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Evidence that a Chloroplast Surface Protein Is Associated with a Specific Binding Site for the Precursor to the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase[1]

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ABSTRACT

Most chloroplast proteins are encoded by nuclear genes and synthesized in the cytoplasm as higher molecular weight precursors. These precursors are imported posttranslationally into the chloroplasts, where they are proteolytically processed, and sorted to their proper locations. The first step of this import process is thought to be the binding of precursors to putative receptors on the outer envelope membrane of chloroplasts. We have investigated the interaction of the precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase with its putative receptor by using a heterobifunctional, photoactivatable cross-linker. The resulting cross-linked conjugate has a molecular weight of 86,000, and is present on the surface of chloroplasts as determined by its sensitivity to digestion with protease. Control experiments demonstrated that the label in the conjugate is derived from small subunit precursor and that the conjugate is formed only when modified precursor is reacted in the presence of chloroplasts. Based on these results, we postulate that a protein on the surface of chloroplasts is part of the receptor which interacts with the small subunit precursor.

Most proteins found in chloroplasts and mitochondria are nuclear encoded and synthesized on cytoplasmic ribosomes. The majority of these proteins are made as higher mol wt precursors which are then imported into the respective organelle, proteolytically processed to the mature size and sorted to their proper location (*cf.* 9 and 21 for recent reviews). The first step of this import process is thought to be the recognition and binding of the precursor protein to a specific receptor on the surface of the proper organelle. The most extensive evidence for the involvement of specific receptors comes from the study of mitochondrial protein import (9). One type of evidence for the involvement of mitochondrial surface proteins comes from the inhibition of protein import by pretreatment of mitochondria with proteases (19, 23, 24). Another more direct approach has been to measure the specific binding of precursor proteins to putative receptors on the surface of isolated mitochondria or on mitochondrial membranes (10, 11, 17, 19). Similar strategies have been used to study the binding of precursor proteins to putative receptors on the surface of chloroplasts. Cline *et al.* (5) examined the binding of two specific precursor proteins, prSS[3] and the precursor to the Chl *a/b* binding protein, to the surface of isolated intact chloroplasts and provided evidence that these interactions were part of the import process. They also demonstrated that protease pretreatment of chloroplasts diminished, but did not eliminate, binding and import of these two precursors. Pfisterer *et al.* (18) examined the binding of a mixture of precursor proteins to isolated chloroplast envelope membranes. More recently, Bitsch and Kloppstech (3) provided evidence that envelope receptor proteins can be solubilized with detergents and reconstituted into synthetic membranes.

Despite the studies described above, we still know very little about the putative receptor proteins or their role in the import process. For example, a receptor protein has not been identified in either mitochondria or chloroplasts. The lack of rigorous proof for the presence of receptors, combined with the observation that precursors can interact with lipids (20), has led to the suggestion that precursor proteins may interact directly with lipid bilayer membranes without the involvement of proteinaceous receptors (20, 22). Thus, it becomes important to determine whether proteinaceous receptors exist, to identify the proteins which function as receptors, and to investigate their involvement in the import process. It should be possible to examine precursor/receptor interactions and to identify the receptor proteins using some of the techniques that have been applied to other receptor/ligand interactions, such as the well characterized interactions between polypeptide hormones and their receptors. One approach which has been successfully applied to the problem of receptor identification is the use of photoaffinity cross-linkers (4, 7, 12, 13). Photoaffinity cross-linkers can be sequentially controlled and therefore are more specific than homobifunctional cross-linkers (7, 12). The general strategy is to react a photoactivatable bifunctional reagent with some functional group on the ligand. The modified ligand is then allowed to interact with its receptor in a specific manner. Photoactivation leads to the formation of a cross-link between the ligand and the receptor. We have employed this strategy toward identifying a putative receptor protein on the surface of the chloroplasts.

