Phytoferritin is synthesized *in vitro* as a high-molecular-weight precursor

Studies on the synthesis and the uptake *in vitro* of the precursors of ferritin and ferredoxin by intact chloroplasts

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Evidence is presented that French-bean (*Phaseolus vulgaris*) seed ferritin is composed of one type of subunit with an apparent M_r of 26 500. In normal and iron-loaded leaf tissues it is detected immunologically with an antiserum raised against purified bean seed ferritin and migrates in SDS (sodium dodecyl sulphate)/polyacrylamide-gel electrophoresis with the same mobility as the bean seed ferritin subunit. The biosynthetic pathway of ferritin in normal and iron-loaded leaves was investigated. RNA was extracted, fractionated into polyadenylated RNA and translated in a cell-free rabbit reticulocyte lysate and a wheat-germ-extract system. The products were identified by SDS/polyacrylamide-gel electrophoresis after indirect immunoprecipitation. In all cases the ferritin product had an M_r 5000 higher than that of the native subunit. Uptake and processing of the precursor form of ferritin from iron-loaded leaves by intact chloroplasts was demonstrated. This indicates that, in iron-loaded leaves, ferritin acts as a chloroplast protein. We propose that the ferritin precursor in normal leaves follows the same biosynthetic pathway. This suggests that the iron-buffering function of ferritin in plants takes place in the chloroplast and that non-functional cellular iron will accumulate in this cell organelle.

The iron-storage protein ferritin is widely distributed in Nature. In mammals it appears to be present in all cell types, and liver, spleen and bone marrow are especially rich in ferritin (Munro & Linder, 1978; Aisen & Listowsky, 1980; Bezukorovainy, 1980). An analogous protein has been found in plants (phytoferritin; Hyde *et al.*, 1963) and in fungi (mycoferritin; David & Easterbrook, 1971). It is also found in several species of bacteria (bacterioferritin; Stiefel & Watt, 1979; Yariv *et al.*, 1981; Chen & Crichton, 1982).

Ferritin heterogeneity

Mammalian ferritin is composed of 24 subunits; these subunits exist in two molecular forms, designated 'H' and 'L'. Different ratios of H- and

L-subunits in holo-ferritin are thought to form various isoferritins (Arosio *et al.*, 1978). Watanabe & Drysdale (1981) showed fairly recently that H- and L-subunits probably originate from distinct mRNA species. So far, the presence of distinct subunits in phytoferritins has not been established. Nevertheless, the existence of isophytoferritins became plausible, since ferritins have been detected in normal and iron-loaded leaves possessing various iron contents (van der Mark *et al.*, 1982). We therefore investigated the subunit structure of phytoferritins from plant tissues with normal and high iron contents with emphasis on the immunological detection of distinct subunits. We present here the characterization of these ferritins by SDS/polyacrylamide-gel electrophoresis, protein blotting and immunodetection with anti-(bean seed ferritin) antibodies.

In contrast with the cytoplasmic localization of mammalian ferritin, phytoferritin is reported to be strictly located in the plastid or chloroplast (Seckbach, 1982). This conclusion is drawn from electron-microscopic studies, by which only ferritin with

Abbreviations used: poly(A)⁺ RNA, polyadenylated RNA; AMV, alfalfa mosaic virus; SDS, sodium dodecyl sulphate; SAC, *Staphylococcus aureus* cells; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; phosphate-buffered saline, 10 mM-phosphate buffer containing 140 mM-NaCl; PMSF, phenylmethanesulphonyl fluoride.

normal and high iron content can be made visible. Since it became clear that normal leaf tissue contains ferritin with a low average iron content (350 iron atoms/molecule; van der Mark *et al.*, 1982), the localization of this ferritin should be considered as unestablished.

