Plant Physiol. (1986) 82, 760–764
0032-0889/86/82/0760/05/$01.00/0

The Fate of Chloroplast Proteins during Photooxidation in Carotenoid-Deficient Maize Leaves[1]

Received for publication June 3, 1986

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ABSTRACT

Maize seedlings, treated with the herbicide norflurazon to produce a deficiency in carotenoid pigments, were grown in low-fluence-rate light. Under these conditions, which induced chlorophyll biosynthesis while minimizing photooxidation, carotenoid-deficient seedlings showed identical patterns of chloroplast protein accumulation compared with normal seedlings. Carotenoid pigments thus play no direct role in regulating the accumulation of chloroplast proteins. When shifted to high-fluence-rate light, chlorophyll was rapidly photooxidized in carotenoid-deficient seedlings. Chloroplast proteins showed varying degrees of sensitivity to photooxidation. The P-700 apoprotein of photosystem I was rapidly degraded. Most stromal and thylakoid proteins either decreased progressively in photooxidative conditions or appeared to be unaffected. The relative quantity of the light-harvesting chlorophyll *a/b*-binding protein of photosystem II increased significantly in the first few hours of high-fluence-rate light. It then appeared to be only minimally affected 18 hours after complete photooxidation of chlorophyll.

Nuclear mutations which completely block the accumulation of carotenoid pigments exhibit a wide range of pleiotropic effects. The most dramatic effect is that Chl fails to accumulate due to rapid photooxidation, resulting in albino seedlings which are incapable of photoautotrophic growth (1, 6). Herbicides which block the accumulation of colored carotenoids exhibit phenotypes very similar to those of the nuclear albino mutants (23). Leaf plastids within these carotenoid-deficient seedlings show little or no development of internal membrane structure (4, 14). These plastids are deficient in plastid ribosomes and lack chloroplastic proteins (18, 21, 28). Cytoplasmic protein synthesis and ribosome accumulation is largely unaffected (10). In spite of the absence of chloroplast development in albino seedlings, leaf morphogenesis appears to proceed normally. Carotenoid-deficient seedlings grow until seed reserves have been exhausted, which in maize occurs 2 to 3 weeks after germination.

Although the structural and functional roles of plastid carotenoids are not well understood, their importance in protecting Chl from photooxidation has been well demonstrated (9 and references therein, 19). Carotenoid-deficient plants grown either in very low-fluence-rate white light or in far-red light, conditions in which photooxidation is minimized, can accumulate low levels of Chl (6, 15). Under either low-fluence or far-red growth conditions, chloroplast development appears normal, with carotenoid-deficient chloroplasts showing the ultrastructure typical of normal chloroplasts grown in low light (5, 18, 30). We (25) have recently shown that a further effect of photooxidative damage is that cytoplasmic mRNA for the LHCP II[4] fails to accumulate. Transcription of the nuclear LHCP II gene family is severely limited in photooxidative conditions (7; DG Burgess, WC Taylor, unpublished data). Growth of carotenoid-deficient seedlings in very low light permits accumulation of normal LHCP II mRNA levels (29). The evidence thus far suggests that photooxidative damage to plastid components is responsible for most, if not all, of the pleiotropic effects of a deficiency in carotenoid pigments (16–18). This is true regardless of the cause of the carotenoid deficiency, herbicide or mutational lesion.

In the present study we examined the extent to which both nucleus- and plastid-encoded proteins accumulate in carotenoid-deficient chloroplasts under nonphotooxidative conditions. The fate of these proteins was then monitored under photooxidative conditions. This was accomplished by growing maize seedlings in the presence of the herbicide norflurazon (Sandoz 9789) in low-fluence-rate white light (nonphotooxidative conditions). Under these conditions, Chl, chloroplastic proteins, and plastid ribosomes, which fail to accumulate in albino seedlings in daylight growth, accumulated to wild type levels (15). However, carotenoid pigments failed to accumulate in the herbicide-treated plants during low-light growth. Exposure of these seedlings to high-fluence-rate light resulted in the photooxidation of greater than 90% of the Chl within 24 h. Under these photooxidative conditions, a specific subset of chloroplastic proteins was rapidly degraded, while another group of proteins remained intact, even after 24 h of photooxidative growth.