MATERIALS AND METHODS

Percoll and thermolysin were purchased from Sigma. Phosphocreatine (di K^+ salt) was from Calbiochem. [^{3}H]Leucine was from New England Nuclear, *p*-APB and all other cross-linking agents used from Pierce Chemical Co., Rockford, IL, and ^{14}C-

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[3] Abbreviations: prSS, precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase; *p*-APB, *p*-azidophenylacyl bromide; SS, small subunit of ribulose-1,5-bisphosphate carboxylase; MOPS, 4-morpholinepropanesulfonic acid.

protein mol wt markers from Bethesda Research Labs.

Chloroplast Isolation. The peas were grown and chloroplasts isolated as previously described by Cline *et al.* (5) with the following modifications. All buffers contained MOPS, at the same concentration and pH, instead of Hepes. The Percoll gradient was formed by mixing 15 ml Percoll and 15 ml 2-fold concentrated grinding buffer in a centrifuge tube and centrifuging at 42,000*g* for 30 min. Chloroplasts were used at a concentration corresponding to a Chl concentration of 1 mg/ml ($2–5 \times 10^8$ chloroplasts per ml).

Precursor Synthesis. Messenger RNA was extracted and hybrid select mRNA for prSS was isolated as described by Cline *et al.* (5). In some cases mRNA coding for prSS was prepared by *in vitro* transcription of a cloned prSS gene. A plasmid containing the gene for pea prSS was obtained from A. Cashmore. A plasmid coding for the mature SS was prepared by T. Moore in our laboratory. Plasmid DNA was purified and the *in vitro* transcriptions were performed as previously described by Lubben and Keegstra (14). Tritium labeled proteins were synthesized in a cell-free wheat germ system as described previously by Cline *et al.* (5) with the following modifications. Phosphocreatine (di K^+ salt) was substituted for phosphocreatine (di tris salt), and protein synthesis was terminated by chilling to 0°C.

Precursor Modification. All the procedures of precursor modification, binding and cross-linking were carried out under dim green light at 20°C unless otherwise noted. Samples of ^{3}H-prSS in 25 μl of wheat germ cell-free translation mixture in 10 × 75 mm glass tubes were reacted with 2.5 μl of various *p*-APB solutions in methanol to give final *p*-APB concentrations of 0.01, 0.1, and 1 mM in 9.1% methanol. The concentrations of other components in the reaction mix were: 7.2 mM Hepes, 1.13 mM DTT, 30.77 μM amino acids, and wheat germ protein ≈ 40 A_{260} units/ml. Control ^{3}H-prSS samples (25 μl) without *p*-APB were treated with 2.5 μl methanol. After the *p*-APB modification reaction had proceeded for 15 min, 27.5 μl of 60 mM leucine in 2× import buffer was added.

Binding and Cross-linking Reactions. Following the addition of import buffer (165 μl) to the *p*-APB modified ^{3}H-prSS, 80 μl of a chloroplast suspension containing 1 mg Chl/ml were added and the modified ^{3}H-prSS was bound to the chloroplasts for 10 min. After centrifugation of the chloroplasts for 2 min at 500*g*, the supernatant, which contained wheat germ extract, and unbound ^{3}H-prSS, was discarded. The chloroplasts were resuspended in 300 μl of import buffer and placed in a glazed porcelain 12 place spot plate (Coors 550). The azido group of the cross-linker was converted to the nitrene form by illumination with short wave UV light (Mineral Light II) (254 nm) at a distance of 3 cm for 5 min. Control samples were also irradiated with UV light, unless otherwise noted.

Analysis of Cross-linked Products. The chloroplasts were repurified by layering on a 40% Percoll solution and centrifuging at 1800*g* for 4 min. The pellet of intact chloroplasts was resuspended in 1 ml import buffer. The chloroplasts were again centrifuged at 500*g* for 2 min, the supernatant was removed and the pellet subjected to SDS-PAGE as described below.