To gain more insight in the localization as well as the genetic origin of the ferritins in normal and iron-loaded leaves, characterized by low and normal iron content respectively, we studied the synthesis *in vitro* of ferritins from both types of leaves, using the cell-free protein-synthesizing systems derived from wheat-germ and reticulocytes. We characterized the primary ^{35}S -labelled translation products by means of indirect immunoprecipitation, SDS/polyacrylamide-gel electrophoresis and fluorography. The results of the present study clearly demonstrate that, firstly, both ferritins are synthesized by poly(A)⁺ mRNA from which we conclude a coding by nuclear DNA. Secondly, both ferritins migrate as a single protein subunit in SDS/polyacrylamide-gel electrophoresis with an identical M_r . When compared with the authentic subunit, ferritin subunits synthesized *in vitro* migrate at somewhat higher M_r , suggesting that ferritin subunits are synthesized as precursors. The uptake and processing of ferritin precursor synthesized *in vitro*, as coded by poly(A)⁺ RNA from iron-loaded leaves, into isolated chloroplasts were studied. It suggests that, in iron-loaded leaves, ferritin is localized in the chloroplast.

Chloroplast ferredoxin is known to be coded for by nuclear DNA (Huisman *et al.*, 1978b). The identification of the precursor form of ferredoxin is still a subject of investigation (Bartlett *et al.*, 1979; J., C. Gray, personal communication). We therefore reinvestigated synthesis *in vitro* of chloroplast ferredoxin, and the uptake and processing of the precursor by intact chloroplasts.

Materials and methods

Plant culture

French-bean (*Phaseolus vulgaris* L., cv. Prelude) plants were cultured as described by van der Mark *et al.* (1982). The secondary leaves of normal plants were harvested 18 days after sowing. Iron-loaded leaves were obtained by replacing the iron-free medium of 16-day-old iron-deficient seedlings with medium containing 90 μM -ferric sodium EDTA. The secondary leaves were harvested 40 h after the start of supplying the iron (van der Mark *et al.*, 1982). Tobacco (*Nicotiana tabacum* L.) plants were cultured in a greenhouse; the youngest leaves were used for the RNA preparation.

Preparation of RNA

The extraction of the leaf material was performed as described by Huisman *et al.* (1978b). After a

second phenol/cresol step the precipitated RNA preparation was washed twice with 70 and 100% ethanol respectively, the pellet was dried and dissolved in 4% (w/v) Sarkosyl and 0.1 M-EDTA in 0.1 M-Tris/HCl, pH 8.0. CsCl was then added to the crude RNA solution to a final concentration of 1% (w/v) and subsequently layered on a 1.2 ml cushion of 5.7 M-CsCl in 0.1 M-EDTA. Centrifugation was performed in a Beckman SW 50 rotor at 140 000 *g* for 16 h at 22°C. The RNA pellet was dissolved in 0.1% SDS, precipitated and washed with 70% and 100% ethanol respectively. Poly(A)⁺ RNA was obtained by chromatography on oligo(dT)-cellulose (Type 3; Collaborative Research, Waltham, MA, U.S.A.) essentially as described by Aviv & Leder (1972).

Translation of RNA *in vitro*

RNA samples were translated in a mRNA-dependent rabbit reticulocyte cell-free system and a cell-free protein-synthesizing system derived from wheat-germ. The reticulocyte lysate was kindly provided by the Plant Virus Group (University of Leiden) and used as described by Van Tol & van Vloten-Doting (1979). The wheat-germ extract was prepared as described by Marcu & Dudock (1974), and had an average $E_{260} = 150 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, the protein content being 70 mg/ml. It was stored in small aliquots under liquid N₂ until use. The initial composition for protein synthesis *in vitro* with the wheat-germ extract was based upon that of Roberts & Paterson (1973) and contained 0.55% (v/v) wheat-germ extract, 25 mM-Hepes/KOH, pH 7.5, 130 mM-K⁺, 2.6 mM-Mg²⁺, 0.75 mM-ATP, 20 μM -GTP, 1.4 mM-dithiothreitol, 15 mM-phosphocreatine, creatine phosphokinase (6.25 $\mu\text{g}/\text{ml}$; 140 units/mg), 15 μM each of the amino acids (minus methionine), 0.375 mM-spermidine (neutralized to pH 7.5), [^{35}S]methionine (1000 Ci/mmol; 250 $\mu\text{Ci}/\text{ml}$) and placental ribonuclease inhibitor (1 $\mu\text{g}/\text{ml}$). The incorporation of radioactivity into trichloroacetic acid-insoluble products was measured as described by Roberts & Paterson (1973). The fidelity of the translation was determined by using AMV RNA 1 and RNA 4 (Rutgers, 1977).

