Plant Physiol. (1994) 106: 293-302

Identification of Peroxisome Membrane Proteins (PMPs) in Sunflower (*Helianthus annuus* L.) Cotyledons and Influence of Light on the PMP Developmental Pattern[1]

Lilian W. Jiang, Jeff Bunkelmann, Leslie Towill, Stefan Kleff[2], and Richard N. Trelease*

Arizona State University, Department of Botany, Tempe, Arizona 85287-1601

Boundary membranes were recovered from glyoxysomes, transition peroxisomes, and leaf-type peroxisomes purified from cotyledons of sunflower (*Helianthus annuus* L.) at three stages of postgerminative growth. After membranes were washed in 100 mM Na_2CO_3 (pH 11.5), integral peroxisome membrane proteins (PMPs) were solubilized in buffered aminocaproic acid/dodecyl maltoside (0.63 M/1.5%) and analyzed by nondenaturing and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Six prominent nondenatured PMP complexes and 10 prominent SDS-denatured polypeptides were identified in the membranes of the three types of peroxisomes. A nondenatured complex of approximately 140 kD, composed mainly of 24.5-kD polypeptides, decreased temporally, independently of seedling exposure to white, blue, or red light; only far-red light seemed to prevent its decrease. PMP complexes of approximately 120 and 70 kD, in contrast, were present at all stages and changed in polypeptide content. It remains to be determined whether these data reflect changes within in vivo complexes or within complexes formed following/during detergent solubilization. Conversion of glyoxysomes to leaf-type peroxisomes in white or red light after a 2-d dark period was accompanied by the appearance of three SDS-denatured PMPs: 27.5, 28, and 47 kD. The former two became part of the PMP120 and 70 complexes, as well as part of a new PMP130 complex that also possessed the PMP47. Growth of seedlings in blue or far-red light did not promote the appearance of PMPs 27.5 or 28. Blue light promoted the appearance of PMP47, and far-red light seemed to prevent its appearance. Chlorophyll likely is not the photoreceptor involved in accumulation of PMPs because the PMP composition is distinctly different in seedlings irradiated with red or blue light of comparable fluence rates. Several lines of evidence indicate that the synthesis and acquisition of membrane and all matrix proteins are not coupled. The data provide evidence for a change in PMP composition when sunflower or any other oilseed glyoxysomes are converted to leaf-type peroxisomes and suggest that the change is regulated by both photobiological and temporal mechanisms.

Germinated oilseeds are an excellent system in which to study the developmental biogenesis of peroxisomes. Following germination, oil stored in cotyledons are converted mostly into carbohydrates for transport to the growing shoot and root (Trelease and Doman, 1984). Afterward, the cotyledons emerge above ground and assume a photosynthetic role while new leaves develop. Glyoxysomes possess enzymes of the glyoxylate cycle and fatty acid β-oxidation and function during the oil-mobilizing growth phase (Breidenbach and Beevers, 1967; Cooper and Beevers, 1969). Glyoxysomes enlarge (Kunce et al., 1984; Chapman and Trelease, 1992) and are converted during a transition period into leaf-type peroxisomes that participate in the glycolate cycle as part of photorespiration (Tolbert et al., 1968). Three models were proposed previously to describe the peroxisome changeover during the transition period; the "one-population" hypothesis (Trelease et al., 1971) is the currently accepted model (Beevers, 1993) and forms the basis for interpretations in this study.

Considerable research has been devoted to the study of matrix proteins of eukaryotic peroxisomes (Huang et al., 1983; van den Bosch et al., 1992; Subramani, 1993). Much less has been accomplished relative to the composition, biogenesis, or function of PMPs. The identification and partial characterization of PMPs by SDS-PAGE has been reported for several different types of peroxisomes, e.g. mammalian peroxisomes (Causeret et al., 1993), yeast peroxisomes (Sulter et al., 1993), castor bean glyoxysomes (Luster et al., 1988; Halpin et al., 1989), cotton cotyledon glyoxysomes (Chapman and Trelease, 1992), and potato tuber peroxisomes (Struglics et al., 1993). In none of the studies were data reported concerning PMPs separated by nondenaturing PAGE. Yeasts are well suited for studying PMPs because both the proliferation and enzyme composition of peroxisomes can be altered by manipulating the growth conditions. For example, in *Hansenula polymorpha*, constituent PMPs are 22, 31 (triplet), 42, 49, and 51 kD. However, PMPs 24, 29, 37, and 62 are induced in ethanol-grown cells but not in methanol (Sulter et al., 1990, 1993).

In plants, SDS-denatured PMPs have been identified mostly in seedling glyoxysomes. Six major PMPs were observed in membranes of enlarging glyoxysomes in cotyledons of cotton seedlings; no function was attributed to any of these polypeptides (Chapman and Trelease, 1992). Mn-superoxide dismutase activity was reported to be associated with an 82-

[1] This research was supported by U.S. Department of Agriculture National Research Initiative Competitive Grants Program grant 92-37304-7755 to R.N.T. and by the Deutsche Akademische Austauschdienst/Germany to S.K.

[2] Present address: University of Münster, Department of Botany, Sclossgarten 3, 48149 Münster, Germany.

* Corresponding author; fax 1-602-965-6899.

Abbreviations: HPR, hydroxypyruvate reductase; MS, malate synthase; PMP, peroxisome membrane protein; TIP, tonoplast intrinsic protein.

kD polypeptide in glyoxysomal membranes of watermelon cotyledons (Sandalio and Del Rio, 1987, 1988). Donaldson's group (Hicks and Donaldson, 1982; Donaldson and Fang, 1987; Fang et al., 1987) demonstrated that Cyt b_5, Cyt P-450, NADH:ferricyanide reductase, NADH:Cyt *c* reductase, and NADPH:Cyt *c* reductase were present in glyoxysomal and ER membranes of castor bean endosperm. These activities co-purified with a glyoxysomal 32-kD membrane protein (Luster et al., 1988; Bowditch and Donaldson, 1990). Alani et al. (1990) showed that redox activities increased in endosperm following germination of castor bean. They proposed that the membrane of the ER and glyoxysomes were being enriched with redox proteins during glyoxysome proliferation.