Chloroplast samples, which were to be protease treated, were centrifuged for 2 min at 500*g*, the supernatant discarded, and the chloroplasts resuspended in 250 μl of import buffer. Thermolysin (2 mg/ml in import buffer with 10 mM $CaCl_2$) was added to give final concentrations of 100 or 200 μg/ml and the incubation was allowed to proceed for 10 min at 20°C. The protease treatment was stopped by the addition of 25 μl of 50 mM EDTA in import buffer. The chloroplasts were then repurified as described above except all buffers contained 5 mM EDTA (6).

Samples were analyzed by SDS-PAGE as in Cline *et al.* (5) except with a 5 to 15% linear acrylamide gradient accompanied by a 5 to 17.5% sucrose gradient. The ^{14}C-labeled mol wt markers used were myosin (205,000), phosphorylase *b* (97,400), BSA (68,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), and lysozyme (14,300). The mol wt of cross-linked products were estimated using a standard curve of R_F *versus* Log M_r for marker proteins. Quantitation of the amount of ^{3}H in gel bands and molecules prSS/chloroplast was conducted as in Cline *et al.* (5).

RESULTS AND DISCUSSION

Formation of a Conjugate by Cross-linking. There were several reasons for choosing prSS for the studies reported here. First, it is probably the most abundant protein imported into chloroplasts and therefore its import system might be expected to be one of the most active and its receptor one of the most abundant. Second, it is possible to prepare radiochemically pure prSS in more than one way. It can be prepared by *in vitro* translation of mRNA purified by hybrid selection (5) or by *in vitro* expression of cloned prSS genes (14). Finally, prSS was chosen because the binding of prSS to isolated chloroplasts has been characterized in some detail (5; A Friedman, K Keegstra, unpublished data).

Radiochemically pure prSS prepared in a wheat germ translation system was used as the starting material for these experiments. The cross-linking process involved three sequential steps (Fig. 1). In the first step a photoactivatable cross-linking reagent, *p*-APB, was reacted in the dark with ^{3}H-prSS. In the second step, modified ^{3}H-prSS was bound to chloroplasts in the dark. Chloroplasts were washed and all unbound material in the supernatant was discarded. Finally, chloroplasts containing bound ^{3}H-prSS were treated with UV light to bring about the cross-linking reaction. Although the cross-linking reagent certainly reacted with many different proteins from the wheat germ extract used for translation, only prSS should bind specifically to the surface of chloroplasts, and only radiolabeled prSS should be observed during fluorographs of the cross-linked products. Several different cross-linking reagents were examined (see below), but the best results were obtained with *p*-APB, a photoactivatable heterobifunctional reagent which reacts with free sulfhydryl groups.

Analysis of the reaction products by SDS-PAGE and fluorography demonstrated that a specific high mol wt conjugate was formed (Fig. 2). The formation of a high mol wt (86,000; Fig. 2B) conjugate was completely dependent upon the presence of *p*-APB in the reaction mixture. Control reactions lacking *p*-APB did not show this conjugate (Fig. 2A, lane 2; Fig. 2C, lane 3). Moreover, conjugate formation was dependent upon the concentration of *p*-APB used in step 1. Increasing the concentration of *p*-APB from 0.01 to 1.0 mM resulted in increased amounts of conjugate (Fig. 2A, lanes 3–5; Fig. 2C, lanes 1 and 2). Raising the *p*-APB concentration to 10 mM caused a decrease in conjugate formation (not shown). The formation of cross-linked conjugate was also dependent upon the presence of chloroplasts. Illumination of the *p*-APB-modified prSS with UV light in the absence of chloroplasts did not lead to the formation of the high mol wt conjugate (Fig. 2A, lane 6; Fig. 2C, lane 5).

