encoded luminal protein from photosystem II. *J. Biol. Chem.* **270**, 12197–12202.
- Karnauchov, I., Cai, D., Schmidt, I., Herrmann, R. G. & Klösgen, R. B. (1994) The thylakoid translocation of subunit 3 of photosystem I, the *psaF* gene product, depends on a bipartite transit peptide and proceeds along an azide-sensitive pathway. *J. Biol. Chem.* **269**, 32871–32878.
- Kirwin, P. M., Meadows, J. W., Shackleton, J. B., Musgrove, J. E., Elderfield, P. D., Mould, R. M., Hay, N. A. & Robinson, C. (1989) ATP-dependent import of a luminal protein by isolated thylakoid vesicles. *EMBO J.* **8**, 2251–2255.
- Klösgen, R. B., Brock, I. W., Herrmann, R. G. & Robinson, C. (1992) Proton gradient-driven import of the 16 kDa oxygen-evolving com-

- plex protein as the full precursor protein by isolated thylakoids, *Plant Mol. Biol.* 18, 1031–1034.
- Knott, T. G. & Robinson, C. (1994) The secA inhibitor, azide, reversibly blocks the translocation of a subset of proteins across the chloroplast thylakoid membrane, *J. Biol. Chem.* 269, 7843–7846.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4, *Nature* 227, 680–685.
- Laidler, V., Chaddock, A. M., Knott, T. G., Walker, D. & Robinson, C. (1995) A SecY homologue in *Arabidopsis thaliana*. Sequence of a full-length cDNA clone and import of the precursor protein into chloroplasts, *J. Biol. Chem.* 270, 17664–17667.
- Li, X., Henry, R., Yuan, J., Cline, K. & Hoffman, N. E. (1995) A chloroplast homologue of the signal recognition particle subunit SRP54 is involved in the posttranslational integration of a protein into thylakoid membranes, *Proc. Natl Acad. Sci. USA* 92, 3789–3793.
- Lill, R., Cunningham, K., Brundage, L. A., Ito, K., Oliver, D. & Wickner, W. (1989) SecA protein hydrolyzes ATP and is an essential component of the protein translocation ATPase of *Escherichia coli*, *EMBO J.* 8, 961–966.
- Lill, R., Dowhan, W. & Wickner, W. (1990) The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins, *Cell* 60, 271–280.
- Mant, A., Nielsen, V. S., Knott, T. G., Møller, B. L. & Robinson, C. (1994) Multiple mechanisms for the targeting of photosystem I subunits F, H, K, L, and N into and across the thylakoid membrane, *J. Biol. Chem.* 269, 27303–27309.
- Mant, A., Schmidt, I., Herrmann, R. G., Robinson, C. & Klösgen, R. B. (1995) Sec-dependent thylakoid protein translocation: Δ pH requirement is dictated by passenger protein and ATP concentration, *J. Biol. Chem.* 270, 23275–23281.
- Michl, D., Robinson, C., Shackleton, J. B., Herrmann, R. G. & Klösgen, R. B. (1994) Targeting of proteins by bipartite presequences: C_FOII is imported by a novel, third pathway, *EMBO J.* 13, 1310–1317.
- Mould, R. M. & Robinson, C. (1991) A proton gradient is required for the transport of two luminal oxygen-evolving proteins across the thylakoid membrane, *J. Biol. Chem.* 266, 12189–12193.
- Mould, R. M., Shackleton, J. B. & Robinson, C. (1991) Transport of proteins into chloroplasts: requirements for the efficient import of two luminal oxygen-evolving complex proteins into isolated thylakoids, *J. Biol. Chem.* 266, 17286–17289.
- Mould, R. M., Knight, J. S., Bogsch, E. & Gray, J. C. (1997) Azide-sensitive thylakoid membrane insertion of chimeric cytochrome *f* polypeptides imported by isolated pea chloroplasts, *Plant J.* 11, 1051–1058.
- Nakai, M., Goto, A., Nohara, T., Sugita, D. & Endo, T. (1994) Identification of the SecA protein homolog in pea chloroplasts and its possible involvement in thylakoidal protein transport, *J. Biol. Chem.* 269, 31338–31341.
- Nielsen, V. S., Mant, A., Knoetzel, J., Møller, B. L. & Robinson, C. (1994) Import of barley photosystem I subunit N into the thylakoid lumen is mediated by a bipartite presequence lacking an intermediate processing site: role of the delta pH in translocation across the thylakoid membrane, *J. Biol. Chem.* 269, 3762–3766.
