Changes in Thylakoid Galactolipids and Proteins during Iron Nutrition-Mediated Chloroplast Development

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ABSTRACT

Changes in the amounts of thylakoid galactolipids and proteins were monitored for 96 hours following iron resupply to iron-deficient sugar beet (Beta vulgaris L. cv F58-554H1) plants. During this period of iron nutrition-mediated chloroplast development, the amount of galactolipid per leaf area increased linearly with time. Assuming galactolipids are an index for the amount of thylakoids, then there was a linear synthesis of thylakoid membranes during regreening. Total thylakoid protein synthesis, however, lagged behind galactolipid synthesis, suggesting that proteins are inserted secondarily into the galactolipid matrix of the thylakoid membrane during development.

Iron deficiency caused an increase in the free chlorophyll band under the conditions of gel electrophoresis used. Of the chlorophyll proteins resolved, the chlorophyll protein associated with photosystem I was most diminished in iron-deficient tissue, and appeared to recover most rapidly. Changes in the light-harvesting chlorophyll proteins are also discussed.

The number of polypeptides resolved by lithium dodecyl sulfate-polyacrylamide gel electrophoresis was higher in iron-deficient thylakoids. During regreening, the number of resolved polypeptides decreased.

Unlike the membrane lipids of mitochondria and the chloroplast outer envelope which are high in phospholipids, thylakoid membrane lipids are comprised mainly of galactolipids (for reviews, see 6, 27). These lipids represent approximately 70% of the non-pigment thylakoid lipids (11) and 43% of all chloroplast lipids (15).

The role of galactolipids in the structure and function of the thylakoid membrane is not well understood (6, 27). Both membrane stability and the correct orientation of pigment molecules for efficient light harvesting may depend on the presence of galactolipids (6). Other studies indicate a role for galactolipids in photosynthetic electron transport, e.g., photosynthetic O₂ evolution, Cyt c photoreduction, and PSI activity (6). When defatted BSA is added to thylakoid preparations, however, large amounts of galactolipids may be removed without severely affecting electron transport (6). In contrast, O₂ evolution and phosphorylation were not as well protected by BSA, but no direct role for galactolipids was suggested (30).

Besides lipids, the other major component is protein which constitutes 48% of the thylakoid membrane (25). Some thylakoid proteins bind Chl and probably function in orienting Chl molecules. Many other proteins, such as Cyt and plastocyanin, play an integral role in photosynthetic electron transport. In bacteria, it is thought that protein and Chl are inserted into preexisting lipid membrane material (16). This view has been supported by studies which have shown that, in synchronously dividing cultures of the photosynthetic bacterium, Rhodopseudomonas sphaeroides, the protein/phospholipid ratio changes sinusoidally over time (17).

In the present study, we explored the idea that a similar developmental process occurs in higher plant thylakoids, i.e., that proteins may be inserted into the lipid matrix of the membrane of thylakoids during their synthesis. To do this, we used the procedure developed in earlier studies in which we followed thylakoid synthesis in iron-deficient plants during iron resupply (22). The synthesis of thylakoid lipid was followed by monitoring galactolipid formation, while the synthesis of thylakoid proteins was followed by measuring total thylakoid protein. Polypeptide composition was examined by LiDS/PAGE.

1 Abbreviations: LiDS, lithium dodecyl sulfate; DGDG, digalactosyldiglyceride; MGDG, monogalactosyldiglyceride; CF, coupling factor; CPI, Chl-protein 1; LHCP, light-harvesting Chl protein; TMBZ, 3,3',5,5'-tetramethylbenzidine.
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MATERIALS AND METHODS

Plant Culture. Sugar beets (Beta vulgaris L. cv F58-554H1) were cultured, made iron deficient, and then resupplied with iron as described previously (22). Harvested leaf samples were immediately placed in a distilled H₂O/ice bath until extraction (within 1 h).