Although the acquisition and composition of lipids (Chapman and Trelease, 1991) and proteins (Chapman and Trelease, 1992) have been examined in membranes of enlarging glyoxysomes in dark-grown cotton seedlings, no data have been reported concerning the composition of membrane proteins in differentiating oilseed peroxisomes relative to light exposure. In this study, we identified six prominent nondenatured PMP complexes and 10 SDS-denatured PMPs from the cotyledons of sunflower (*Helianthus annuus* L.) seedlings in glyoxysomes, transition peroxisomes, and leaf-type peroxisomes. Our studies showed that exposure of seedlings to varied qualities of light affects the change in developmental pattern of PMPs that occurs when sunflower glyoxysomes are converted to leaf-type peroxisomes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Sunflower seeds (*Helianthus annuus* L. cv large grey) were purchased at Tempe Feed & Tack (Tempe, AZ). Sunflower achenes were soaked for 12 h (30°C) in distilled water with aeration and then scrolled in a double layer of Whatman No. 1 sheets. They were germinated and grown in the paper scrolls at 30°C for 1 d. Afterward, the seedlings were selected for uniform size and individually transferred under a dim green fluorescent light to distilled water-saturated vermiculite in trays. Seedlings were placed in a chamber at 30°C in continuous darkness or light for various numbers of days depending on the experiments.

Light Conditions

Fluorescent bulbs (eight, 40 W, cool-white) with a fluence rate of 150 μmol m^{-2} s^{-1} (Quantum Sensor; Li-Cor, Lincoln, NE) were the white light source. A series of cheesecloth screens were used to decrease fluence rates to 33, 15, or 1.5 μmol m^{-2} s^{-1}. The same fluorescent bulbs, which emit little far-red light, were used with blue and red acetate filters in the lighted chamber, giving fluence rates of 23 and 29 μmol m^{-2} s^{-1}, respectively. Incandescent bulbs (four, 100 W) together with the eight fluorescent bulbs and the combined blue and red filters were used to produce far-red light with a fluence rate of 45 W m^{-2} (YSI Radiometer; Yellow Springs Instruments, Yellow Springs, OH) The lighted chamber was kept at 30°C with a cooling system. Figure 1 shows the transmission characteristics of the acetate filters. The peak transmission of light with blue acetate filter was at 480 nm, and 0% transmission was apparent between 580 and 720 nm. Because of the nature of the acetate filter, light also was transmitted in the 800-nm region, but without the incandescent bulbs on in the lighted chamber, only light in the blue region was transmitted. The red filter allowed transmission of light from about 600 to 800 nm but not between 400 and 560 nm. When the blue and red filter were combined, light was transmitted only in the far-red region (>720 nm).

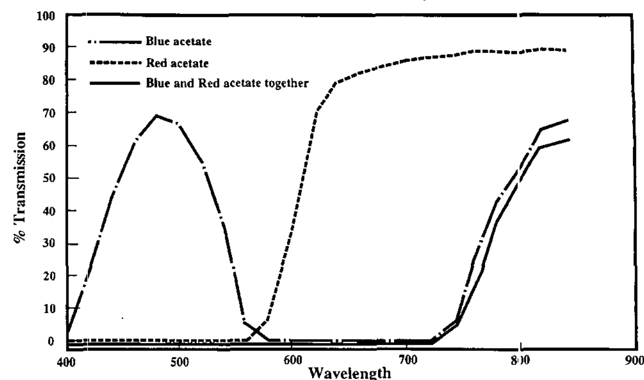

Figure 1. Transmission characteristics of blue only, red only, and blue plus red acetate filters determined with a spectrophotometer. These filters were used in a growth chamber to test the effect of light quality and intensity on changes in relative amounts of PMPs during the glyoxysome/peroxisome changeover in sunflower seedlings.

Preparation of Crude Extracts

Sunflower cotyledons harvested at different times were frozen in liquid N_2 and then stored at −80°C. Cotyledons to be assayed for MS (EC 4.1.3.2) and HPR (EC 1.1.1.81) activities were homogenized in homogenizing buffer containing 87.5 mM Tris-HCl, 7 mM $MgCl_2$, 1.75 mM EDTA, pH 7.7, 1 mM PMSF, and 2 mM DTT (did not interfere with the assay for MS activity). Triplicate homogenizations were done for each time period. Ten frozen cotyledon pairs per replicate were ground to a powder in a pre-chilled mortar with a pestle, and the powder was suspended in 3 mL of homogenizing buffer. Homogenization was performed with a motor-driven Teflon pestle in a glass vessel at 4°C. Homogenates were centrifuged for 30 min at 27,000*g* in a Beckman JA-20 rotor at 4°C. The supernatants were used for enzyme assays; activities were measured at room temperature (22°C) in a DU-64 spectrophotometer (Beckman, Fullerton, CA).

Enzyme, Chl *a*, and Protein Assays

Enzymes used as organelle markers were assayed for MS according to the method of Trelease et al. (1987) for glyoxysomes, HPR was measured using the method of Titus et al. (1983) for leaf-type peroxisomes, and catalase (EC 1.11.1.6) was measured according to the method of Ni et al. (1990) for

both organelles. Cyt *c* oxidase (EC 1.9.3.1) assayed according to the method of Tolbert et al. (1968) marked mitochondria. Chl *a* content was measured in the Suc gradient fractions. To prepare a standard curve, Chl from 10 sunflower cotyledon pairs (2 d in dark plus 3 d in white light) were extracted into 6 mL of 80% acetone, and total Chl concentration (mg/mL) was determined from the *A* at 652.5 nm divided by 34.5 (Vernon, 1960) using a Beckman DU-64 spectrophotometer. These Chl samples were diluted in 80% acetone to a range of 0 to 0.4 μg Chl mL^{-1}. Chl *a* fluorescence at 438 nm was measured with a Spex FluoroMax (DM 3000 software) fluorescence spectrophotometer. Gradient fraction samples (5–20 μL) were dissolved into 3 mL of 80% acetone, and the amount of Chl *a* was calculated from the standard curve values in the range of 0.05 to 0.25 μg Chl mL^{-1}. The amount of protein in gradient fractions was measured by the method of Bradford (1976) in the range of 0.2 to 20 μg of protein with bovine γ-globulin (Bio-Rad) as standard. The amount of PMP was measured by the bicinchoninic acid method as described in the Pierce instruction pamphlet (1991) with BSA in 42 mM Tris-HCl, 0.6 M aminocaproic acid, pH 7.2, and 1.6% (w/v) dodecyl maltoside (Calbiochem) as standard.

