Localization of Ferredoxin Isoproteins in Mesophyll and Bundle Sheath Cells in Maize Leaf

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ABSTRACT

Four ferredoxin isoproteins were identified in the C₄ plant Zea mays L. by analysis of extracts from leaves, mesocotyls, and roots of the young seedlings. The relative amounts of the isoproteins isolated from the photosynthetic and nonphotosynthetic organs were different. All the isoproteins present in the leaves of green and etiolated plants, whereas two out of the four isoproteins were not detected in the roots or in the mesocotyls. During the greening of etiolated seedlings, the level of the two isoproteins unique to the leaf increased markedly. Analysis of the cellular and subcellular distribution of the two major leaf isoproteins showed that one isoprotein was present in the chloroplasts of both mesophyll and bundle sheath cells, whereas the other was only found in the chloroplasts of bundle sheath cells. This is the first report of the cell-specific expression of ferredoxin isoproteins in the leaves of a C₄ plant.

The presence of different molecular species of plant-type Fd in one organism has been reported in a variety of higher plants and algae (4, 7, 11, 15, 17), and variations in the Fd isoproteins with stages and conditions of growth have been observed (4, 17, 20). Although several attempts have been made to examine whether the Fd isoproteins have different functions in different aspects of Fd-linked metabolism, no conclusive evidence has been obtained for the biological significance of the isoproteins (4, 7, 19). Recently, the heterocyst of a blue-green alga, Anabaena variabilis, was shown to contain a specific Fd which is distinct from the Fd found in vegetative cells. The heterocyst Fd seemed to be involved in a specific interaction with nitrogenase (15).

In the leaves of C₄ plants, the metabolic process of photosynthesis is partitioned between two types of cell, BSC¹ and MC, and the intercellular compartmentalization of the assimilation of carbon and nitrogen is well documented (5). Furthermore, in the leaves of NADP-malic enzyme types of species, such as maize, almost all of capacity for non-cyclic electron flow is localized in the MC, and the PSII activity of the chloroplasts in the BSC is weak (5). In the course of a survey of Fd isoproteins in C₄ plants, we have found that maize has at least four Fd isoproteins. We report here that the relative amount of Fd isoproteins in MC and BSC are different. The cell-specific expression of the isoproteins may be related to metabolic and photochemical differences between the two types of cell.

† Abbreviations: BSC, bundle sheath cell; MC, mesophyll cell; PEP, phosphoenolpyruvate.

MATERIALS AND METHODS

Plant Growth

Maize (Zea mays L. cv Golden Cross Bantam T51) seedlings were grown in vermiculite at 25 to 28°C in a dark room for 8 to 10 d. Five-day-old etiolated seedlings were greened under a light source consisting of fluorescent tubes with an intensity of about 300 μE/m²s for 3 d. When mature leaves were required, the plants were grown under natural illumination in a greenhouse for 2 to 3 weeks.

Extraction and Partial Purification of Fd

Dark-grown seedlings were divided into three parts, leaves including coleoptiles, mesocotyls and primary roots. Mesocotyl is the first internodal part formed above the seed storage tissues, which elongates considerably during growth in darkness. Ten g each of these tissues were ground with a mortar and pestle with 1 g of polycolar AT and a small amount of quartz sand in 40 ml of ice-cold extraction buffer (20 mM Tris-HCl [pH 7.5]), 100 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM PMSF, and 1%[v/v] 2-mercaptoethanol). The homogenate was filtered through two layers of cheese cloth and centrifuged at 12,000g for 10 min. The resulting supernatant was mixed with an excess amount of DEAE-cellulose (about 20 g wet weight) and the suspension of resin was packed in a column. After the column was washed with 20 mM Tris-HCl (pH 7.5) and 100 mM NaCl, the adsorbed proteins were eluted with elution buffer (20 mM Tris-HCl [pH 7.5], 0.7 M NaCl). The eluate was fractionated by addition of ammonium sulfate to 70% saturation and the precipitate was discarded after centrifugation at 12,000g for 10 min. The supernatant containing Fd was passed through a small column of DEAE-cellulose equilibrated with a 70% saturated solution of ammonium sulfate, and Fd was eluted with elution buffer, as above, in a small volume. As an estimate of the recovery of Fd by this method, we found that loss of spinach Fd that had been added to an initial homogenate was less than 20%.