Preparation of antibodies and immunoprecipitations of cell-free translation products

Antiserum against purified bean seed ferritin and tobacco ferredoxin was raised as described by van der Mark *et al.* (1981) and Huisman *et al.* (1977) respectively. SAC, strain Cowan I, were grown as described by Kessler (1975) and fixed with formaldehyde. Before immunoprecipitation of products synthesized *in vitro*, the translation mixture was adjusted to 1% SDS and boiled for 3 min. The mixture was diluted to 0.1% SDS with phosphate-buffered saline containing 1% Triton X-100,

0.3% bovine serum albumin, 100 μ M-PMSF and 1 mM-methionine. Before specific immunoprecipitation, a preimmune precipitation was performed; preimmune serum together with freshly washed SAC suspension were added to the translation mixture and the suspension was allowed to rotate for 30 min. After centrifugation, a specific immune precipitation was performed by the addition of specific antiserum and incubating end-over-end for 16 h at 4°C. A titrated amount of SAC suspension was added followed by an incubation of 30 min at 4°C. The bacteria-bearing immune complexes were centrifuged in an Eppendorf Microfuge for 60 s at room temperature, followed by three washings with phosphate-buffered saline/1% (v/v) Triton and two washings with phosphate-buffered saline. After washing, the antigen-antibody complexes were dissolved in SDS/sample buffer and boiled for 3 min. SAC were removed by centrifugation immediately before electrophoresis. If non-specific binding of products to the antigen-antibody complexes, was present at this stage of the procedure the entire immunoprecipitation was repeated, starting with boiling the antigen-antibody complexes in 1% SDS.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis on 10–20% acrylamide gradient/SDS slab gels was performed as described by Laemmli (1970). Stained polyacrylamide gels were treated with En³Hance (New England Nuclear), according to the specifications of the manufacturers and, after drying, fluorographed at –70°C. Protein standards (Pharmacia) were used for calculation of molecular weights.

Immunodetection of ferritin subunits after SDS/polyacrylamide-gel electrophoresis and protein blotting

Protein blotting on nitrocellulose and immunodetection of protein was carried out as described by Towbin *et al.* (1979). Iodinated protein A and ferritin were prepared with the chloramine-T method (Greenwood & Hunter, 1963).

Uptake of products of translation in vitro into intact chloroplasts

Intact chloroplasts were isolated from spinach (*Spinacia oleracea*) leaves by Percoll-gradient centrifugation as described by Mills & Joy (1980). Chloroplasts used in the present study were at least 90% intact as estimated by the ferricyanide test (Lilley *et al.*, 1975). The chlorophyll content was determined according to Bruinsma (1963). For reconstitution of protein transport, 100 μ l of ³⁵S-labelled product was adjusted to 0.33 M-sorbitol/30 mM-Hepes/KOH, pH 8.0, and 25 mM-methionine, and mixed with 200 μ l of intact chloroplasts