Isolation of Peroxisomes

Peroxisomes were isolated from cotyledons of sunflower seedlings grown for different times and under different light conditions by essentially the same procedure described by Eising and Gerhardt (1986). All operations were performed at 4°C. Cotyledons (amounts given in Table I) were homogenized with a mortar and pestle in 30 mL of 1.0 M Suc, 170 mM Tricine, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.9% (w/v) BSA, pH 7.5, 14.7 mM β-mercaptoethanol (10 μL of stock per 10 mL of medium), and 1 mM PMSF. The homogenate was filtered through three layers of cheesecloth and centrifuged in two 30-mL tubes for 10 min at 1500*g* in a Beckman JA-20 rotor. The supernatant was centrifuged for 20 min at 14,000*g* in the same rotor to obtain peroxisome-enriched pellets. The pellets were gently suspended with a camel hair brush in 11 mL of homogenizing medium. A 5.5-mL suspension of organelles was applied on top of each step Suc gradient composed of 4 mL of 60% (w/w), 5 mL of 57%, 7 mL of 51%, 5 mL of 47%, 5 mL of 42%, and 4 mL of 35% Suc. Suc solutions were prepared in 1 mM EDTA, pH 7.5. Fifty-one percent Suc was substituted for the 52.5% Suc prescribed by Eising and Gerhardt (1986). The gradients were centrifuged for 1.5 h at 70,000*g* with a Beckman SW 28 rotor in a Beckman XL-80 ultracentrifuge at settings of No. 5 acceleration and No. 5 deceleration. After centrifugation, three gradient fractions were collected with a large bore Pasteur pipet from the following interphases: 42 to 47%, 47 to 51%, and 51 to 57%.

Preparation of PMPs

PMPs were prepared using the methods described by Fang et al. (1987) with minor modifications. Peroxisomes were burst by hypotonic shock in 2 volumes of 100 mM K phosphate, 3 mM $MgCl_2$, 2 mM EDTA, pH 7.2, 1 mM PMSF, and 2 mM DTT. The suspension was mixed and held at 4°C for 30 min and then centrifuged for 45 min at 100,000*g* with a Beckman 70.1Ti rotor to sediment peroxisomal membranes. The membrane pellets were completely resuspended in 4 mL of 100 mM Na_2CO_3, pH 11.5, by repeated mixing in a pipet tip, and the mixture was incubated on ice for 30 min before centrifugation for another 45 min at 100,000*g*. The membranes were resuspended in a solution made by adding 56 μL of 10% dodecyl maltoside to 300 μL of 50 mM Tris-HCl and 0.75 M aminocaproic acid, pH 7.2. By mixing in a pipet tip, the membrane proteins were solubilized in the detergents. The mixture was centrifuged for 30 min at 100,000*g*, and the pellet was treated again as described above with 237 μL (same ratio) of detergent solution. The supernatants, containing aminocaproic acid/dodecyl maltoside-solubilized PMPs, were combined for further analyses.

Electrophoresis

Nondenatured PMP complexes were separated by modifications of the methods described by Schägger and von Jagow (1991). Taurodeoxycholate (4 μL of 2% per 50 μL of sample) was added to create a charge shift on the proteins. Tris-HCl was substituted for Bis-Tris in the native gel buffer. Electrophoresis in 5 to 10% gradient gels (Protean II, 20-cm plates, Bio-Rad) was for 15 h at constant voltage (70 V), followed by 1 h at 200 V (constant voltage). Jack bean urease and BSA from Sigma were used as high molecular mass standards to assign estimated kD values to nondenatured protein complexes. PMP subunits were separated by SDS-PAGE using a discontinuous buffer system (Laemmli, 1970). The aminocaproic acid/dodecyl maltoside-solubilized membrane proteins were added to an equal volume of 2× SDS treatment buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, and 20% glycerol), and 10 mM DTT (final concentration) was added from a 0.5 M DTT stock. The samples were heated (not boiled) at 45°C for 15 min and then applied to 10 to 15% gradient gels. Electrophoresis (Protean II, 20-cm plates, Bio-Rad) was for 1 h at a constant current (15 mA), followed by 8 h at 20 mA constant current. Low molecular mass standards from Bio-Rad were used for estimation of molecular mass.

Two-dimensional gel separations were accomplished with essentially the same results by two different methods. For the first method, PMP complexes (20 μg of protein/lane) were separated in 1.0-mm-thick nondenatured gradient gels described above. One of the duplicate lanes was silver stained (see below) to visualize relative migrations of the complexes, and the other excised lane was incubated in 1× SDS buffer without DTT at 45°C for 45 min. The gel lane (slice) was carefully placed on the top of a 10 to 15% SDS gel (1.5 mm thick), and electrophoresis was conducted as described for SDS-PAGE. SDS-denatured polypeptides contained within each nondenatured complex were visualized in a vertical row after silver staining; figures illustrating these results are not shown. For the second method, nondenatured PMP complexes were separated in four lanes (20 μg of protein/lane) for each stage (12 lanes total) in 1.5-mm-thick nondenaturing gradient gels as described above. Gels were stained with a

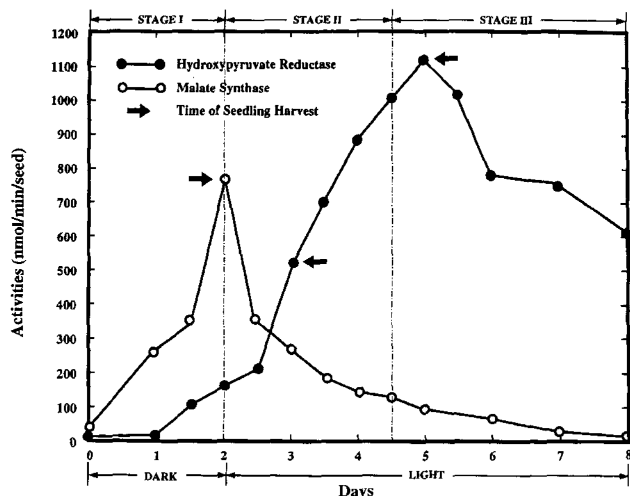

Figure 2. Time course of MS (glyoxysome) and HPR (leaf-type peroxisome) activities in cotyledons of sunflower seedlings grown in the dark for 2 d and then transferred to continuous white light. The periods established for stages I, II, and III are indicated by vertical dashed lines. The solid arrows indicate the times when seedlings were harvested.