Other bands, for example fainter bands at 100, 77, and 45 kD can also be seen in *p*-APB cross-link experiments (Fig. 2A, lanes 4 and 5; Fig. 2C, lanes 1 and 2). Similar bands are sometimes also seen in control (no *p*-APB) experiments, although at a much lower intensity (Fig. 2A, lane 2; Fig. 2C, lane 3). The 86 kD band has never been observed in any of the control samples. The faint band at 45 kD rarely showed enhancement with increasing concentration of *p*-APB (it does in Fig. 2A, but not in Fig. 2C and in several other experiments). The 77 kD and 100 kD bands always increased with increasing *p*-APB. Because similar bands are occasionally seen in controls, it is possible they represent some form of aggregate of prSS, and that formation of this aggregate is enhanced by the presence of *p*-APB. It is also possible that the 77 and 100 kD bands produced by *p*-APB are related to

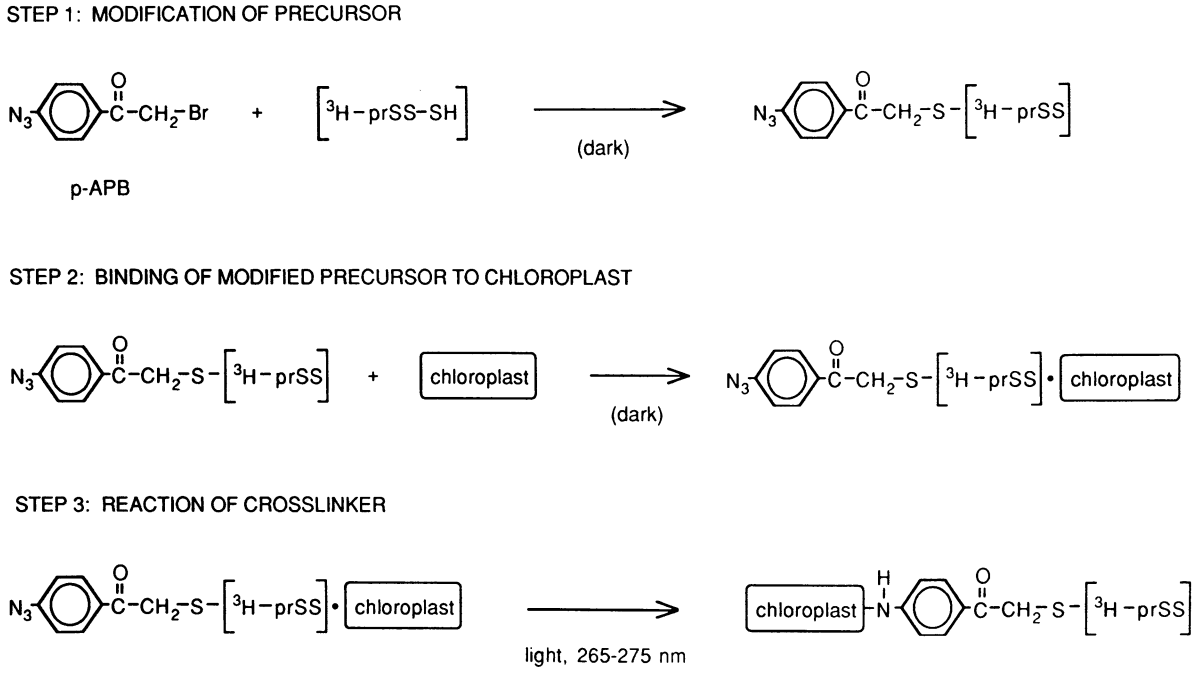

FIG. 1. Diagram depicting the strategy for the cross-linking experiments.

the 86 kD conjugate, but migrate differently because of a different conformation caused by multiple cross-links or by cross-links between different residues on the respective proteins.

It was important to ensure that the radiolabel in the conjugate is derived from prSS and not from some contaminant in the translation reaction or from a translation product arising from some minor mRNA present in the purified prSS mRNA. To investigate the first point, an *in vitro* translation reaction without any mRNA was used in a cross-linking reaction. Under these conditions, no conjugate is formed (data not shown). From this result we conclude that the labeled conjugate is not formed by a contaminant in the translation mixture. To investigate the second possibility, prSS was produced by *in vitro* translation of mRNA produced by *in vitro* transcription of a cloned prSS gene (14). Under these conditions the 86 Kd conjugate is also formed (Fig. 2, panel C). The amount of conjugate formed was always lower when cross-linking was performed with prSS produced by expression of a cloned gene (compare panels A and C of Fig. 2). The reason for this decrease is unknown, but one possible explanation is that some component from the *in vitro* transcription reaction inhibits cross-linking. Despite the quantitative difference, qualitatively the same pattern was seen, *i.e.* the 86 kD conjugate was the major product and the 77 and 100 kD bands were minor products. The observation that the 86 kD conjugate is formed regardless of the method used to produce prSS, allows us to conclude that the label in the conjugate is not derived from a contaminant in the hybrid select mRNA.