(200 μ g of chlorophyll). The samples were illuminated with 8000 lx (Philips TL33 lamp) during an incubation period of 1 h at 25°C. For re-isolation of the intact chloroplasts the entire reaction mixture was underlayered with 2 ml of 40% Percoll containing 0.33 M-sorbitol/50 mM-Hepes/KOH, pH 8.0, and centrifuged at 3000 g for 3 min. The supernatant was tested for the presence of non-incorporated translation products by immunoprecipitation. The pellet of chloroplasts was resuspended in 0.3 ml of 0.33 M-sorbitol/50 mM-Hepes/KOH, pH 8.0, containing 15 μ g of proteinase K/ml and kept at room temperature for 10 min. Subsequently the chloroplast suspension was adjusted to 2 mM-PMSF, washed with 0.33 M-sorbitol, 50 mM-Hepes/KOH, pH 8.0, and 2 mM-PMSF, and finally the chloroplasts were lysed by resuspending the pellet in 2 mM-PMSF. Cell debris was spun down and the supernatant was tested for the presence of incorporated labelled products.

Results

Purified bean seed ferritin

Ferritin was isolated from bean seeds (van der Mark *et al.*, 1981). On SDS/polyacrylamide-gel electrophoresis it shows one band with a mol.wt. of 26 500, indicating that this ferritin is composed of one type of subunit (Fig. 1, lane 1). The migration of the H- and L-subunits from rabbit liver ferritin in the gel system used is shown in lane 2. Antibodies raised against purified bean seed ferritin were further analysed in the crossed-immunoelectrophoresis test. These antibodies reacted with ferritin from bean seeds and leaves in an identical manner (van der Mark *et al.*, 1981).

Immunological characterization of ferritin subunits after SDS/polyacrylamide-gel electrophoresis

To investigate the presence of different subunits in ferritin from normal and iron-loaded leaves, crude leaf extracts were subjected to SDS/polyacrylamide-gel electrophoresis and the gels blotted on to nitrocellulose paper. Immunodetection showed that ferritin subunits derived from the different tissues and bean seed ferritin migrated identically on SDS/polyacrylamide-gel electrophoresis (Fig. 2).

Translation in vitro

Addition of poly(A)⁺ RNA isolated from bean leaves to a reticulocyte lysate resulted in the incorporation of 10–12% of the input of [³⁵S]-methionine into trichloroacetic acid-precipitable products. In the optimized wheat-germ translation system (see the Materials and methods section) the efficiency of [³⁵S]methionine incorporation for AMV RNA 1 and RNA 4 was 20%, the optimal RNA

Fig. 1. SDS/10–20% polyacrylamide-gradient-gel electrophoresis of ferritins

1, Purified bean seed ferritin; 2, purified rabbit liver ferritin; 3, marker proteins.

concentration being higher than found by Rutgers (1977). With poly(A)⁺ RNA from bean leaves the concentration optima were at 250 µg of RNA/ml of translation mixture. The efficiency of [³⁵S]methionine incorporation of these RNA species was between 14 and 18%.

Immunological characterization of ferritin subunits synthesized *in vitro*

The products synthesized under direction of poly(A)⁺ RNA from normal and iron-loaded leaves were used for immunoprecipitation with antiserum raised against bean seed ferritin. The immunoprecipitated products were further characterized by SDS/polyacrylamide-gel electrophoresis and their molecular weights were compared with those of authentic ferritin subunits. Fig. 3 shows a representative analysis of ferritin subunits synthesized *in vitro* by SDS/polyacrylamide-gel electrophoresis and fluorography. The immunoprecipitation of products synthesized *in vitro* from poly(A)⁺ RNA isolated from normal and iron-loaded leaves resulted in one band (Fig. 3, lanes 1 and 4). The immunological specificity of antibody binding was established by addition of an excess of homogeneous unlabelled ferritin in the immunoprecipitation re-

Fig. 2. Identification of ferritin subunits in leaf tissues by SDS/polyacrylamide-gel electrophoresis followed by protein blotting and immunodetection

Extracts of bean leaves were made as described by van der Mark *et al.* (1982). Samples with an equal amount of ferritin were subjected to SDS/polyacrylamide-gel electrophoresis. After blotting on nitrocellulose, ferritin subunits were detected by incubation with an antiserum against bean seed ferritin and [¹²⁵I]-protein A followed by autoradiography. The autoradiogram shows: 1, extracts of leaves, dark-grown; 2, normal; 3, iron-loaded; 4, bean seed ferritin (0.2 µg).