low concentration of Coomassie blue (0.003% Coomassie brilliant blue R-250 in 30% ethanol and 10% acetic acid) for about 4 h and not destained (Chen et al., 1993). This procedure revealed all of the prominent bands observed with silver staining. The same prominent molecular mass bands from each of four lanes for each stage were cut out and mixed with 100 μL of 2× SDS treatment buffer and 4 μL of 500 mM DTT in 2.5-mL microfuge tubes. The excised bands (four per tube) were homogenized with a tapered Teflon pestle by hand and then incubated at 45°C for 15 min. After centrifugation at about 13,000g for 10 min, the supernatants were applied to lanes in a 10 to 15% (1.0 mm thick) SDS gel without measuring the amount of protein per sample (because of low concentration and limited sample volume). Data from these experiments are included in Figure 5.

Silver staining of SDS-denaturing and nondenaturing gels was done by modifications of the method described by Heukeshoven and Dernick (1985). Each gel was placed in a glass tray containing 100 mL of 30% (v/v) ethanol and 10% (v/v) acetic acid and gently agitated for 3 h. Afterward, the gel was neutralized with repeated rinses in water and placed in an aqueous reducing solution (15 min) containing 1% (w/v) K ferricyanide and 1.6% (w/v) sodium thiosulfate. The yellow color of the reducer was completely rinsed out of the gel with water before the gel was immersed in 0.1% (w/v) silver nitrate. After 30 min, the silver nitrate solution was discarded, and 50 mL of developer solution (0.3 M sodium carbonate and 0.02% formaldehyde) was poured over the gel. The resulting dark brown precipitate was quickly discarded, and an additional 100 mL of developer were added to the gel. When the desired protein staining was attained, the developer was discarded and the precipitation reaction was stopped with 100 mL of 3% (v/v) glacial acetic acid.

RESULTS

Time Course of Glyoxysomal and Leaf-Type Peroxisomal Enzyme Activities

Figure 2 illustrates the results of measuring MS and HPR activities in cotyledons of sunflower seedlings. Three stages were defined during germination and seedling growth. During stage I (2 d in the dark), the glyoxysomal enzyme activity increased substantially to a peak from d 0, whereas the leaf-type peroxisomal activity remained undetectable for 1 d and then increased during the next day. Transfer of the seedlings after 2 d of darkness to white light initiated stage II, in which the transition occurred between glyoxysomes and leaf-type peroxisomes. The activity of MS declined to less than one-fourth of the maximal activity, whereas HPR activity increased continuously to near peak activity. Early in stage III, HPR activity reached a peak and then declined slowly, whereas the low activity of MS continuously declined to zero. Leaf-type peroxisomes became the predominant type of peroxisomes. For analyses of PMPs, seedlings were harvested at d 2, 3, or 5 to obtain cotyledons representative of the three stages.

Isolation of Peroxisomes in Suc Gradients

Table I shows the distribution of proteins, Chl *a* contents, and marker enzymes in the major fractions recovered from

Table I. *Enzyme-specific activities and Chl fluorescence in step Suc gradient fractions obtained from sunflower cotyledons harvested at three stages of growth*

Data for stage I are from pooled samples from two Suc gradients, and those for stage II and III were from four Suc gradients. Starting material for stage I was 15 g (105 cotyledon pairs) per gradient and was 13 g for both stages II and III (72 cotyledon pairs for stage II and 43 cotyledon pairs for stage III) per gradient. ND, Not determined.

	Stage I			Stage II			Stage III		
	42–47%	47–51%	51–57%	42–47%	47–51%	51–57%	42–47%	47–51%	51–57%
Protein (mg)	1.09	0.63	1.56	3.08	2.53	4.20	3.22	0.73	0.21
Catalase (μmol min^{-1} mg^{-1})	1,650	3,810	22,190	7,100	6,700	30,900	6,300	9,900	11,800
MS (nmol min^{-1} mg^{-1})	4,340	4,590	15,640	986	756	1,479	84	718	713
HPR (nmol min^{-1} mg^{-1})	Trace	Trace	Trace	29	36	73	180	407	356
Cyt c oxidase (nmol min^{-1} mg^{-1})	159	49	42	187	35	6	357	35	17
Chl (μg)	ND	ND	ND	140	10	2	29	4	3

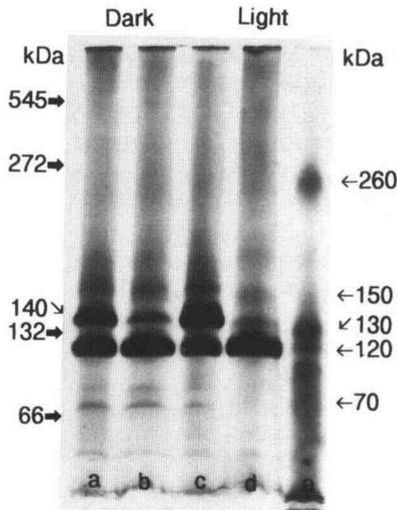

Figure 3. Nondenaturing PAGE (5–10% linear gradient) separations of PMP complexes extracted from glyoxysomes, transition, and leaf-type peroxisomes collected in the 51 to 57% gradient fractions. Ten micrograms of protein were applied to each lane. Thin arrows mark positions of PMP complexes; thick arrows mark positions of molecular mass standards (in kD: jack bean urease, 545; BSA tetramer, 272; BSA dimer, 132; BSA monomer, 66). Lanes: a, 2 d dark; b, 3 d dark; c, 1 d dark, 1 d light; d, 2 d dark, 1 d light; e, 2 d dark, 3 d light.