Thylakoid Extraction. All extractions were done at 0 to 4°C. Leaf samples were homogenized in a Waring Blender (using short bursts of 1-3 s duration) in a buffer composed of 400 mm sorbitol, 50 mm Tricine, 10 mm NaCl, 1 mm MnCl₂, 5 mm MgCl₂, 1 mm EDTA, and 10 mm Na-asorbate, pH adjusted to 7.6 at 2°C. The brei was filtered through four layers of Miracloth, and the filtrate centrifuged at 1200g for 1 to 2 min. The pellet was resuspended in a buffer of 20 mm Tricine, 10 mm NaCl, and 5 mm MgCl₂, pH 7.8 at 2°C, and centrifuged at 19,000g for 10 min. The wash and centrifugation were repeated, and the pellet resuspended in a solution containing 65 mm Tricine, 12% glycerol, and 5 mm DTT, pH 7.0 at 5°C. DTT was not included if the extracts were to be used immediately. An aliquot was removed for Chl determination, and the rest stored as small aliquots in liquid N₂ until used.

Galactolipid Analysis. Lipids were extracted from isolated thylakoids according to Roughan and Batt (28), and separated on a silica gel G TLC plate with acetone:benzene:water (91:30:8) (24). Lipid bands were identified by exposure to I₂ (vapor), and galactolipid levels determined colorimetrically (28).

Total Thylakoid Protein. Protein contents were determined by a modified Lowry procedure (20), except reagent A contained 0.25% instead of 1.0% SDS. To eliminate Chl interference, absorbance was measured at 730 nm.

Gel Electrophoresis. Polypeptides were separated by the method of Delepelaire and Chua (5) modified as follows: the 7.5 to 15% gradient gel was stabilized with a 10 to 24% glycerol gradient. Both the stacking and running gel contained 0.1% LiDS. The thylakoid preparations (in 65 mm Tris-Cl [pH 7.0 at 5°C] and 12% glycerol) were solubilized by the addition of 20% LiDS and 10% Triton X-100 at a ratio of 4:2:1 (LiDS:Triton X-100:protein).

Cyt Identification. Possible Cyt polypeptides were localized by combining the heme-dependent peroxidase activity procedures of Thomas, Ryan, and Levin (31) and Guikema and Sherman (9). Gels were immersed in equal parts of 4 mm TMBZ and 2 mm Na-acetate (pH 5.0). After 2 h in darkness, H₂O₂ was added to a concentration of 0.5%. Photographs were taken (wire inserted to mark stain location), and the gels stained in 10% isopropanol, 10% acetic acid, and 0.5% Coomassie brilliant blue R.

Chl. Chl content was determined in 80% acetone according to the method of Arnon (1).

RESULTS

In Fe-deficient plants, the total galactolipid/area was 34 nmol cm⁻², about 25% of the amount of galactolipid in the control.

FIG. 2. Changes in MGDG and DGDG with time after resupply of iron to Fe-deficient plants. A, MGDG/area (Δ) and DGDG/area (●); B, MGDG/DGDG (n = 4 with five pooled leaves per sample as in Fig. 1A).

FIG. 3. Changes in thylakoid protein content with time after resupply of Fe to Fe-deficient plants: A, Thylakoid protein/area (n = 6 with at least five leaves per sample; control leaves contained 475 μg cm⁻²); B, protein/galactolipid (n = four pooled samples); C, protein/Chl (n = 10, 7, 6, 6, and 4 for 0, 24, 48, and 96 h, and control, respectively; samples pooled as described above).
almost tripled from 14 to 41 nmol cm⁻² during the 96-h resupply period. The more rapid increase in MGDG content resulted in an increase in the ratio of MGDG to DGDG (Fig. 2B).

Total thylakoid protein content, unlike the galactolipid content, exhibited a lag phase during the first 24 h of thylakoid synthesis following Fe resupply (Fig. 3A). The ratio of protein/galactolipid decreased over the first 24 h (although the result was not statistically significant) (Fig. 3B). Thylakoid protein content was less affected by Fe deficiency than was Chl content (protein levels of Fe-deficient leaves were about 40% of the control compared to 10% for Chl). The protein/Chl ratio was about 40 in Fe-deficient plants and decreased to 11 after regreening for 96 h (Fig. 3C).