In an effort to determine whether the cross-linking was specific to prSS, cross-linking was conducted with mature SS. Tritium-labeled mature SS was prepared by *in vitro* transcription and translation of a cloned prSS gene that had the transit sequence removed. *In vitro* transcription of this gene yielded a truncated RNA that had the methionine codon at the amino terminus of mature SS as the first methione codon in the transcript. Translation of this RNA yielded a protein identical in size to mature SS. When cross-linking experiments were conducted with this form of SS, no binding of SS was detected nor was any conjugate formed (data not shown). From these results, we conclude that the presence of the transit peptide is essential for the formation of the conjugate. Since the transit peptide is essential for the import process, but not for other interactions of SS (*e.g.* assembly with LS), this result provides evidence that the observed conjugate is an interaction formed during the import process.

Protease Sensitivity of the Conjugate. We sought to determine whether the conjugate was still on the surface of the chloroplast or whether some or all of it might have been internalized. Isolated chloroplasts containing the conjugate were subjected to protease treatment under conditions that had previously been shown to digest only outer membrane proteins (6). Mild protease treatment resulted in digestion of the 86 kD conjugate as well as the other bands which appear at higher and lower mol wt (Fig. 3, lane 4). From this result we conclude that the conjugate was still present on the surface of chloroplasts.

Influence of Precursor Modification upon Import. The reaction of prSS with *p*-APB decreases its ability to be imported. This conclusion can be seen from the results presented in Figure 3. In control reactions some mature SS was observed inside the chloroplasts in a protease protected form (Fig. 3, lanes 1 and 3). This import was caused in part by the UV light treatment since controls without UV treatment showed reduced or no import (not shown). In prSS samples treated with *p*-APB, the extent of import was greatly reduced (Fig. 3, lanes 2 and 4). From this we conclude that the *p*-APB modified precursors are imported poorly or possibly not at all. Because modified precursors still are able to bind to the surface of chloroplasts, further studies will be required to understand the step in import which is blocked by modification of the precursors.

Efforts to Increase the Yield of Conjugate. In the experiments described above, only 3 to 5% of the 3H-prSS used in an experiment is found in the cross-linked conjugate. Several explanations for this low recovery are possible. First, DTT present in the translation mixture reacts directly with *p*-APB, lowering the yield of modified precursor. Second, the arylazide is unstable in the presence of DTT, and is reduced to the amine which is no longer photoreactive (7). These problems could be overcome by performing the modification reaction in the absence of DTT.