Suc gradients. It is apparent that peroxisomes were recovered in all three fractions at all three stages. Those in the 42 to 47% step from all three stages were mixed with substantial amounts of mitochondria (Cyt *c* oxidase) and with plastids (stages II and III). The 42 to 47% fractions always were visibly green, whereas the 47 to 51% fractions usually were pale green, and the 51 to 57% fraction was colorless. These observations are substantiated by the very low Chl *a* contents, especially in the 51 to 57% fractions. The amount of peroxisome protein recovered in the 51 to 57% fraction was at least 20-fold lower per gradient at stage III than at stage I or II. Specific enzyme activities also indicate that peroxisomes in the 51 to 57% fractions were the most highly purified at all three stages. The data for stage III, however, indicate that peroxisomes were highly purified in both the 47 to 51% and 51 to 57% fractions at this stage. Banding patterns in SDS gels of PMPs recovered from these two fractions substantiate these data (Fig. 7).

Nondenatured PMP Complexes

Figure 3 shows the results of electrophoresing PMPs solubilized from peroxisomes isolated from dark- and dark/light-grown seedlings in nondenaturing gels. Six bands (Fig. 3, thin arrows) were apparent in the gels containing samples from all three stages. When results from seedlings grown in 2 or 3 d of darkness (lanes a and b) were compared, the relative amount of the PMP120 complex remained essentially constant, whereas the amount of PMP140 complex decreased after 3 d of darkness (lane b). When the seedlings were exposed to white light (lanes c–e), the amount of PMP120 complex still remained relatively constant, whereas the PMP140 complex decreased with time until it was not visible at stage III (lane e). The banding patterns of samples from 2 d in darkness and from 1 d in darkness and then 1 d in light (cf. lanes a and c) were essentially the same, indicating that light did not promote the disappearance of the PMP140 complex. Comparisons of lanes b (3 d dark) and d (2 d dark, 1 d light) revealed similar patterns for the relative abundance of PMPs 120 and 140, again indicating that light did not cause the disappearance of PMP140. Rather, the decrease of the PMP140 complex seemed to be time dependent. After 3 d of light (lane e), at least two new major complexes became apparent, i.e. PMP complexes 260 and 130. The PMP130 complex consistently appeared at a position lower than the PMP140 complex in adjacent lanes. The compositional difference between these two complexes was determined by two-dimensional gel analyses (Fig. 5). The "PMP70 complex," a designated area constituting approximately a 1-cm-wide region of the gels in the vicinity of 70-kD proteins, consistently became more prominent in the region of gels below the PMP120 complex at stage III.

Denatured PMPs

Figure 4 shows the results of electrophoresing five samples similar to those shown in Figure 3 in SDS gels. Ten denatured PMP polypeptides were identified (Figs. 4 and 5, thin arrows) in samples from all three stages. When gel separations of PMPs from seedlings grown in dark and dark/light conditions were compared, three PMPs (57, 49, and 31) consistently

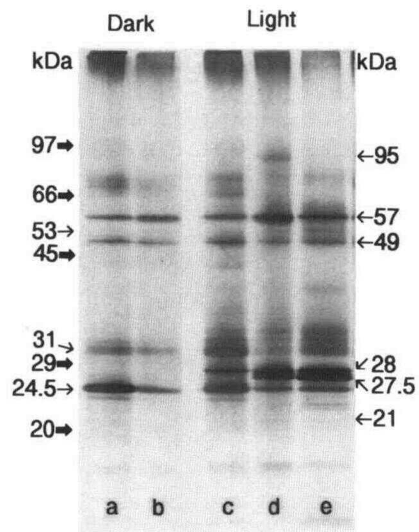

Figure 4. SDS-PAGE (10–15% linear gradient) separations of PMP subunits extracted from glyoxysomes, transition, and leaf-type peroxisomes collected in the 51 to 57% gradient fractions. Ten micrograms of protein were applied to each lane. Thin arrows mark positions of polypeptides; thick arrows mark positions of molecular mass standards. Lanes: a, 2 d dark; b, 3 d dark; c, 1 d dark, 1 d light; d, 2 d dark, 1 d light; e, 2 d dark, 3 d light.

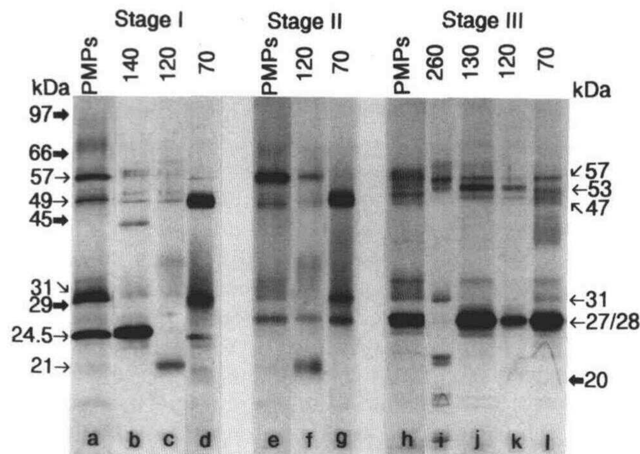

Figure 5. SDS-PAGE (10–15% linear gradient) separations of polypeptides in PMP complexes excised from nondenaturing gels (see Fig. 3). Four lanes of PMPs from each stage (20 μg of protein in each lane) were separated in the nondenaturing gels. Each prominent nondenatured PMP complex (from four lanes) was cut from the gel and homogenized (four bands), and supernatants were applied to SDS gels. Total PMPs from each stage were applied to wells (lanes a, e, and h) to provide a reference for PMP migrations. Lanes: a, stage I PMPs; b, stage I 140-kD complex; c, stage I 120 kD; d, stage I 70 kD; e, stage II PMPs; f, stage II 120 kD; g, stage II 70 kD; h, stage III PMPs; i, stage III 260 kD; j, stage III 130 kD; k, stage III 120 kD; l, stage III 70 kD.

were recovered from all types of peroxisomes (Fig. 4, lanes a–e; Fig. 5, lanes a, e, and h). PMP31 appeared to be relatively more abundant after 1 d in darkness and then 1 d in light (Fig. 4, lane c). In seedlings grown in 2 or 3 d of darkness (Fig. 4, lanes a and b), four prominent PMPs (57, 49, 31, and 24.5) were identified; only the PMP24.5 decreased in relative amount at 3 d of darkness (Fig. 4, lane b). When the seedlings were exposed to white light (Fig. 4, lanes c–e), the amount of PMP24.5 after 1 d of darkness and 1 d light (Fig. 4, lane c) was essentially the same as after 2 d of darkness (Fig. 4, lane a), whereas this PMP decreased after 3 d whether the seedlings were in darkness (Fig. 4, lane b) or exposed to light (Fig. 4, lanes d and e). In most of our experiments, the amount of PMP24.5 decreased to the extent that it was barely detected (cf. lanes a, e, and h in Fig. 5). In concert with the appearance of nondenatured complexes after light exposure (Fig. 3), four new denatured PMPs (53, 47 [difficult to see in Fig. 4], 28, and 27.5) appeared after exposure to light. PMP28 appeared after only 1 d of darkness followed by 1 d of light (Fig. 4, lane c), whereas PMP27.5 appeared after 2 d of darkness followed by either 1 d of light (Fig. 4, lane d) or 3 d of light (Fig. 4, lane e). PMP53 appeared only after 3 d of light (Fig. 4, lane e; Fig. 5, lanes h, j, and k).