action, which resulted in the disappearance of the labelled band, owing to the competition by the unlabelled ferritin for the amount of antibody used (Fig. 3, lanes 2 and 5). The same results were obtained with the wheat-germ-extract system (results not shown). The bands of the products synthesized *in vitro* correspond to an apparent M_r of 31 500, about 5000 higher than authentic ferritin subunits in this gel system (Fig. 3, lane 3). These data indicate that, in both normal and iron-loaded leaves, ferritin subunits are coded for by nuclear DNA and synthesized by poly(A)⁺ RNA in the cytoplasm as higher M_r precursors. Entering the chloroplasts the ferritin precursor should be cleaved to release the native protein (Dobberstein *et al.*, 1977). To investigate this part of the biosynthetic pathway we studied the uptake, by isolated intact chloroplasts, of the ferritin precursor synthesized *in vitro*.

Fig. 3. Identification of the precursor of the subunit of ferritin in bean leaves by SDS/polyacrylamide-gel electrophoresis and fluorography

[35 S]Methionine products, synthesized *in vitro* in a reticulocyte system programmed with poly(A) $^{+}$ RNA from normal or iron-loaded leaves, were used for indirect immunoprecipitation with an antiserum raised against bean seed ferritin. The fluorogram shows: 1, iron-loaded leaves; 2, as 1, with excess native ferritin (10 μ g); 3, 125 I-labelled bean seed ferritin; 4, normal leaves; 5, as 4, with excess native ferritin (10 μ g). Abbreviations used: pFt, ferritin precursors; Ft, ferritin.

Uptake of precursors by intact chloroplasts in vitro

The uptake *in vitro* of the most abundant chloroplast proteins synthesized as cytoplasmatic precursors has been extensively studied. These are the precursor for the small subunit of ribulose 1,5-bisphosphate carboxylase (pS) with M_r 20000 (Dobberstein *et al.*, 1977; Chua & Schmidt, 1978; Highfield & Ellis, 1978), and the precursor for the light-harvesting chlorophyll *a/b* protein (pLHGP), with M_r 29500 (Apel & Kloppstech, 1978; Schmidt *et al.*, 1980). Recently, using optimal conditions for post-translational uptake of polypeptides, the transport of less-abundant polypeptides was also observed (Grossman *et al.*, 1982). So far, wheat-germ translation products labelled *in vitro* and programmed by RNA species from normal leaves and cells were always used in the chloroplast uptake studies. In the present study we used the total labelled products from the translation programmed by poly(A) $^{+}$ RNA from iron-loaded leaves, and could confirm the abovementioned results. Fig. 4(b) (lane 2) shows the M_r distribution of the [35 S]-methionine-labelled products. As judged by their M_r ,

pS and pLHCP were also the most abundant precursors among the products of this translation procedure. We incubated the products synthesized *in vitro* with isolated intact chloroplasts; the chloroplasts were then re-isolated, proteinase-treated and lysed and finally separated by centrifugation into soluble and membrane fractions. Fig. 4 shows the stained SDS/polyacrylamide gel (a) and the fluorogram (b) of these fractions. The uptake *in vitro* and processing by the chloroplasts is proven by the fact that newly imported proteins co-migrate with the stained bands of the spinach chloroplast proteins (compare Fig. 4b, lanes 2 and 3 with Fig. 4a, lanes 2 and 3). The most abundant newly imported stromal polypeptide is pS (Fig. 4b, lane 3), whereas pLHCP is clearly present among the polypeptides that are associated with the thylakoid membranes (Fig. 4b, lane 4).