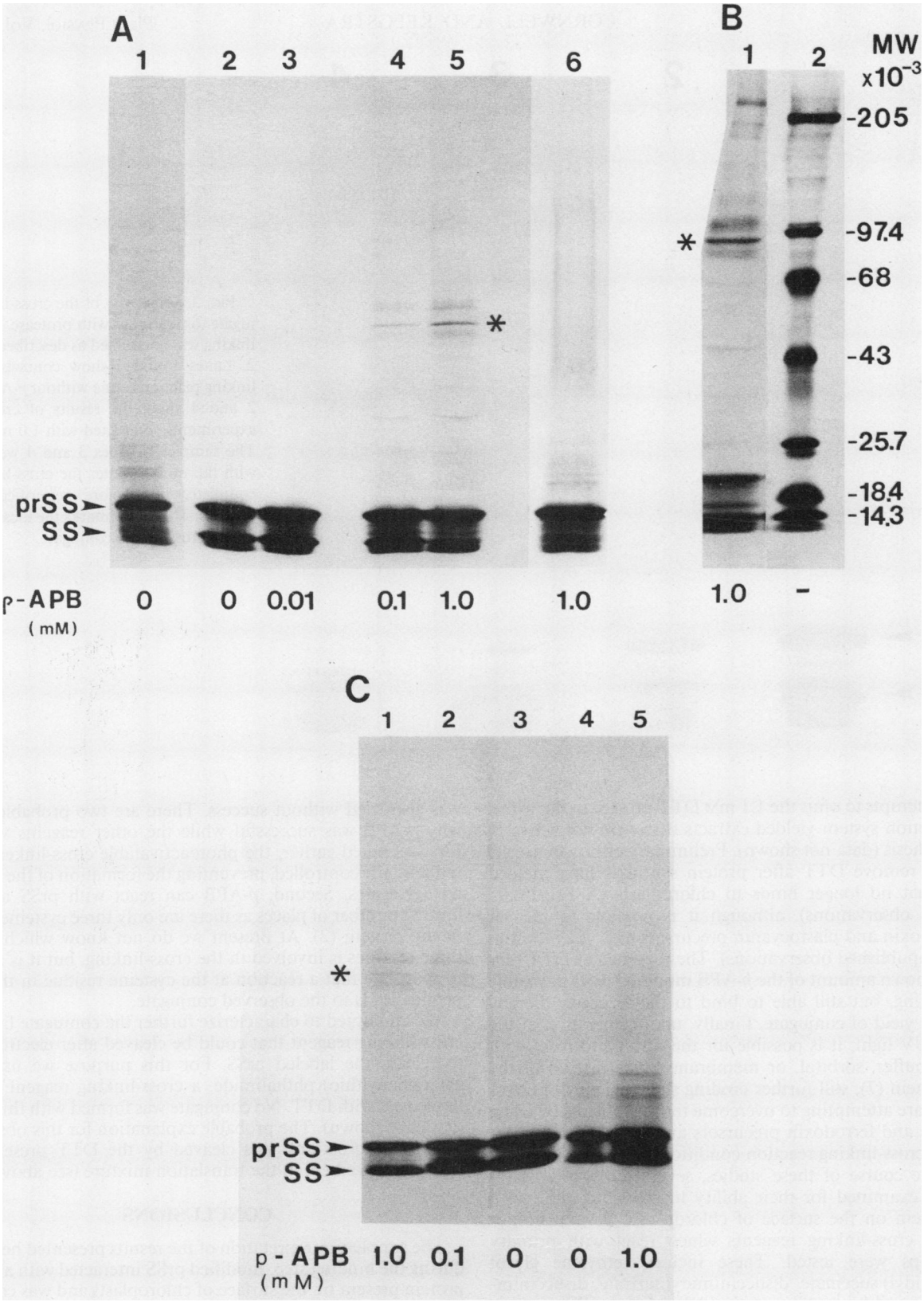

FIG. 2. Fluorograms of SDS-PAGE analysis of cross-linking experiments. Panel A, tritium-labeled precursor protein was prepared by translation of hybrid-selected mRNA. Lane 1 contains prSS that has received no further treatment. Lane 6 contains prSS that has been modified with 1.0 mM *p*-APB and then photoreacted in the absence of chloroplasts. Lanes 2 to 5 contain prSS samples that were modified with various concentrations of *p*-APB (0–1 mM, as shown below each lane). Modified prSS was bound to isolated chloroplasts in the dark and the chloroplasts were washed to remove unbound molecules. Chloroplasts containing bound prSS were subjected to UV light treatment, repurified to remove any broken chloroplasts, and subjected to SDS-PAGE and fluorography. Panel B, lane 1 contains a duplicate sample of the conditions described for panel A, lane 5. Lane 2 contains ^{14}C-labeled mol wt markers. The asterisk marks the location of the 86 kD cross-linked conjugate (panels A + B are fluorograms of two different gels). Panel C, the experiment is a duplicate of that shown in panel A, except that labeled precursor was prepared by *in vitro* transcription/translation of a cloned SS gene. Lanes 1 to 3 contain prSS samples that were modified with various concentrations of *p*-APB and then used in cross-linking experiments as described in panel A. Lane 4 contains prSS from the translation reaction that received no other treatment. Lane 5 contains prSS that has been modified with 1.0 mM *p*-APB and then photoreacted in the absence of chloroplasts.