Polypeptide Composition of PMP Complexes

Figure 5 shows the SDS-denatured polypeptide composition of the major PMP complexes identified in Figure 3. Visibility of the PMP140 complex was sufficient for excision only from stage I gels. It was composed mostly of a 24.5-kD polypeptide (lane b), which was not found within any other PMP complex (the weak band in lane d is from lateral diffusion of an adjacent band, not shown, from a PMP140 sample). Recently, Bunkelmann and Trelease (1993) and Bunkelmann et al. (1994) came to the conclusion that a prominent low molecular mass polypeptide recovered from glyoxysome membranes of four different oilseed species, including the 24.5-kD protein in sunflower, was a protein body membrane protein. They discovered that an antibody to bean TIP (Johnson et al., 1989) cross-reacted on western blots with a low molecular polypeptide specific to each oilseed, including the sunflower 24.5-kD polypeptide. Only this polypeptide was found to be a contaminant.

The PMP120 complex was composed of 21- and 57-kD polypeptides in glyoxysomes (stage I) and transition peroxisomes (stage II) (lanes c and f), whereas it was composed mostly of light-induced PMPs 27.5, 28, and 53 in leaf-type peroxisomes (stage III, lane k). The prominent PMPs 27.5 and 28 also constituted a major portion of PMP130 and PMP70 complexes (lanes j and l) in leaf-type peroxisomes and were in the PMP120 and PMP70 complexes of transition peroxisomes (lanes f and g). They were not within any of the glyoxysome complexes. As mentioned before, the PMPs 130 and 140 complexes consistently migrated differently in adjacent lanes; they also were composed of different polypeptides (cf. lanes b and j). The PMP70 complex from glyoxysomes and transition peroxisomes (lanes d and g) possessed mainly PMPs 49 and 31. These polypeptides also were in the stage III PMP70 complex but were less prominent because of the accumulation of PMPs 27.5 and 28.

Influence of Light Quality and Intensity on PMPs

When making comparisons with the 3-d dark-grown seedlings, only PMP49 was not affected by either light quality or light intensity (Figs. 6). Exposure of seedlings to red, blue, or far-red light seemed to increase the relative amount of PMP57. The response of PMP31 to light was difficult to ascertain because it was not sharply defined in all gels (Fig. 6). Red or white light exposure promoted the appearance of PMPs 47, 28, and 27.5. Seedlings exposed to blue light did not promote the appearance of PMPs 28 or 27.5 but promoted the appearance of PMP47. Far-red light seemed to prevent the appearance of the PMP47 and the temporal decrease of PMP24.5 (lane c). PMPs 28 or 27.5 also did not appear after exposure to far-red light.

To do the experiments described above, sufficient quantities of PMP samples often were difficult to obtain per experiment, especially for analyses of stage III PMPs. Figure 7 shows that the SDS polypeptide patterns are the same for fractions collected from the 47 to 51% step as those collected from the 51 to 57% step at all three stages. Lanes b and e containing stage II samples are presented together to show the identity of banding patterns. These data allowed us to select organelles in either or both gradient steps for analyzing polypeptide patterns, especially for the experiments on light quality and intensity.

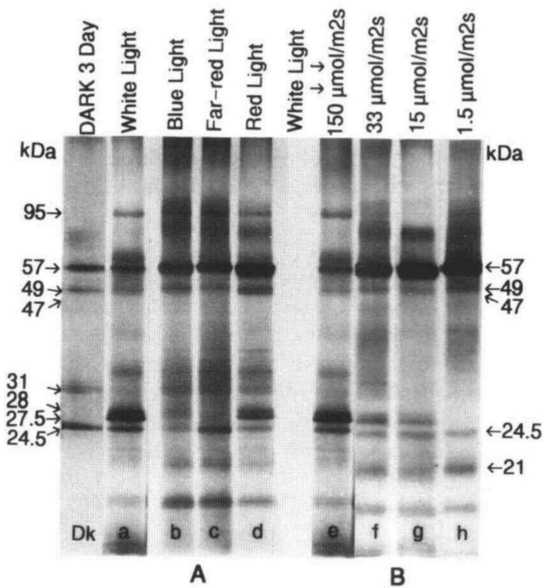

Figure 6. Influence of light quality (A, lanes a–d) or light intensity (B, lanes e–h) on the relative amount of stage II PMP subunits. The seedlings were kept in the dark for 2 d and then transferred to the varied light conditions for 1 d. Comparisons are made relative to the PMPs in 3-d dark-grown seedlings (lane Dk). Peroxisomes were collected in the 51 to 57% gradient fractions. Ten micrograms of protein were applied to each lane.

DISCUSSION

Sunflower seedlings were kept in complete darkness for 2 d and then transferred to continuous white light for several days to experimentally emphasize the transition from glyoxysomes to leaf-type peroxisomes in a relatively short growth period (Fig. 2). Eising and Gerhardt (1987) grew sunflower seedlings similarly and set up their three stages based on data with three different peroxisomal enzymes (catalase, isocitrate lyase, and glycolate oxidase). For their stage I, seedlings were kept in darkness for 2.5 d. However, activity peaks of isocitrate lyase and glycolate oxidase occurred at 2 and 5 d, respectively, as were the peaks of MS and HPR shown in Figure 2. Their stage II also was defined as 2.5 d of light after 2.5 d of darkness. The declining HPR activity observed during stage III (Fig. 2) likely was not indicative of cotyledon senescence for the reasons documented by Eising and Gerhardt (1987), i.e. activities of other peroxisomal enzymes involved in photorespiration, activities of Calvin cycle enzymes, Chl content, and photosynthetic capacity of the cotyledons were nearly constant during stage III.