MATERIALS AND METHODS

Plant Materials. Seeds of the maize inbred line B73 (Pioneer HiBred International, Johnston, IA) were planted on soil, covered with 1 cm of vermiculite and grown in a growth chamber at 30°C with 12 h of low-fluence-rate light (0.011 μE m^{-2} s^{-1}) and 12 h of darkness at 22°C. These conditions were chosen as those which permitted maximum Chl accumulation without evidence of photooxidation. Seedlings were germinated and grown in either water (untreated) or a solution of 100 μM norflurazon. After 7 d of growth, when seedlings were 10 to 14 cm in height, the light was increased to the high fluence rate (96 μE m^{-2} s^{-1}) and the temperature kept constant at 24°C. Seedlings were harvested at intervals directly into liquid N_2 and kept at −70°C until used.

[1] Supported by grants from the Competitive Research Grants Office of the United States Department of Agriculture to W. T.

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[4] Abbreviations: LHCP II, light-harvesting chlorophyll *a/b*-binding protein of photosystem II; norflurazon, 4-chloro-5-(methylamino)-2-(a,a,a-trifluro-*m*-tolyl)-3(2H)-pyridazinone.

Protein Gel Blotting. PAGE of leaf homogenates, protein blotting and antibody reactions were as described (21). Samples from herbicide-treated and untreated plants were always compared on the same blot of the same gel so that autoradiographic intensity is an accurate measurement of the relative amount of a given polypeptide in each sample. Dilution series of homogenates of untreated leaves from high-fluence-rate grown seedlings were reacted with each antiserum to demonstrate a linear relationship between autoradiographic intensity and relative amount of polypeptide.

Preparation of Antisera. All antisera were raised against isolated protein complexes or individual proteins from maize in white New Zealand rabbits as previously described (21). NADP-malic enzyme was prepared according to Collins and Hague (11).

Antisera against maize Cyt *f* and PSI polypeptides were generously provided by Alice Barkan of this laboratory. Maize Cyt b_6/f complex was prepared by the method of Hurt and Hauska (22) followed by SDS-PAGE fractionation to purify Cyt *f*. PSI was prepared as a by-product of the Cyt b_6/f preparation. After octylglucoside solubilization of the Cyt complex, the membrane pellet was resuspended in water at a Chl concentration of 1 mg/ml. PSI was solubilized by the addition of Triton X-100 to 0.7% with constant stirring for 30 min. Following pelleting of the membranes (38,000*g* for 30 min), the supernatant was layered onto a 5 to 25% sucrose gradient in 0.1% Triton X-100, 20 mM Tris-HCl (pH 7.8). Centrifugation was for 15 h at 4°C at 40,000 rpm in an SW41 rotor (Beckman Instruments). The lower green band had a Chl *a/b* ratio of 6 to 7 and a Chl/P700 ratio of 120 to 200. This preparation yielded polypeptides of 70, 20, 18, 11, 10, and 9 kD. Based on these properties, this complex seems to correspond to the spinach PSI_{110} or PSI_{200} described by Mullet *et al.* (27). The complex was concentrated, denatured in 8 M urea, 10% SDS, 10% 2-merceptoethanol at room temperature for 1 h and injected into rabbits as described (21).

Antiserum for the light-harvesting Chl *a/b*-binding polypeptides of PSI from spinach was generously provided by Eric Lam (University of California, Berkeley) (24).