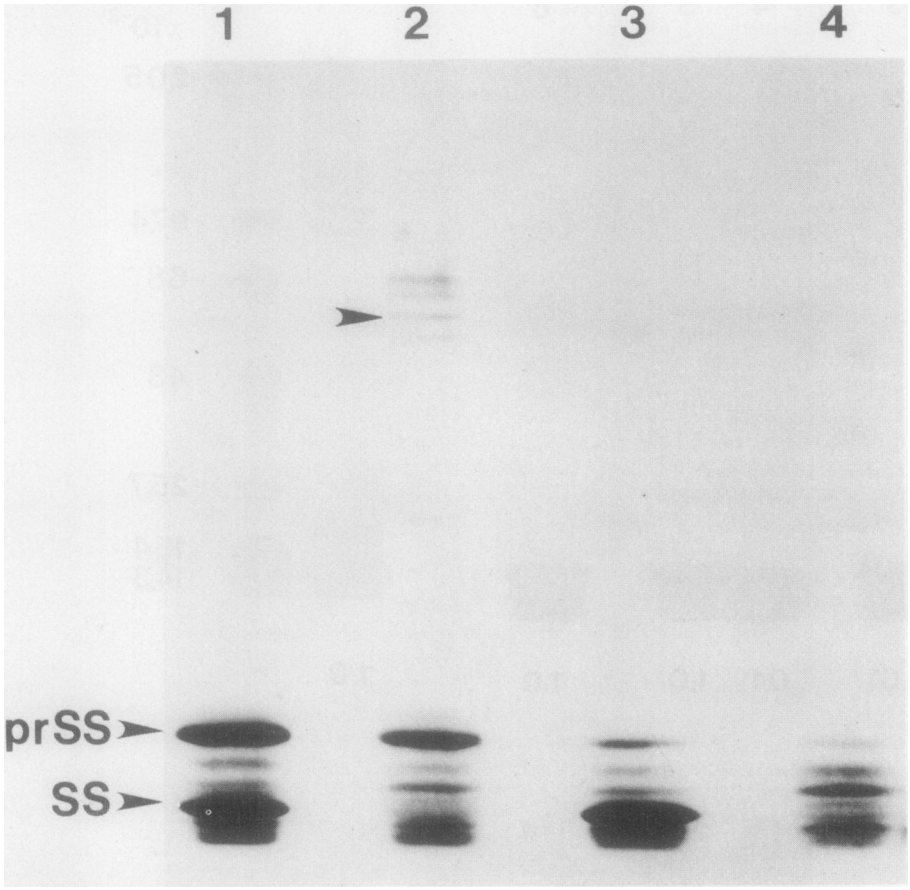

FIG. 3. Sensitivity of the cross-linked conjugate to treatment with protease. The cross-linking was conducted as described in Figure 2. Lanes 1 and 3 show controls of cross-linking reactions done without *p*-APB. Lanes 2 and 4 show the results of cross-linking experiments conducted with 1.0 mM *p*-APB. The samples in lanes 3 and 4 were treated with thermolysin after the cross-linking was complete, but before repurification and analysis. The arrow marks the location of the 86 kD cross-linked conjugate.

However, attempts to omit the 1.1 mM DTT present in the wheat germ translation system yielded extracts that were not active in protein synthesis (data not shown). Preliminary efforts to use gel filtration to remove DTT after protein synthesis have yielded precursor that no longer binds to chloroplasts (A Friedman, unpublished observations), although it is possible to recover active ferredoxin and plastocyanin precursors after gel filtration (L Olsen, unpublished observations). The presence of DTT renders an unknown amount of the *p*-APB modified prSS incapable of cross-linking, but still able to bind to the receptor, thereby lowering the yield of conjugate. Finally, upon generation of the nitrene by UV light, it is possible for the nitrene to react with the MOPS buffer, sorbitol, or membrane lipids instead of the receptor protein (7), still further eroding the efficiency of cross-linking. We are attempting to overcome these problems by using plastocyanin and ferrodoxin precursors and by using modifications of the cross-linking reaction conditions.