The data in Table I show that peroxisomes isolated from dark- and light-grown cotyledons were highly purified. The Suc gradient method was designed and perfected by Eising and Gerhardt (1986), who were interested in measuring changes in catalase-specific activity and hematin content during the peroxisome changeover. Continuous Suc gradients typically are used for peroxisome isolation because they produce high-resolution separations of glyoxysomes from contaminants such as protein bodies and etioplasts and separation of thylakoids/plastids from leaf-type peroxisomes (Vigil, 1983). However, for resuspended sunflower cotyledon organelles, the steps designed by Eising and Gerhardt accomplished separations from contaminants, especially in the 52.5 to 57% step. We changed one of their steps slightly (51 for 52.5%) because we often observed a diffuse distribution of organelles between the 47 and 52.5% and 52.5 and 57% interphases with our variety of sunflower seeds. The results shown in Figure 7 are especially relevant to this study because integral membrane proteins, other than those found in the organelles from the 52.5 to 57% step, were not found in samples from the 47 to 52.5% step. Hence, we could, and sometimes did, use samples from both steps for our experiments.

The change in distribution and amount of PMPs during the transition from glyoxysomes to peroxisomes also was addressed in an ultrastructural study. Vigil and Fahimi (1990) did a quantitative freeze-fracture investigation of intramembranous particles in peroxisomes of watermelon cotyledons. They found that the number of integral membrane proteins per μm^2 in protoplasmic and ectoplasmic faces of leaf-type peroxisomes decreased by 32 and 40%, respectively, from those in corresponding faces of glyoxysomal membranes. This implies that the composition of membrane proteins is less complex in leaf-type peroxisomes. From our results, the amount of nondenatured PMP120 complex remained relatively constant throughout all three stages, whereas PMP140 complex decreased temporally (independently of light exposure).

As stated in "Results," however, we have data that indicate that the PMP140 complex, composed almost exclusively of 24.5-kD polypeptide, is a protein body membrane protein or TIP. This complex, not associated with any detergent re-

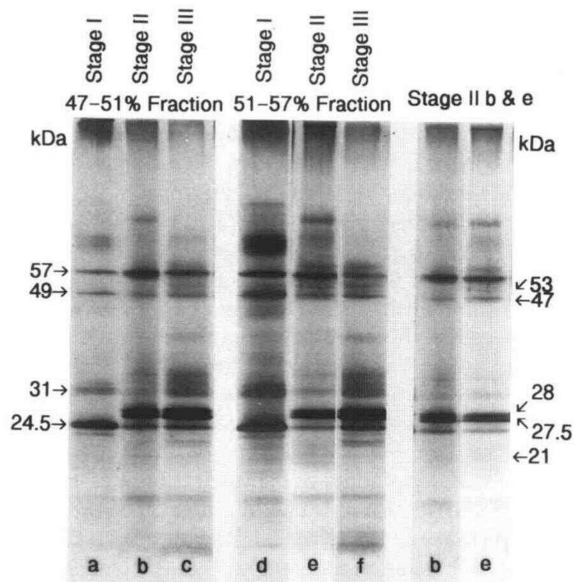

Figure 7. SDS gel comparisons of PMP patterns from peroxisomes collected in the 47 to 51% or 51 to 57% steps of Suc gradients. Samples were either from stage I, II, or III sunflower seedlings. Ten micrograms of protein were applied to each lane.

associated PMPs, apparently was derived from protein body vesicles possessing an extraordinary concentration of the TIP (Bunkelmann et al., 1994). The other three PMP complexes, 260, 130, and 70 kD, however, appeared (or became more prominent, i.e. PMP70) when sunflower seedlings were exposed to light for 3 d. The PMP260 complex possessed 57- and 31-kD polypeptides that were identified in glyoxysomes and transition peroxisomes, but it also possessed some lower molecular mass polypeptides (Fig. 5, lane i) that were not observed in membrane preparations of the other peroxisomes. The 27.5- and 28-kD polypeptides were associated with three different complexes (PMPs 130, 120, and 70). We do not currently believe that the complexes observed by nondenaturing PAGE are functional in vivo entities. Rather, our belief is that these complexes formed as a consequence of hydrophobic membrane proteins associations that separated in nondenaturing gels by charge differences because of bound taurodeoxycholate (Schägger et al., 1994). Nevertheless, our data indicate a net increase in different integral membrane proteins in leaf-type peroxisomes. This seems inconsistent with interpretations of the freeze-fracture data for watermelon. However, until one can determine which proteins are associated with membrane protein complexes in vivo, comparisons of changes in gel patterns with changing numbers of complexes observed with freeze-fracture must be guarded.

The concurrent appearance of PMP polypeptides 27.5, 28, and 47 and the substantial increase in HPR activity during the 24-h, continuous, white-light exposure of 2-d dark-grown seedlings (Figs. 2 and 4) suggest a coupled accumulation of membrane and matrix proteins within enlarging transition peroxisomes. Some HPR, however, was acquired by glyoxysomes in the dark after a 24-h dark period (Fig. 2) without the detectable appearance of any of the above-mentioned PMP polypeptides. Occurrence of low levels of HPR (and glycolate oxidase) activities is well documented for cotyledon glyoxysomes of dark-grown oil seedlings (Schnarrenberger et al., 1971; Huang et al., 1983; Titus et al., 1983). We also found that the appearance of these PMPs after exposure of seedlings to light varied with the duration of the prior dark period, i.e. PMP28 appeared when HPR activity increased after 1 d of darkness but without the appearance of either PMP27.5 or PMP47 (Fig. 4).