- Nohara, T., Nakai, M., Goto, A. & Endo, T. (1995) Isolation and characterization of the cDNA for pea chloroplast SecA. Evolutionary conservation of the bacterial-type SecA-dependent protein transport within chloroplasts, *FEBS Lett.* 364, 305–308.
- Nohara, T., Asai, T., Nakai, M., Sugiura, M. & Endo, T. (1996) Cytochrome *f* encoded by the chloroplast genome is imported into thylakoids via the SecA-dependent pathway, *Biochem. Biophys. Res. Commun.* 224, 474–478.
- Oliver, D. B., Cabelli, R. J., Dolan, K. M. & Jarosik, G. P. (1990) Azide-resistant mutants of *Escherichia coli* alter the SecA protein, an azide-sensitive component of the protein export machinery, *Proc. Natl Acad. Sci. USA* 87, 8227–8231.
- Oliver, D. B. (1993) SecA protein: autoregulated ATPase catalysing pre-protein insertion and translocation across the *Escherichia coli* inner membrane, *Mol. Microbiol.* 7, 159–165.
- Partis, M. D., Griffiths, D. G., Roberts, G. C. & Beechey, R. B. (1983) Cross-linking of protein by ω -maleimido alkanoyl *N*-hydroxysuccinimido esters, *J. Protein Chem.* 2, 263–277.
- Payan, L. A. & Cline, K. (1991) A stromal protein factor maintains the solubility and insertion competence of an imported thylakoid membrane protein, *J. Cell Biol.* 112, 603–613.
- Rajapandi, T. & Oliver, D. (1996) Integration of SecA protein into the *Escherichia coli* inner membrane is regulated by its amino-terminal ATP-binding domain, *Mol. Microbiol.* 20, 43–51.
- Rothstein, S. J., Gatenby, A. A., Willey, D. L. & Gray, J. C. (1985) Binding of pea cytochrome *f* to the inner membrane of *Escherichia coli* requires the bacterial SecA gene product, *Proc. Natl Acad. Sci. USA* 82, 7955–7959.
- Scaramuzzi, C. D., Hiller, R. G. & Stokes, H. W. (1992a) Identification of a chloroplast-encoded *secA* gene homologue in a chromophytic alga: possible role in chloroplast protein translocation, *Curr. Genet.* 22, 421–427.
- Scaramuzzi, C. D., Stokes, H. W. & Hiller, R. G. (1992b) Characterisation of a chloroplast-encoded *secY* homologue and *atpH* from a chromophytic alga, *FEBS Lett.* 304, 119–123.
- Schiebel, E., Driessen, A. J. M., Hartl, F.-U. & Wickner, W. (1991) $\Delta\mu_{H^+}$ and ATP function at different steps of the catalytic cycle of preprotein translocase, *Cell* 64, 927–939.
- Söllner, T., Rassow, J., Wiedmann, M., Schlossmann, J., Keil, P., Neupert, W. & Pfanner, N. (1992) Mapping of the protein import machinery in the mitochondrial outer membrane by cross-linking of translocation intermediates, *Nature* 355, 84–87.
- Swidersky, U. E., Hoffschulte, H. K. & Müller, M. (1990) Determinants of membrane-targeting and transmembrane translocation during bacterial protein export, *EMBO J.* 9, 1777–1785.
- Valentin, K. (1993) SecA is plastid-encoded in a red alga: implications for the evolution of plastid genomes and the thylakoid protein import apparatus, *Mol. Gen. Genet.* 236, 245–250.
- Voelker, R., Mendel-Hartvig, J. & Barkan, A. (1997) Transposon-disruption of a maize nuclear gene, *thal1*, encoding a chloroplast SecA homologue: *in vivo* role of chloroplast SecA in thylakoid protein targeting, *Genetics* 145, 467–478.
- Wickner, W. & Leonard, M. R. (1996) *Escherichia coli* preprotein translocase, *J. Biol. Chem.* 271, 29514–29516.
- Yuan, J. & Cline, K. (1994) Plastocyanin and the 33-kDa subunit of the oxygen-evolving complex are transported into thylakoids with similar requirements as predicted from pathway specificity, *J. Biol. Chem.* 269, 18463–18467.
- Yuan, J., Henry, R., McCaffery, M. & Cline, K. (1994) SecA homolog in protein transport within chloroplasts: evidence for endosymbiont-derived sorting, *Science* 266, 796–798.