Immunological characterization of specific transported proteins

The uptake and processing of the [35 S]methionine-labelled ferritin precursor synthesized *in vitro* directed from iron-loaded leaves was examined with intact spinach chloroplasts. After the incubation of chloroplasts with the wheat-germ translation products, the chloroplasts were re-isolated by centrifugation through a layer of 40% Percoll. To remove all proteins potentially stuck to the chloroplast envelopes a proteinase treatment was also performed. The chloroplasts were then lysed and immunoprecipitated with antiserum against bean seed ferritin. The immunoprecipitate was analysed by SDS/polyacrylamide-gel electrophoresis. The fluorogram shows that the ferritin precursor was transported into the chloroplast and apparently processed by a specific proteinase to its mature size (M_r 26500; Fig. 5, lane 2). The chloroplast-free solution remaining as a supernatant on the 40% Percoll layer after centrifugation was also used for the detection of ferritin. As is apparent from Fig. 5 (lane 1) the immunoprecipitated ferritin product remaining in the supernatant still existed as precursor and was not affected by the incubation with intact chloroplasts. The apparent absence of proteolytic activity in the supernatant yields extra evidence that the processing took place after uptake into the chloroplasts (Fig. 5, lane 1).

Among the iron-containing chloroplast proteins that are known to be synthesized in the cytoplasm is ferredoxin. Using a wheat-germ translation system, Huisman *et al.* (1978a, b) reported that ferredoxin is synthesized as a precursor having an M_r 3000–4000 higher than the authentic protein. Since we were able to study the uptake and processing of specific polypeptides by intact chloroplasts, we re-examined and extended the preliminary studies on the ferre-

Fig. 4. Uptake of [^{35}S]methionine-containing polypeptides synthesized *in vitro* by intact chloroplasts

Products of a wheat-germ translation mixture programmed with poly(A)⁺ RNA of bean leaves were incubated with intact chloroplasts. After the incubation the chloroplasts were re-isolated and lysed. (a) SDS/polyacrylamide-slab-gel electrophoresis; the gel was stained for proteins; (b) fluorogram. In both (a) and (b): 1, M_r standards + ^{125}I -labelled ferritin; 2, wheat-germ ^{35}S -labelled translation products; 3, spinach chloroplast stromal proteins after incubation with [^{35}S]methionine-labelled translation products; 4, spinach chloroplast thylakoid membranes after incubation with [^{35}S]methionine-labelled translation products. pLHCP indicates precursor of light-harvesting chlorophyll *a/b* protein. pS is the precursor of small subunit of ribulose 1,5-bisphosphate carboxylase.

doxin precursor. For this purpose poly(A)⁺ RNA was isolated from tobacco leaves and translated in the wheat-germ extract. The [^{35}S]methionine-labelled products were incubated with intact spinach chloroplasts (see the Materials and methods section). The intact chloroplasts were reisolated, lysed and the supernatant of the reaction mixture was used for the immunochemical detection of the ferredoxin synthesized *in vitro* remaining outside the chloroplast. Fig. 5 (lane 3) shows that ferredoxin migrated in the gel system used with an apparent M_r of 27 000, whereas unlabelled pure tobacco ferredoxin migrated to M_r 22 000. When the lysed chloroplasts were used for immunoprecipitation with the antiserum raised against tobacco ferredoxin, the immunoprecipitate resulted in a band with the same mobility as authentic ferredoxin. This result clearly supports previous observations that ferredoxin is synthesized as a higher- M_r precursor.

Discussion

Ferritin isolated from bean seeds shows, on SDS/polyacrylamide-gel electrophoresis, one band, indicating that this ferritin is composed of one type of subunit. This is in agreement with the data obtained for other isolated seed ferritins, e.g. pea (*Pisum sativum*) and lentil (*Lens esculenta*) (Crichton *et al.*, 1978).