Extraction of Fd from MC protoplasts and BSC strands was carried out by homogenizing them in 5 to 10 volumes of the extraction buffer in a tightly fitting glass homogenizer. The extract was centrifuged at 12,000g for 10 min and the Fd contained in the supernatant was semipurified on a small scale as described above.

Isolation of Protoplasts

Protoplasts of MC were prepared by an enzymic procedure essentially, according to the method of Kanai and Edwards
Isolation of Chloroplasts

Chloroplasts from MC and BSC were prepared by the mechanical method of Jenkins and Russ (9) and by the enzymic digestion of mechanically prepared BSC strands as described by Jenkins and Boag (8), respectively. Crude preparation of chloroplasts from both types of cell were suspended in a medium composed of 0.35 M sorbitol, 25 mM Hepes-KOH (pH 7.8), 5 mM EDTA, and 0.1% (w/v) BSA and layered on a solution of 30% Percoll in the same medium. The intact chloroplasts were sedimented through the Percoll by centrifugation at 700 g for 3 min. The yields (as Chl) of chloroplasts of MC and BSC were approximately 2 mg and 0.9 mg Chl from 10 g fresh weight of leaf materials, respectively.

Electrophoresis and Western Blot

Fd isoproteins were analyzed by PAGE using the buffer system (pH 8.0 in the separation gel) of Williams and Reisfeld (22). A slab gel (13.5 × 15 × 0.1 cm) was prepared using linear gradient of acrylamide from 15 to 25%. All electrophoretic separations were performed in a cold chamber, at 4°C with prechilled buffer and gel at a constant current of 20 mamp for 2.5 h. Gels were stained with Coomassie brilliant blue R-250 and if necessary, proteins containing non-heme iron were visualized with α,α-dipyridyl and thioglycolate as described by Brill et al. (2). For Western blot analysis of the electrophoresis gel using a rabbit antibody raised against the purified Fd I, proteins were electrophoretically transferred to Immobilon (Millipore) after the gel is boiled for 10 min to denature Fd, and the polypeptides bound by the antibody are visualized with 125I-protein A.

Assay of Enzymic Activity and Measurement of Chl

Assays of PEP carboxylase (18), NADP-malic enzyme (10), and NADP-malate dehydrogenase (10) were based on published procedures, and Chl was determined according to Arnon (1).

RESULTS AND DISCUSSION

Fd Isoproteins in Leaf, Mesocotyl, and Root

Figure 1 shows that young leaves of etiolated seedlings contained four acidic proteins with electrophoretic mobilities similar to that of the purified spinach Fd. We have identified all of the proteins as isoproteins of plant-type Fd by the following criteria and designated them, respectively, Fd I, Fd II, Fd III, and Fd IV: (a) the proteins once separated by chromatography on DEAE-cellulose are not interconvertible during electrophoresis (Fig. 2A); (b) each of the proteins contains non-heme iron (Fig. 2B) and gives absorption maxima around 420 and 460 nm (data not shown), which are characteristics typical of plant-type Fd; (c) the sizes of the proteins appear to be the same as or very close to that of the authentic Fd from spinach, as judged by their mobilities on SDS-PAGE (data not shown). To examine immunological cross-reactivity of these Fd isoproteins, we applied a Western blot procedure to analyze polyacrylamide gel using an antiserum against Fd I. As seen in Figure 2C, Fd II as well as Fd I reacted with the antiserum, but Fd III and Fd IV did not. This indicates that Fd I is antigenically, thus structurally more similar to Fd II than Fd III and Fd IV, although the final conclusion must be awaited until direct structural information for the proteins becomes available.