Changes in thylakoid polypeptide composition were examined by gel electrophoresis. The unstained native gel revealed five Chl-protein bands (in addition to free pigment which on an equal Chl basis was greater in chlorotic than in greened tissue) (Fig. 4). The largest Chl-protein (apparent mol wt, 110 kD), which did not fluoresce, is presumed to be CP1 (Fig. 4). On an equal protein and Chl basis (equal Chl not shown), CP1 was depleted in Fe-deficient plants and appeared to be synthesized rapidly during the first 24 h of regreening. It continued to increase throughout the 96-h period.

The oligomer of LHCP (apparent mol wt, 64 kD) was diminished in Fe-deficient plants and increased throughout the greening period. The spectrum of the largest of the Chl-proteins running in the LHCP monomer region (apparent mol wt, 31 kD) indicates that it is not an LHCP, but is similar to CP29 as reported by Green and Camm (8), and by Abadia, Nishio, and Terry (unpublished). On an equal protein basis, this green band (CP29) increased slightly during the later stages of regreening, while on an equal Chl basis it did not appear to change (it may have decreased). On an equal protein and Chl basis, the larger LHCP (apparent mol wt 29.6 kD) increased most during the later stages of regreening. The smallest LHCP band (apparent mol wt, 28.6) was somewhat diffused and changes in this band are not apparent on the green gels.

After staining, polypeptide bands (Fig. 5) were identified by comparing relative mobilities and apparent mol wt as reported by Metz and Miles (21). Two polypeptides with apparent mol wt of 43.8 and 52.7 kD were present in chlorotic tissue, and had relative mobilities previously reported to be associated with PSII (21). The 43.8 and 52.7 kD polypeptides appeared to decrease during the regreening period, both on an equal protein (Fig. 5) and equal Chl basis (Fig. 6A). Changes in the apoprotein of CP1 were difficult to see because it comigrated with other polypeptides in the region of CF α (data not shown).

To determine the changes on an areal basis, varying amounts of protein were loaded as shown in Figure 7. In this gel CP1 (polypeptide 4) and the LHCP (polypeptides 29? and 31?) are not clearly evident in the Fe-deficient or 24-h resupplied plants due to the small amount of thylakoids loaded into the zero and 24-h lanes. The oligomer of LHCP (polypeptide 11) increased significantly between 48 and 96 h (see also the unstained gel, Fig. 4). The PSII polypeptide (polypeptide 16 from Fig. 5) was present at the 0- and 24-h time points, but one of the other PSII polypeptides (polypeptide 19) was less apparent (Fig. 7). During regreening both polypeptides increased, but polypeptide 16 appeared to stain most heavily at the 48-h time-point.

Some polypeptides, for example Cyt f and polypeptides 28, 33, and 43, increased during the regreening period, while many others decreased (e.g. polypeptides 5, 9, 24, and 39) (Fig. 5). Still others, such as CF, were present in large amounts in the Fe-deficient plants, and although they decreased, they remained high throughout regreening. Interestingly, one large mol wt protein (polypeptide 1) appeared to increase during the early regreening period and decrease during the later 48-h period.
FIG. 5. Iron nutrition-mediated changes in thylakoid polypeptides: gel shown in Figure 4A stained with Coomassie blue.

The results of the heme-dependent peroxidase activity staining are shown in Figure 6B. When equivalent amounts of Chl per sample were loaded into the gel, Cyt f( apparent mol wt, 38.4) in the 24-h sample appeared to stain more heavily than the others. The subunit of Cyt b6 (apparent mol wt, 24.1) appeared to increase rapidly during the first 24 h of regreening and decreased thereafter. One unidentified band (apparent mol wt, 41.1) stained most heavily after 48 h of regreening. This band may be a peroxidase, noncovalently bound heme (12) or possibly another form of Cyt f (Sherman, personal communication).

DISCUSSION

The total amount of galactolipids per area increased linearly with time during iron resupply while thylakoid protein lagged behind galactolipid synthesis in the early stages of thylakoid development. This suggests that proteins may be inserted secondarily into the lipid matrix of the thylakoid membrane in higher plants, thus paralleling the apparent mode of development of photosynthetic membranes in synchronized bacterial cell cul-
deficient
deficiency.
to protein
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increased
free (Fe-deficient plants pared to have been may
labile due to the decrease in the galactolipid to protein ratio or some other metabolic lesion caused by Fe
deficiency.