During the course of these studies, several cross-linking reagents were examined for their ability to cross-link prSS to a specific protein on the surface of chloroplasts. Several homobifunctional cross-linking reagents which react with primary amino groups were tested. These include: ethylene glycol bis(succinimidyl) succinate, disuccinimidyl tartrate, disuccinimidyl suberate, and bis (sulfosuccinimidyl) suberate. When these reagents were reacted with prSS that was bound to the surface of isolated chloroplasts, a high mol wt aggregate was formed. It remained at the top of the gel during subsequent analysis by SDS-PAGE. The most likely explanation is that not only was prSS cross-linked to its receptor, but the conjugate was also cross-linked to other outer membrane proteins. A heterobifunctional photoactivatable reagent that reacts with primary amino groups, sulfosuccinimidyl-6-(4′-azido-2'nitrophenylamino) hexanoate, was also tried without success. There are two probable reasons why *p*-APB was successful while the other reagents were not. First, as noted earlier, the photoactivatable cross-linkers can be sequentially controlled, preventing the formation of the high mol wt aggregates. Second, *p*-APB can react with prSS at only a limited number of places as there are only three cysteine residues in the protein (2). At present we do not know which of these three residues is involved in the cross-linking, but it is tempting to speculate that a reaction at the cysteine residue in the transit peptide leads to the observed conjugate.

We attempted to characterize further the conjugate by using a cross-linking reagent that could be cleaved after electrophoresis to release the labeled prSS. For this purpose we used *N*-(4-azidophenylthio) phthalimide, a cross-linking reagent that can be cleaved with DTT. No conjugate was formed with this reagent (data not shown). The probable explanation for this observation is that the cross-link was cleaved by the DTT present as an essential ingredient in the translation mixture (see above).

CONCLUSIONS

The simplest interpretation of the results presented here is that during the binding step, modified prSS interacted with a receptor protein present on the surface of chloroplasts and was covalently attached to this receptor protein during the photoreaction. This interpretation is supported by the fact that conjugate formation is dependent upon the concentration of *p*-APB, upon the presence of chloroplasts and the presence of prSS as opposed to mature SS. The conjugate must be at the chloroplast surface because subsequent protease treatment can degrade the conjugate. Other interpretations are still possible. The protein involved in conjugate formation may be an outer envelope protein that is part of the import apparatus, but is not a receptor. Alternatively,

it is possible that the conjugate observed in our studies represents a covalent connection between prSS and some required soluble factor from the wheat germ translation system. Protein import into mitochondria has been reported to be dependent upon soluble (presumably cytosolic) factors that are provided by the *in vitro* translation system (1, 8, 15, 16). However, this latter possibility seems unlikely in view of the observation that the 86 kD conjugate is not formed when modified prSS is photoactivated in the presence of wheat germ translation mixture, but in the absence of chloroplasts (Fig. 2A, lane 1; Fig. 2C, lane 5).

If we make the assumption that the 86 kD conjugate represents a single prSS molecule cross-linked to a single receptor, the mol wt of the putative receptor can be calculated to be 66,000. We have examined stained electrophoretograms of outer envelope membrane proteins and have not found a protein at that mol wt. It is possible that the mol wt estimate obtained by the above calculation is incorrect because this calculation makes some assumptions about the shape of the conjugate that may not be correct. Another explanation for the inability to see a stained band at the proper mol wt is based on the observation that chloroplasts contain approximately 3000 functional receptors per chloroplast (A Friedman, unpublished observations). Using this number it is possible to calculate that a protein present in this quantity would be a very minor band on stained gels and probably would be below the limit of detection.

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