Chlorophyll and Carotenoid Determination. Frozen leaf tissue was ground to a fine powder in a coffee grinder chilled with dry ice. The tissue was thawed in 100% acetone in the dark, filtered through sintered glass and the spectra recorded on a Cary 920 visible spectrophotometer from 350 to 720 nm. Chl *a* and *b* were determined using the equations of Arnon (3) and the carotenoid content (including xanthophylls) estimated from the *A* at 480 nm using the equations of Davies (12).

RESULTS

Chlorophyll Accumulation in Carotenoid-Deficient Seedlings Grown under Low-Fluence White Light. In normal daylight, maize seedlings germinated and grown in the presence of 100 μM norflurazon (Sandoz 9789) fail to accumulate either Chl or carotenoids (15, 18, 23, 25). These seedlings also fail to accumulate any of the major chloroplast proteins found in normal green seedlings (15). Growth of norflurazon-treated seedlings in low-fluence white light (0.011 $\mu E\ m^{-2}\ s^{-1}$) is sufficient for the conversion of Pchlide to Chl(ide) but insufficient for the complete photooxidation of Chlorophyll (15). Chl accumulates in treated seedlings to about two-thirds of the level in untreated seedlings also grown in low light (Table I). There appeared to be a low level of carotenoids in low-light-grown treated seedlings, but this level was close to the limit of sensitivity of detection and may not be entirely accurate.

Untreated seedlings accumulated both Chl and carotenoids during low-light growth, and after a short lag period rapidly accumulated greater quantities during high-light growth. In low light the Chl *a/b* ratio is high in both the norflurazon-treated and untreated seedlings. Exposure of untreated seedlings to high light resulted in the accumulation of Chl *b* at a faster rate than Chl *a*, and resulted in the lowering of the Chl *a/b* ratio to that of daylight-grown seedlings (3.5) within 24 h (Table I).

Table I. *Quantities of Chl and Carotenoids in Herbicide-Treated and Untreated Seedlings*

Plants were grown for 7 d in low light, then shifted either to high light or complete darkness for the indicated time. All measurements are listed in $\mu g\ (g\ fresh\ wt)^{-1}$.

h in High Light (H) or Dark (D)	Carotenoids	Total Chl	Chl *a*	Chl *b*	*a/b*
	μg g⁻¹ fresh weight				*ratio*
Norflurazon treated					
0 h	42.85	40.93	1.92	21.3	1.07
1 h H	33.34	32.05	1.29	24.8	0.27
6 h H	9.39	8.84	0.55	16.0	0.18
24 h H	3.36	2.44	0.92	2.8	0.25
6 h D	46.41	43.69	2.72	16.0	0.96
24 h D	27.77	25.86	1.91	13.6	0.87
Untreated					
0 h	72.06	69.49	2.57	27.0	13.69
1 h H	71.17	68.99	2.18	31.6	12.13
6 h H	277.45	190.84	36.61	5.2	15.26
24 h H	1078.79	842.02	236.77	3.5	30.12
6 h D	41.68	39.88	1.80	22.2	9.64
24 h D	34.76	33.34	1.33	25.1	9.40

Exposure of the norflurazon-treated seedlings to high-fluence-rate white light (96 $\mu E\ m^{-2}\ s^{-1}$) resulted in the rapid loss of Chl from the carotenoid-deficient seedlings due to photooxidation. The amount of Chl *b* dropped markedly for the first 6 h of growth, and then appeared to increase to approximately 50% of low light levels after 24 h. Whether this was due to greater stability of Chl *b* in high light is difficult to determine as the overall amount of Chl is so low after 24 h of high-light growth that the determination of Chl *a/b* ratios may not be accurate in these seedlings (Table I).

Chl and carotenoid levels dropped in both the treated and untreated seedlings when they were placed into darkness. However, both norflurazon-treated and untreated seedlings retained greater than 50% of the Chl, and the untreated seedlings retained greater than 75% of the carotenoids, after 24 h of dark growth (Table I).

