Plastid Import and Iron-Sulfur Cluster Assembly of Photosynthetic and Nonphotosynthetic Ferredoxin Isoproteins in Maize

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ABSTRACT

We have previously isolated and characterized two cDNAs of maize (Zea mays) ferredoxin (Fd) isoproteins, which are differentially expressed in photosynthetic and nonphotosynthetic organs, and in response to illumination of the developing seedlings (Hase T, Kimata Y, Yonekura K, Matsumura T, Sakakibara H [1991] Plant Physiol 96: 77–83). To clarify the biosynthetic pathway of Fds present in the different organs, precursors of the two Fd isoproteins synthesized by in vitro transcription and translation were examined to determine whether they are imported to chloroplasts and etioplasts. Precursors for both Fd I and Fd III, a leaf-specific, photosynthetic isoprotein and a constitutive, nonphotosynthetic one, respectively, were imported into chloroplasts and processed to the mature size. Some of the mature-sized molecules inside the organelles were found to be assembled with an iron-sulfur cluster. The cluster assembly occurred without tight coupling to the translocation and processing steps of the protein import, and the process was temperature dependent and did not require light. Etioplasts were also capable of importing the precursor of Fd III and assembling the cluster. These combined data show that the constitutive, nonphotosynthetic Fd has the ability to become localized in plastids as a functional molecule.

Fds are acidic, low mol wt, soluble iron-sulfur proteins found in various organisms, and act as multifunctional electron carriers in diverse redox systems. In higher plants, Fds are mainly distributed in photosynthetic organs and occupy key positions for partitioning photo-reducing power to many metabolic processes (2). They are also present in nonphotosynthetic organs such as roots (12, 25, 27), and it is generally assumed that nonphotosynthetic Fds are probably involved in electron transfer from pyridine dinucleotides to some Fd-linked enzymes (25, 27). Although the primary structures of nonphotosynthetic Fds differ considerably from those of photosynthetic Fds (9, 28), it is still speculative whether any functional difference exists between the two types of Fds.

Biosynthesis of photosynthetic Fds has been studied using an in vitro reconstituted system. They are synthesized in cytosol as a larger precursor with an amino-terminal extension (10, 22), transported post-translationally into the chloroplast stroma, and processed to mature size (21). In addition to these general steps of protein import, Fds have to be assembled with a redox center composed of two atoms each of iron and sulfur to become a functional molecule. Current evidence suggests that insertion of the prosthetic group occurs inside the chloroplast (14, 26). In contrast to the accumulated data on photosynthetic Fds, information on the biosynthetic process of nonphotosynthetic Fds is completely lacking. Such information would be important for clarifying the subcellular compartmentation of nonphotosynthetic Fds and for understanding their possible physiological role(s) in nonphotosynthetic cells.

We reported previously that maize has at least four Fd isoproteins (Fd I to Fd IV), which are divided into two groups based on their organ distributions and light responses (9, 12). Fd I and Fd II are distributed only in leaves, being localized in chloroplasts, and their steady-state levels of protein and mRNA increase in a light-inducible manner. On the other hand, Fd III and Fd IV show neither organ specificity nor light induction. They are expressed constitutively in various parts of seedlings including leaves, mesocotyls, and roots. Whether the constitutive Fds are also localized in plastids is not known. Sequencing of Fd cDNAs has shown that Fd I and Fd III have precursors with an amino-terminal extension of 52 and 55 amino acid residues, respectively, but that the presequences share little sequence homology (9). These results imply that Fd III may be localized to a specific organelle, but do not necessarily lead us to the conclusion that Fd III is a plastid protein.

In the present study, we showed that the precursor of maize constitutive, nonphotosynthetic Fd, as well as that of leaf-specific, photosynthetic Fd, is imported into chloroplasts and assembled as a holo-Fd carrying an iron-sulfur cluster using an in vitro assay system. It was also found that the same or an analogous biosynthetic process is present in etioplasts. We propose that the subcellular location of nonphotosynthetic Fd is the plastid.