Ferritin subunits from normal and iron-loaded leaves were characterized by separation of crude extracts of these tissues on SDS/polyacrylamide-gel electrophoresis, followed by preparation of protein blots from these gels and immunological detection with antiserum raised against bean seed ferritin. It appeared that all tissues examined contained only one subunit, of M_r 26 500, comparable with bean seed ferritin. Although this result does not exclude the existence of other subunit types in phytoferritin, not detected by the antiserum, the use of this

Fig. 5. Uptake of precursors for ferritin and ferredoxin by intact chloroplasts

Wheat-germ ^{35}S -labelled translation products were incubated with intact chloroplasts. After the incubation the chloroplasts were reisolated and treated with proteinase. The supernatants of the chloroplast incubation mixture and the lysed chloroplasts were used for immunoprecipitation. The immunoprecipitates were analysed by SDS/polyacrylamide-slab-gel electrophoresis followed by fluorography. The fluorogram shows: immunoprecipitates with anti-serum raised against bean seed ferritin (a) and ferredoxin (b) of the supernatants of the chloroplast incubation mixtures (lanes 1 and 2) and of the lysed chloroplasts (lanes 2 and 4). Abbreviations used: pFt, ferritin precursor; Ft, ferritin; pFd, ferredoxin precursor; Fd, ferredoxin.

antiserum to detect ferritin in leaf tissues with variable iron content and purified bean seed ferritin as marker for the mature protein subunit in leaf tissue is justified.

In the present study we show that ferritin subunits are synthesized when poly(A)⁺ RNA species from normal and iron-loaded leaves, are used as templates in the protein-synthesizing reticulocyte-lysate or wheat-germ-extract systems. The results indicate that ferritin is synthesized in the cytoplasm and is coded for by nuclear DNA. The polypeptide synthesized *in vitro* has, in both cases, an M_r about 5000 higher than that of the native ferritin subunit.

By using a reconstituted system for polypeptide transport *in vitro* we are able to demonstrate the uptake of the ferritin precursor, synthesized *in vitro* on poly(A)⁺ RNA from iron-loaded leaves, into intact chloroplasts. Furthermore, the precursors

remaining outside the chloroplasts were not attacked; however, once inside the chloroplasts the precursors are processed to the mature size of the ferritin subunit. The ferritin synthesis in iron-loaded leaves is stimulated as compared with its synthesis in normal leaves (Seckbach, 1972; van der Mark *et al.*, 1982), but RNA species from both kinds of leaves synthesize the same precursor for ferritin. Although only the uptake of the ferritin precursor as synthesized by RNA from iron-loaded leaves is proven, we expect that in normal leaves the same biosynthetic pathway occurs.

The uptake of spinach ferredoxin synthesized *in vitro* by intact chloroplasts was studied by Bartlett *et al.* (1979). However, they did not demonstrate a precursor form of ferredoxin as synthesized by the wheat-germ-extract. The identification of the precursor form of ferredoxin is complicated by the anomalous migration of the mature protein on SDS/polyacrylamide-gel electrophoresis, probably due to the high negative charge, which prevents proper binding of SDS (Williams & Grutzer, 1971; Huisman *et al.*, 1978a). With the gel system used in the present study we found a difference in migration of mature ferredoxin and its precursors that is somewhat greater than that reported in a previous study (Huisman *et al.*, 1978b). We were also able to show the uptake of the ferredoxin precursor by intact chloroplasts and its concomitant processing to a molecule of M_r comparable with that of authentic protein. We may conclude that for ferredoxin biosynthesis the same events take place as already described for the small subunit of ribulose 1,5-bisphosphate carboxylase (Highfield & Ellis, 1978; Chua & Schmidt, 1978) and for ferritin.

In the present study the chloroplast localization of ferritin with normal iron content was established, and such a localization seems likely for the low-iron ferritin found in normal leaves. This implies that the buffering function performed by ferritin takes place inside the chloroplast or plastid. Probably all iron entering the cell and not used outside the plastid is taken up by this organelle. It is known that ferrochelatase is localized inside the plastid (Jones, 1968; Castelfranco & Jones, 1975), and also the incorporation of iron into non-haem iron proteins is thought to occur in the chloroplast. Iron not directly used for incorporation in photosynthetic electron-carriers is then stored by ferritin.

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