LHCP II. While the level of Chl dropped dramatically in the norflurazon-treated seedlings during the first 6 h of high-light growth, LHCP II actually increased during this same period (Fig. 1A). This LHCP II was membrane associated and exhibited the same electrophoretic mobility as the mature apoprotein from light-grown untreated plants. The relative amount of LHCP II remained fairly constant for an additional 24 h of high-light illumination, although only a very small amount of Chl *b* remained at this point. Initially the untreated seedlings grown in low light had levels of LHCP II similar to those of the treated plants. Exposure of these seedlings to high light resulted in a rapid accumulation of LHCP II after a lag of between 1 and 6 h (Fig. 1B). After 24 h of high-light growth the untreated seedlings had levels of LHCP II many times greater than those of the treated or low-light-grown seedlings.

PSI Polypeptides. The P-700 apoprotein of the PSI reaction center accumulated in both carotenoid-deficient and normal seedlings during growth in low light. Exposure of the norflurazon-treated seedlings to high light resulted in the rapid loss of P-700 apoprotein such that it was undetectable after 1 to 6 h of high light illumination (Fig. 1A). Untreated seedlings showed rapid accumulation after 1 to 6 h of high-light illumination.

All of the polypeptides associated with PSI did not decrease concomitantly in the norflurazon-treated seedlings during high-

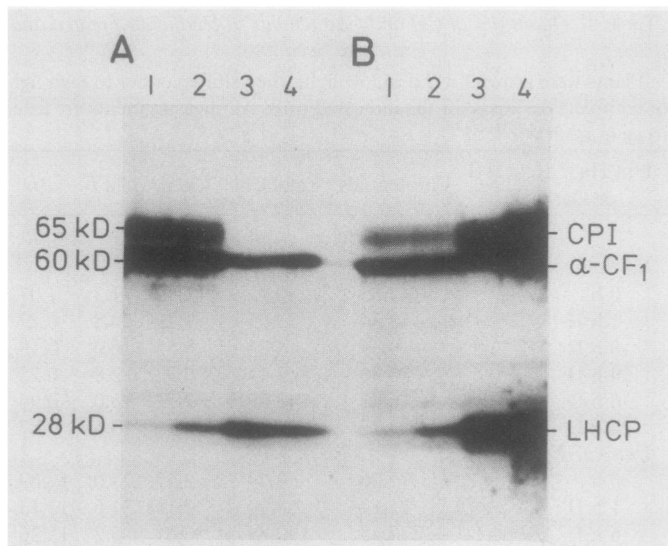

FIG. 1. Thylakoid proteins in low-light-grown seedlings transferred to high light. A blot of membrane associated polypeptides was reacted with antisera for LHCP II (LHCP), P-700 apoprotein (CPI), and α-subunit of ATP synthetase (α-CF_1). Equal amounts of protein were loaded in each gel lane. Herbicide treated (A) or untreated (B) seedlings were sampled after 7 d growth in low light (1), or with an additional 1 h (2), 6 h (3), or 24 h (4) growth in continuous high light.

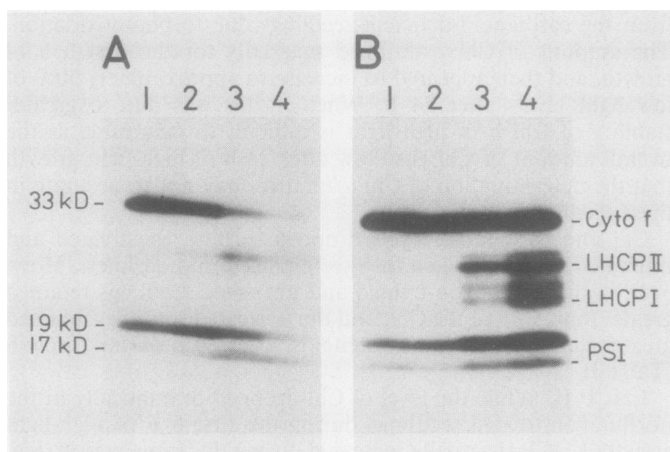

FIG. 2. Thylakoid proteins in low-light-grown seedlings transferred to high light. A blot of membrane associated polypeptides was reacted with antisera for Cyt *f* and PSI. Equal amounts of protein were loaded in each gel lane. Herbicide treated (A) or untreated (B) seedlings were sampled after 7 d growth in low light (1), or with an additional 1 h (2), 6 h (3), or 24 h (4) growth in continuous high light.