Of the Chl-proteins resolved, CP1 was diminished most in Fe-deficient plants. It also appeared to increase faster during the first 24 h of regreening than the other Chl-proteins. These results suggest that PSI is synthesized or assembled rapidly during regreening.

As discussed in “Results”, polypeptides 16 and 19 may be associated with PSII. On an equal Chl or protein basis, both polypeptides were present in larger amounts than CP1 in Fe-deficient leaves. On an areal basis, polypeptides 16 and 19 were diminished in Fe-deficient tissue and upon resupply followed a pattern similar to that of CP1. However, on a per Chl or protein basis, 16 and 19 appeared to decrease during regreening which suggests that they increased less rapidly than CP1. Based on the data of polypeptides 16 and 19, it appears that PSII was less affected by Fe deficiency than PSI and increased at a slower rate during regreening. The decrease in these two polypeptides on a Chl basis appears to be consistent with the earlier study (22) which showed that the Q/Chl ratio was high in Fe-deficient plants and decreased by half during resupply.

The pattern of appearance of PSI and the LHCP provides a possible explanation for the increase in the Chl a/b ratio which occurred over the first 24 h as reported earlier (22) and as occurred in this study (data not shown). The present results suggest that this may be a result of the more rapid accumulation over the first 24 h of CP1 (which contains mainly Chl a) compared to LHCP which contains both Chl a and b. After 24 h regreening, the Chl a/b ratio decreased as LHCP and CP1

Fig. 6. Iron nutrition-mediated changes in thylakoid polypeptides. A, Gel shown in Figure 4B stained with Coomassie blue; B, TMBZ stained (heme-associated peroxidase activity).
accumulated at similar rates. In addition, the heaviest heme staining of the Cyt f band at 24 h appears to corroborate our earlier finding that the Chl/Cyt f ratio decreased during the first 24 to 48 h of regreening (22).

While changes in the LHCP (polypeptide 31) correlate well with changes in the Chl a/b ratio, changes in CP29 do not. CP29 is considered to be an internal antenna for PSII (8), and changes in CP29 correlate better with changes in the PSII polypeptides (polypeptides 16 and 19), which decreased on an equal Chl and protein basis. Thus, the synthesis of CP29, the core antenna of PSII, appears to occur independently of the bulk LHCP.

Others have also reported that Fe deficiency causes a decrease
in all the Chl-proteins in higher plants (18). Euglena (26), and cyanobacteria (10). In studies where it was possible to tell, a larger amount of free pigment was also associated with Fe-chlorosis. Since it is presumed that most Chl is associated with proteins (19) the question remains — with what polypeptides is the Chl associated in Fe-deficient plants? The stained gels indicated that Fe deficiency increases the number of polypeptides resolved by LiDS/PAGE. In bean (18) and Euglena (26), Fe deficiency apparently reduced the number of polypeptides. Similarly, etioplasts have a decreased number of polypeptides (13). During regreening of Fe-deficient plants, a number of polypeptides decreased or disappeared suggesting that they may: (a) be a result of a lesion in protein synthesis; (b) appear in response to Fe limitation; (c) be precursors of other membrane proteins; or (d) function in membrane assembly. The increase in the ratio of mono to digalactolipids appears to correspond with increased grana stacking in the regrowing plants (see the electron micrographs of Platt-Aloia et al. [23]) Similar correlations between the MGDG/DGDG ratio and stacking have been reported for etioplast transformations (4). However, others have found no change in the MGDG/DGDG ratio during chloroplast development (29). Stacking has also been correlated with a decrease in the ratio of galactolipid to Chl, e.g. maize and sorghum (3), and in developing bean (7). A similar correlation was obtained during Fe nutrition-mediated chloroplast development, i.e. the galactolipid to Chl ratio decreased from 8 to 3 after 96 h of regreening. Correlations between grana stacking and fatty acids and phospholipids have also been discussed; however, this area remains an open question (14).

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