Recently, Fd or a Fd-like protein has been shown to be present in the nonphotosynthetic tissues of several plants (16, 21). Leaves used in this experiment included coleoptile, but the content of Fd in coleoptiles separated from leaves was less than detectable level on the same fresh weight basis as leaves (data not shown). We have also examined extracts from mesocotyls and roots of maize seedlings to see whether and which Fd-isoproteins are present. As shown in Figure 1, appreciable quantities of Fd III were detected in both mesocotyl and root, and a small but consistent trace of Fd IV was found in the mesocotyl. Fd III was apparently distributed in all parts of the seedling, whereas Fd I and Fd II were only found in leaves. Therefore, the organ-specific expression of Fd isoproteins appears to function in leaves but not in nonphotosynthetic tissues. Suzuki et al. (16) reported that a
Localization of Maize Fd Isoproteins

Figure 2. Separation of Fd isoproteins and detection by staining of non-heme iron and Western blot. Four Fd isoproteins separated on a column of DEAE-cellulose developed with a linear gradient of NaCl from 0.2 to 0.3 M in 0.1 M Tris-HCl (pH 7.5) were subjected to electrophoresis as in Figure 1. The gel was stained with Coomassie brilliant blue (A) or with α,α-dipyridyl (B) as described in (2), or analyzed by immunoblotting with an antiserum against Fd I (C). Lane 1, extract of the etiolated leaves; Lanes 2 to 5, Fd I to Fd IV, respectively; lane 6, mixture of 4 Fd isoproteins. The amount of Fd in lanes 2 to 5 is 3 μg, 9 μg, and 0.1 μg in A, B, and C, respectively.

Figure 3. Changes in the levels of Fd isoproteins with age of seedlings, and the influence of illumination. The seedlings were grown in the dark for 8 d after germination or in the dark for the first 5 d and then in the light for the following 3 d. Fd isoproteins obtained from shoots of 0.7 g fresh weight were analyzed by PAGE as in Figure 1.

Fd-like protein in maize root was able to substitute for spinach Fd in NADPH-Cyt c reduction catalyzed by Fd-NADP reductase. Fd III, prepared from roots in this study, had the same ability as this Fd-like protein (Y. Kimata and T. Hase, unpublished results), suggesting that Fd III may correspond to the Fd-like protein.

We have studied changes in the relative abundance of Fd isoproteins during growth of seedlings and the effect of illumination on the level of each isoprotein. Fd contained in the etiolated or greening seedlings was analyzed at daily intervals from the 3rd to the 8th d after germination (Fig. 3). At earlier developmental stages, Fd I and Fd II were hardly detectable and then their levels increased gradually in the dark. This increase became quite marked in the light and the level reached at least five times that of the levels in dark-grown seedlings. By contrast, levels of Fd III and Fd IV remained almost constant or decreased slightly throughout the same period. These observations suggest that the increase in levels of Fd I and Fd II is somehow related to the appearance of photosynthetic ability, although light is not necessarily required for the biosynthesis of Fd I and Fd II. Indeed, the enzymic activities involved in the photosynthetic fixation of carbon, such as NADP-malic enzyme and PEP carboxylase, increased about 10-fold during the greening of the etiolated leaves in our experiments (data not shown).