light growth. Two low-molecular-mass polypeptides previously identified as constituents of maize PSI, 17 kD and 19 kD, (A Barkan, R Malkin and WC Taylor, unpublished data) were measured using an antiserum raised against a purified maize PSI preparation. The 19 kD polypeptide was present in significant quantities in low-light-grown seedlings. It showed a major decrease between 6 and 24 h of high-light illumination (Fig. 2A). The 17 kD polypeptide was barely detectable in low-light-grown plants, increasing after 6 h of high light and then decreasing. These smaller molecular mass polypeptides accumulated along with P-700 apoprotein in the untreated seedlings exposed to high light (Fig. 2B).

The 24 to 26 kD Chl *a*/*b*-binding polypeptides of the PSI light-harvesting complex were not detectable in either herbicide-treated or untreated plants grown in low light. They were first detectable in untreated plants after 6 h of high-light illumination (Fig. 2B). Identification of these polypeptides was made by their ability to cross-react with antiserum prepared from the PSI light-harvesting complex polypeptides of spinach (24). Although the antiserum was made from a purified PSI preparation, that preparation contained a small amount of contaminating LHCP II, as is evident in the low level of reactivity of the antiserum to LHCP II polypeptides (Fig. 2).

Cytochrome *f*. Cyt *f* exhibited a progressive decrease to very low levels after 24 h of high-light exposure in treated seedlings (Fig. 2A). Untreated seedlings contained relatively large amounts of Cyt *f* during low-light growth. The relative amount remained constant after 24 h of high-light exposure (Fig. 2B).

Coupling Factor. The α-subunit of ATP synthetase was present in the carotenoid-deficient seedlings after 24 h of high-light growth (Fig. 1A), although at levels several-fold less than in low-light-grown seedlings. Untreated seedlings contained α-subunit of ATP synthetase when grown in low light. The relative amount increased slightly after growth in high light (Fig. 1B).

Enzymes of Photosynthetic Carbon Fixation. Similar amounts of both CO_2-fixing enzymes, ribulose 1,5-bisP carboxylase and P-enolpyruvate carboxylase, were found in both herbicide-treated and untreated seedlings grown in low light (Fig. 3). The same was true for two enzymes involved in the C_4 carbon reduction cycle, pyruvate orthophosphate dikinase and NADP-malic enzyme. All four enzymes showed slight increases in untreated plants after 24 h of high-light illumination. When herbicide-treated seedlings were illuminated with high light, pyruvate orthophosphate dikinase decreased significantly. The other three either decreased slightly or remained at about the same relative quantity. It should be mentioned that this protein blot shows only the large subunit of ribulose 1,5-bisP carboxylase. Our particular protein blotting system does not efficiently transfer the small subunit (26). In all cases, the original gel was stained with Coomassie blue to show that large and small subunits were detectable in equal molar quantities.

Soluble Leaf Proteins. The profiles of total stainable soluble leaf proteins did not show many significant differences when herbicide-treated and untreated seedlings were compared (Fig. 4). Nor were there significant changes when low-light-grown plants were transferred to high light.