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MATERIALS AND METHODS

Plasmid Construction

DNA fragments coding for precursors of maize Fd I and Fd III were inserted behind the SP6 promoter of an in vitro transcription plasmid, pGEM3Z or pGEM4Z (Promega). A scheme of the genetic manipulations is shown in Figure 1. The EcoRI fragment excised from pFD1, which contained coding sequences of Fd I (9), was inserted into the EcoRI site of pGEM3Z. Plasmid carrying the insert with the desired orientation was selected, and a short region from HindIII to Smal in the polylinker sites of pGEM3Z was deleted to remove initiation and termination codons present between the SP6 transcription start site and initiation codon of the Fd cDNA. The Sau3AI/Xhol fragment containing the coding region of Fd III was obtained from pFD3-1, a full-length cDNA 154 base pairs longer in the 5′ region than pFD3 reported previously (9). The fragment was inserted into pGEM42 after cleavage with BamHI and SalI. The resulting plasmids, pGEM3Z/FD1 and pGEM4Z/FD3-1, were propagated and purified on a CsCl density gradient. All these conventional DNA techniques were used as described by Sambrook et al. (19).

In Vitro Transcription and Translation

The two plasmids, pGEM3Z/FD1 and pGEM4Z/FD3-1, were linearized with PvuII and transcribed with SP6 RNA polymerase (Promega). The transcription buffer contained 40 mM Tris-HCl (pH 7.8), 6 mM MgCl2, 2 mM spermidine, 10 mM DTT, 0.5 mM each of ATP, GTP, CTP, and UTP, and 0.2 units/μL RNasin. The transcription reaction was carried out in 50 μL of the buffer containing 10 μg of the linearized DNA and 10 units of SP6 RNA polymerase at 37°C for 60 min. The mixture was phenol-extracted, ethanol-precipitated, and dissolved in 50 μL of sterile water. The generated RNAs were translated in a wheat germ system (Promega). The translation mixture (50 μL) contained 25 μL of the germ extract, 10 μL of the transcript aliquot, 10 units of RNasin, 10 μCi L-[4,5-3H]leucine (140 Ci/mmol, Amersham), and 2.5 μL of a mixture of other amino acids (1 mM each). The translation mixture was incubated at 30°C for 40 min, and kept at -80°C until use.

Isolation of Intact Plastids

Intact chloroplasts were isolated from spinach leaves as described previously (3). After centrifugation in 30% (v/v) Percoll, the chloroplast pellets were washed once with import buffer (50 mM Hepes-KOH [pH 8.0], 0.33 mM sorbitol), and resuspended in the same buffer at a Chl concentration of about 2 mg/mL. Maize (Zea mays L. cv Golden Cross Bantum T-51) seedlings were grown on vermiculite for 12 to 14 d at 28°C in the dark, and etioplasts were prepared from the etiolated leaves essentially by the same method as that above except that 20% (v/v) Percoll was used for separation of intact etioplasts. Isolated etioplasts were suspended in import buffer at a protein concentration of about 2 mg/mL. Chl was measured according to the method of Arnon (1) and protein with a dye-binding assay kit (Bio-Rad).

Import Experiment

Standard experiments on chloroplast import were carried out in import buffer containing 8 mM leucine, 0.74 to 1.2 mg Chl/mL chloroplasts, and 1000 to 1800 cpm/μL translation products. The volume of the import mixture varied between 250 and 1000 μL according to experimental purpose. Each sample prepared in a glass test tube on ice was incubated with gentle shaking at 30°C under illumination for the desired time. After the incubation, the mixture (200 μL aliquot) was immediately chilled on ice and treated with trypsin at a final concentration of 10 μg/mL for 5 min to cleave any polypeptide present outside the organelles. After the protease treatment, soybean trypsin inhibitor was added at a concentration of 30 μg/mL, and the intact chloroplasts were reisolated by spinning them through a 30% (v/v) Percoll cushion in import buffer. Recovered chloroplasts were then lyzed on ice by suspending them in 100 μL of 5 mM Tris-HCl (pH 7.2), and respun with a microcentrifuge at 15,000 rpm for 1 min to obtain the supernatant (stomatal fraction). Reactions for chloroplast or etioplast import in the dark were carried out by adding 10 mM ATP to the above mixture. The concentration of etioplasts in the mixture was about 1 mg protein/mL.