Cellular and Subcellular Localizations of Fd Isoproteins in the Leaf

The localizations of the Fd isoproteins in the MC and BSC of green leaves was studied. Figure 4A shows that Fd II was found only in the BSC, within the limits of the sensitivity of our protein staining, whereas Fd I was found in both MC and BSC. The levels of the isoproteins were determined densitometrically on a Chl basis using the authentic spinach Fd (0.2–3 μg) as a standard (Table I). The levels of Fd III and Fd IV in green leaves were too low to be determined. In the preparations of the MC and BSC used in these experiments, the cross-contamination was estimated to be less than 5% as judged by a comparison of activities of marker enzymes (Table I). The ratio of Chl a to Chl b, taken together with microscopic analysis of the preparations, also indicated a reasonably effective separation of the two types of cells. The level of Fd II in the BSC was about two-fold higher than in leaves and this enrichment was comparable with that found in the activity of NADP-malic enzyme. These results strongly suggest that substantially all of Fd II is localized in BSC. Fd I was distributed in MC and BSC with a slight higher level in MC. However, the levels in both fractions were significantly lower than in leaf, although the marker enzymes accumulated in the corresponding fractions with higher specific activities than in leaf. The meaning of this observation is obscure at the moment.

Intact chloroplasts were prepared separately from MC and BSC using a single batch of plants. The purity of the preparations of the two types of chloroplast was estimated by measuring activities of the marker enzymes NADP-malic enzyme and NADP-malate dehydrogenase; contamination of MC chloroplasts by BSC chloroplasts was about 6% and that of BSC chloroplast by those from MC was about 20%. Stromal fractions obtained after rupturing the organelles by a hypotonic treatment were directly analyzed by PAGE in order to determine the levels of the Fd isoproteins. As shown in Figure 4B, each of the stromal fractions showed essentially the same relative amounts of isoproteins as the total extracts of MC and BSC. The levels of Fd I and Fd II were, respectively, as follows: 28 and 39 μg/mg Chl in BSC chloroplasts, and 28 and 2.9 μg/mg Chl in MC chloroplasts. The amount of Fd I in BSC chloroplasts seems to be overestimated due to the
contamination by MC chloroplasts. However, the level of overestimation is considered to be at most 10\% as evaluated from the contents of Fd I in the two types of cells shown in Table I. A detectable level of Fd II in MC chloroplasts (about 7\% of the Fd II found in BSC chloroplasts) can be attributed to contamination by BSC chloroplasts. Thus, these results are consistent with virtually all of Fd I of the MC and BSC, and all of Fd II of the BSC being located in the chloroplast stroma.

The occurrence of a particular species of Fd in BSC chloroplasts gives rise to some speculations on the possible significance of Fd isoproteins. It seems likely that Fd II may be adapted to some requirement(s) of BSC and this hypothesis implies two further possibilities. First, Fd II has a function distinct from that of Fd I in electron transfer and is closely associated with the unique metabolic processes in BSC. Second, the expression of Fd II is not due to a specific function of the molecule, but to the quantitative requirement for a particular amount of Fd in BSC. In fact, the total amount of Fd in BSC is about two times higher than in MC. Although the intracellular compartmentalization of the metabolism of nitrogen and sulfur as well as the assimilation of carbon in C4 plants, in the MC and BSC has been widely studied, there is no known Fd-linked enzyme restricted to or mainly found in BSC chloroplasts; nitrite reductase is predominantly localized in MC (6, 13, 14), sulfite reductase is present in both MC and BSC (14), and glutamate synthase has not been clearly shown to be only in MC or to be in both MC and BSC (6, 13). In C4 plants of the NADP-malic enzyme group, MC chloroplasts are the primary site of photoreduction of NADP+ (5) and the level of Fd-NADP reductase is much higher in MC than in BSC in maize (THase, unpublished results). At present, therefore, there is no concrete evidence to correlate the expression of Fd with the localization of any particular Fd-linked enzymes in BSC. Ferredoxin is also known to be involved in the light modulation of enzymes through the Fd/thioredoxin system and this system is functional in the photoregulation of C3 and C4 photosynthesis (3). One could speculate that the Fd isoproteins may be related to the regulation of chloroplastic enzymes differentially distributed in MC and BSC. In future, analyses of the physicochemical and biosynthetic properties of the Fd isoproteins, in relation to the physiological aspects of the two types of cell will be required. Some of investigations of these properties are now in progress.

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**LITERATURE CITED**