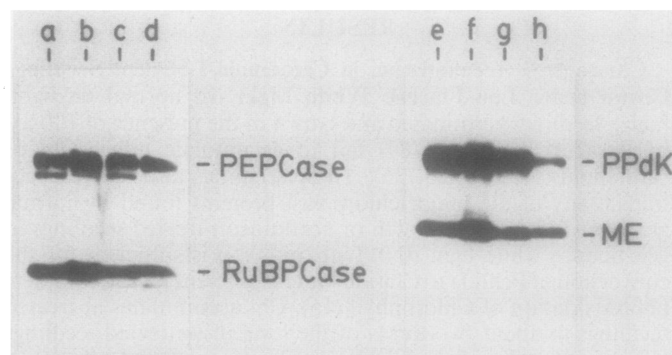

FIG. 3. Soluble carbon fixation enzymes in low-light-grown seedlings transferred to high light. Blots of soluble proteins were reacted with antisera for ribulose 1,5-bisP carboxylase (RuBPCase), P-enolpyruvate carboxylase (PEPCase), pyruvate orthophosphate dikinase (PPdK), and NADP-malic enzyme (ME). Equal amounts of protein were loaded in each gel lane. Herbicide treated (c, d, g, h) or untreated (a, b, e, f) seedlings were grown in low light for 14 d (a, c, e, g), then transferred to continuous high light for 24 h (b, d, f, h).

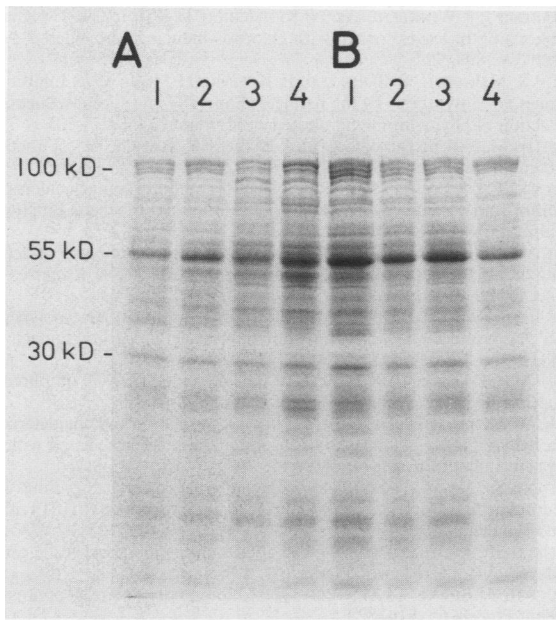

FIG. 4. Total soluble proteins in low-light-grown seedlings transferred to high light. Soluble proteins were stained with Coomassie blue after electrophoretic fractionation through a 10% polyacrylamide gel. Equal amounts of protein were loaded in each gel lane. Herbicide treated (A) or untreated (B) seedlings were sampled after 7 d growth in low light (1), or plus an additional 1 h (2), 6 h (3), or 24 h (4) in continuous high light.

DISCUSSION

Using sensitive immunological probes, we show that specific polypeptide components of chloroplast thylakoid complexes and the soluble carbon fixation pathway enzymes accumulate in the absence of carotenoid pigments, when maize seedings are grown in low-fluence-rate white light. The relative quantities of these polypeptides were remarkably similar to those found in untreated plants grown under identical low-light conditions. We have previously shown that the same relative quantities of LHCP II mRNA are present in herbicide-treated and untreated seedlings which had been grown under low-light conditions (29). Carotenoid pigments thus play no direct role in the regulation of accumulation of chloroplast proteins. Although low-light-grown herbicide-treated plants show the same complement of chloroplast proteins as untreated plants, we have not determined whether these proteins are organized into functional units, nor have we measured photosynthetic rates. Frosch *et al.* (18) reported that ribulose 1,5-bisP carboxylase activities were similar in mustard seedlings treated with norflurazon and untreated seedlings when grown in low-fluence-rate far-red light. Feieraband *et al.* (17), however, found no detectable photosynthetic O_2 evolution in low-light-grown rye seedlings treated with Sandoz 6706, a pyridazinone herbicide closely related to Sandoz 9789.