PAGE and Fluorography

Stromal fractions from the import experiments were denatured by boiling for 5 min in SDS-sample buffer, and separated on a 12.5% (w/v) polyacrylamide gel using the buffer.
system of Laemmli (13). The same aliquots of the stroma were also directly electrophoresed on a nondenaturing polyacrylamide gel as described previously (12). Both gels were then treated with EN'ANCE (New England Nuclear) and fluorographed.

RESULTS

In Vitro Chloroplast Import of Fd Isoproteins

To investigate whether each Fd isoprotein is a chloroplast-targeted protein, we performed in vitro transport experiments with isolated chloroplasts. Precursors of Fd I and Fd III synthesized by in vitro transcription and translation were separately incubated with isolated chloroplasts, and polypeptides taken up in the stroma were analyzed by SDS-PAGE. Figure 2A shows that the precursors of Fd I and Fd III were imported into chloroplasts and processed to mature polypeptides, whose mobilities on the gel were the same as those of the authentic Fd I and Fd III, respectively. The same aliquots of stromal fractions were also analyzed by native PAGE. By this gel system, holo-Fd associated with the iron-sulfur cluster and apo-Fd free of the cluster are easily distinguishable (14, 26); the holo-form runs fast as a single band, whereas the apo-form migrates more slowly and is separated into several bands. Furthermore, the respective holo-forms of maize Fd isoproteins show mobilities distinct from one other on the gel due to their subtle differences in molecular configuration and intrinsic electrical charge (12). As shown in Figure 2B, a proportion of the mature-sized polypeptides run with exactly the same mobility as the holo-forms of the corresponding authentic Fd isoproteins, suggesting that some of the molecules were correctly assembled with the iron-sulfur cluster in isolated chloroplasts. A time-course experiment (Fig. 3) showed that the mature-sized polypeptides and holo-forms of Fd I and Fd III accumulated in a time-dependent manner, and that there was no significant difference between Fd I and Fd III in their rates of accumulation. These combined results demonstrate that both leaf-specific and constitutive Fds are capable of being imported into chloroplasts, processed correctly to mature size, and assembled into the holo-form.

Analysis of the Iron-Sulfur Cluster Assembly Process

The results described above confirmed previous observations that a metabolic pathway for introducing the iron-sulfur cluster to Fd is present in isolated chloroplasts (26), and that assembly of the cluster occurs in an in vitro import system (14). We further investigated when the cluster is formed during the import process and how environmental factors, such as temperature and light, influence the assembly process.

Under the experimental conditions described in the preceding section, we were unable to analyze the assembly process alone, because the processes of translocation, processing, and
cluster assembly could occur simultaneously. In the experiment shown in Figure 4A, addition of trypsin to the import assay mixture after preincubation for 10 min stopped any further accumulation of the mature-sized Fd III, most probably because of cleavage of the precursor outside the organelle. Even after the import had been blocked by this procedure, the amount of holo-Fd III continued to increase during further incubation (Fig. 4B). Without the trypsin treatment, both the mature-sized Fd III and holo-Fd III increased time dependently (data not shown). This result demonstrated that the conversion of apo-Fd to holo-Fd occurs after, but independently of, the translocation and processing steps.

The "import-independent" assembly of the cluster was carried out at low temperature. As shown in Figure 5, the conversion of apo- to holo-forms observed at 30°C was significantly retarded by transfer of the assay mixture to ice.

In the in vitro reconstitution experiments described above, import reactions were always conducted in light. We were also interested in examining whether light is required for assembly of the cluster. It has been established that ATP is the only source of energy needed for protein translocation (11). When import reactions were carried out in the light or in the dark in the presence of 10 mM ATP, formation of holo-Fd was observed under both conditions, as shown in Figure 6. The total amount of imported protein was lower, but the ratio of the holo-form to the apo-form was significantly higher in the dark than in the light. Thus, light is not essential for assembly of the cluster.
Ability of Etioplasts to Assemble the Iron-Sulfur Cluster

In leaves of young etiolated seedlings, Fd III is the major component among the four Fd isoproteins (12). Schindler and Soll (20) demonstrated that the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase was imported into pea etioplasts in the presence of ATP. We expected that the precursor of Fd III might be imported into etioplasts and assembled with the iron-sulfur cluster, if the subcellular location of Fd III were plastids in nonphotosynthetic cells. An import experiment for Fd III was carried out using maize etioplasts under similar conditions to those for chloroplast import in the dark. The result (Fig. 7) showed that the precursor of Fd III was taken up into etioplasts and processed to mature size, and that more than half of the mature-sized molecule was converted to the holo-form. This suggests that etioplasts are not only able to import the precursor of Fd III, but also to convert apo-Fd III to holo-Fd III.