When carotenoid-deficient seedlings were transferred to high-fluence-rate white light, the most dramatic effect was the rapid loss of Chl, which occurred within several hours. Most of the polypeptides measured in this study decreased concomitantly with Chl and were significantly reduced after 24 h of high-light growth. During this same period, however, two thylakoid polypeptides, LHCP II and the 17 kD polypeptide of PSI, increased in relative amounts for up to 6 h after transfer to high light. There was no net accumulation of any polypeptide in the carotenoid-deficient seedlings after 6 h of high-light growth, while the untreated seedlings rapidly accumulated all chloroplastic proteins after transfer to high light.

It is intriguing that the relative quantity of LHCP II actually increases when low-light-grown seedlings were transferred to high light. It has been repeatedly observed that the accumulation of LHCP II correlates well with the accumulation of Chl (*e.g.* 21). This observation led Apel and Kloppstech (2) and Bennett (8) to propose that LHCP II requires the binding of Chl to facilitate its stable integration into the thylakoid membrane. In the absence of Chl the LHCP II precursor is rapidly degraded so that no net accumulation of the apoprotein occurs.

The present data are not necessarily inconsistent with the conclusions of Apel and Kloppstech (2) and Bennett (8). Our data suggest that Chl is not required for the maintenance of LHCP II once the polypeptide has been integrated into the thylakoid membrane. When carotenoid-deficient seedlings are transferred from low to high light there are two opposing effects on the net accumulation of Chl. One effect of the higher fluence rate is to increase the rate of Chl synthesis and especially increase the rate of Chl *b* synthesis. The opposing effect is to increase the rate of Chl photooxidation. The net effect is a decrease in the total amount of Chl over a several hour period. It may be that a transitory increase in the rate of Chl synthesis provides a pool of free Chl which is rapidly bound to existing LHCP II precursor polypeptides before the Chl is photooxidized. After 24 h of high-light growth the Chl *b* content of herbicide-treated seedlings is about 50% of the low-light level, while the Chl a content is about 5%.

We considered two possibilities for the loss of thylakoid proteins under photooxidative conditions. First, the observed decrease represents the normal turnover rate of the polypeptide in the absence of *de novo* synthesis. Second, polypeptides may be directly damaged by photooxidation and thus susceptible to proteolytic degradation. Our data cannot rigorously distinguish among these possibilities. In another study, the loss of chloroplast NADP-glyceraldehydephosphate dehydrogenase activity in herbicide-treated rye seedlings was shown to occur at 0°C and therefore concluded to be due to photooxidative damage (15).

An interesting observation is the extent of variability in protein sensitivity to photooxidative conditions. The P-700 apoprotein of PSI and Cyt *f* both disappear rapidly from the thylakoid in high light. The disappearance of the P-700 apoprotein is not surprising, given that superoxide has been shown to be produced by PSI (13, 20). However, the rapid loss of Cyt *f* from the thylakoids is not as easily rationalized. Also difficult to explain is that two other components of PSI do not show the same rapid rate of degradation as the P-700 apoprotein. The 17 kD even increases slightly in quantity. The ability to conditionally control photooxidation by changing light intensity may allow us to better dissect the process of assembly of the photosystem complexes and to assay structural and functional relationships between them.

From these and other studies, one can conclude that normal chloroplast morphogenesis can proceed in the absence of carotenoid pigments if photooxidation of chloroplastic components is otherwise limited (*e.g.* 5, 18, 28, 30). As we have shown in this study, individual chloroplastic components are differentially sensitive to photooxidative damage, with some perhaps directly damaged by photooxidation and others unaffected.

Acknowledgments—We are especially grateful to Drs. Alice Barkan and Eric Lam for providing us with antisera, and the Zoecon Corporation, Palo Alto, CA for a gift of Sandoz 9789. We thank Drs. Kenneth Sauer and Richard Malkin for helpful discussions.

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