DISCUSSION

We have demonstrated in this study that the precursor of maize Fd III, a constitutive, nonphotosynthetic Fd, is imported into isolated chloroplasts and etioplasts, where it is proteolytically processed to mature size and converted to holo-Fd carrying the iron-sulfur cluster. Since the structure of the amino-terminal transit peptide of Fd III is very different from those of leaf-specific, chloroplast-localized Fds from maize (9) and other plants (7, 22, 23, 29), and since no data have been obtained on the subcellular localization of Fd in nonphotosynthetic cells, it is difficult to predict whether the transit peptide may function as a signal for targeting to plastids or other organelles. This study presents the first biochemical evidence that Fd distributed in various parts of the plant, including nonphotosynthetic organs, is able to become localized in plastids. Because our import experiment was not done using root plastids, one might argue that Fd III could be localized in some other compartment of root cells. However, this seems unlikely, because several reports have demonstrated that various plastids, such as etioplasts (20), amyloplasts (24), and leucoplasts (5), have abilities to import chloroplastic proteins, similar to chloroplasts.

In general, it is established that roots contain a set of enzymes for assimilatory reduction of nitrate and sulfate (16, 18). Among these enzymes, nitrite reductase, glutamate synthase, and sulfite reductase require Fd as a reductant, and have been shown to be located in root plastids (6, 8). The oxidative pentose-phosphate pathway has been considered to be the initial source of reductant (16). The oxidation of glucose-6-phosphate with concomitant formation of NADPH is capable of sustaining nitrate reduction in isolated plastids from barley (17) and pea (4) roots. A Fd-NADP⁺ oxidoreductase-like enzyme purified from radish root tissues catalyzes electron transfer from NADPH to Fd in vitro (15), and the presence of a similar enzyme in maize roots is also suggested (25). All this evidence reinforces the idea that in maize, Fd III capable of localization in plastids are indeed involved in nitrate and sulfate assimilation in nonphotosynthetic cells. On the other hand, in maize leaves, Fd I and Fd II are major components, and their biosynthesis is strictly correlated with the appearance of photosynthetic activity during seedling growth (9, 12). Thus, these Fds seem most probably to function as major electron carriers for photosynthetic electron transport and allocation of electrons for various metabolic pathways, although Fd III and Fd IV are also present. It is an interesting question whether Fd III and Fd IV are present in the same cells as Fd I and Fd II in leaves, and if this is the case, whether the two groups of Fd are functionally differentiated. At present, we have no definite answers to these questions, although the two types of Fd seem to have slightly different NADP⁺ photoreduction activity in an in vitro assay (S Mizutani, T Hase, unpublished data).

In addition to the above considerations of the subcellular localization of Fd, this study has obtained several pieces of data concerning the biological formation of the iron-sulfur cluster of Fd in plastids. We have been able to show that the cluster is assembled to the mature-sized polypeptide in a time- and temperature-dependent manner without coupling to the translocation and processing steps. This suggests that the cluster assembly proceeds independently of the import reaction, and we expected that most of the imported Fd might be converted to holo-Fd by longer incubation. However, under the experimental conditions we employed, 10 to 20% of the imported Fd at most was converted to the holo-form. This suggests that the components of the cluster, such as sulfide...
and iron or their donor derivatives, may be limited in isolated chloroplasts, or that the period for which apo-Fd is kept competent for conversion to holo-Fd is rather short.

Nonrequirement of light has been shown for cluster assembly in chloroplasts. This was also the case for etioplast import, suggesting that neither light nor any components of the photosynthetic apparatus are directly involved in cluster assembly. In both experiments, it was necessary to add ATP as an energy source for protein translocation, and therefore we cannot determine at present whether ATP is an essential factor. One interesting observation was that the efficiency of cluster assembly in etioplasts seemed to be very high; more than 50% of apo-Fd was usually converted to holo-Fd. This would therefore provide a new experimental system for investigating in more detail the processes involved in biological formation of the iron-sulfur cluster.

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LITERATURE CITED