We attempted to correlate developmental changes in MS and HPR activities with the appearance/nonappearance of PMPs in relation to the varied quality of light exposures (Fig. 5) to help assess the coupled accumulation of matrix and membrane proteins. In the case of MS activities, interpretations were quite simple because none of the light regimes (white, blue, red, far-red) imposed during a 4-d growth period (after 2 d in darkness) had any perceptible effect on the decline in MS activity (data not shown). In the case of HPR, its activity increased for at least 2 d under all of the imposed light regions (data not shown), even when the PMPs did not appear (e.g. in blue and far-red light, Fig. 6). Hence, we were unable to make any definitive correlations between acquisition (or lack thereof) of PMPs with differentiation (matrix enzyme accumulation) of the leaf-type peroxisomes. Also, several lines of evidence indicate that the synthesis and acquisition of all matrix and membrane proteins are not coupled, e.g. the temporal separation of an increase in HPR activity and PMP appearance, the dark-period dependence of PMP appearance, and the negative correlation between the appearance of certain PMPs and the increase in HPR activity.

The white-light stimulation of the developmental increase in HPR (Fig. 2) and other matrix enzymes in leaf-type peroxisomes is well documented, e.g. in mustard (Schopfer et al., 1976; Hong and Schopfer, 1981), watermelon (Kagawa et al., 1973; Kagawa and Beevers, 1975), and cucumber seedlings (Becker et al., 1978). Hondred et al. (1987) suggested that the increase in peroxisomal enzyme activities depended on an increase in translatable mRNAs for these enzymes and that they were regulated at a pretranslational level, most likely involving transcription of new mRNA. Continuous blue, red, and far-red irradiation were shown to be effective in stimulating HPR activities in etiolated wheat leaves (Feierabend, 1975) and etiolated cotyledons of *Pharbitis nil* (Tchang et al., 1984a, 1984b).

From our results, continuous blue, red and far-red irradiation also influenced the appearance of PMPs 27.5, 28, and 47. Chl likely is not the photoreceptor involved in the accumulation of PMPs 27.5 and 28 because distinctly different gel patterns were observed after irradiation of seedlings with red and blue irradiations. Tchang et al. (1984a, 1984b) also concluded that Chl was not the photoreceptor for stimulation of HPR activity in etiolated *P. nil* seedlings in red and blue light. Rather, they suggested that it was a blue-absorbing pigment plus phytochrome. However, Chl might be involved in accumulation of PMP47 because the same gel pattern was apparent after exposure of light in both red and blue regions and because far-red light did not promote its appearance. The proportion of red spectrum in white light seems to be the component that promotes the appearance of PMPs 27.5, 28, and 47 because the gel pattern of the proteins from seedlings exposed to red and white light is the same. However, far-red light prevented the appearance of PMPs 27.5, 28, and 47. van Poucke and Barthe (1970), Schopfer et al. (1976), and Feierabend (1975) reported that phytochrome may play a role in the response of peroxisomal matrix enzymes to light. Bertoni and Becker (1993) demonstrated that phytochrome was involved in regulation of the levels of mRNA encoding HPR in cucumber cotyledons. In our case, phytochrome might be involved in the red and far-red effects, but more data are needed to further demonstrate phytochrome-mediated effects on PMPs.

To our knowledge, the results reported here provide the first evidence for a change in PMP composition when glyoxysomes are converted to leaf-type peroxisomes and also indicate that the differentiation of peroxisomes in sunflower cotyledons during seedling growth involves both photobiological and temporal regulatory mechanisms. However, specific functions for these PMPs remains to be discovered.

ACKNOWLEDGMENTS

We sincerely thank Dr. F. Javier Corpas for his many insightful discussions during the course of this research and Tom Collela for his technical assistance.

Received February 25, 1994; accepted May 23, 1994.
Copyright Clearance Center: 0032-0889/94/106/0293/10.

LITERATURE CITED

Alani AA, Luster DG, Donaldson RP (1990) Development of endoplasmic reticulum and glyoxysomal membrane redox activities during castor bean germination. Plant Physiol **94:** 1842–1848

Becker WM, Leaver CJ, Weir EM, Riezman H (1978) Regulation of glyoxysomal enzymes during germination of cucumber. 1. Developmental changes in cotyledonary protein, RNA, and enzyme activities during germination. Plant Physiol **62:** 542–549

Beevers H (1993) Forty years in the new world. Annu Rev Plant Physiol Plant Mol Biol **44:** 1–12

Bertoni GP, Becker WM (1993) Effects of light fluence and wavelength on expression of the gene encoding cucumber hydroxypyruvate reductase. Plant Physiol **103:** 933–941

Bowditch MI, Donaldson RP (1990) Ascorbate free-radical reduction by glyoxysomal membranes. Plant Physiol. **94:** 531–537

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Breidenbach RW, Beevers H (1967) Association of glyoxylate cycle enzymes in a novel subcellular particle from castor bean endosperm. Biochem Biophys Res Commun **27:** 462–469

Bunkelmann J, Corpas FJ, Trelease RN (1994) A protein body membrane protein (TIP) is a prominent contaminant among oilseed glyoxysome membrane proteins (abstract No. 805). Plant Physiol **105:** S-147

Bunkelmann J, Trelease RN (1993) Immunological cross-reactivities among membrane proteins in oilseed glyoxysomes and mitochondria (abstract No. 762). Plant Physiol **102:** S-134

Causeret C, Bentejac M, Bugaut M (1993) Proteins and enzymes of the peroxisomal membrane in mammals. Biol Cell **77:** 89–104

Chapman KD, Trelease RN (1991) Acquisition of membrane lipids by differentiating glyoxysomes: role of lipid bodies. J Cell Biol **115:** 995–1007

Chapman KD, Trelease RN (1992) Characterization of membrane proteins in enlarging cottonseed glyoxysomes. Plant Physiol Biochem **30:** 1–10

Chen HY, Cheng H, Bjerknes M (1993) One-step Coomassie brilliant blue R-250 staining of proteins in polyacrylamide gel. Anal Biochem **212:** 295–296

Cooper TG, Beevers H (1969) Mitochondria and glyoxysomes from castor bean endosperm. J Biol Chem **244:** 3514–3520

Donaldson RP, Fang TK (1987) B-oxidation and glyoxylate cycle coupled to NADH: cytochrome *c* and ferricyanide reductase in glyoxysomes. Plant Physiol **85:** 792–795

Eising R, Gerhardt B (1986) Activity and hematin content of catalase from greening sunflower cotyledons. Phytochemistry **25:** 27–31

Eising R, Gerhardt B (1987) Catalase degradation in sunflower cotyledons during peroxisome transition from glyoxysomal to leaf peroxisomal function. Plant Physiol **84:** 225–232

Fang TK, Donaldson RP, Vigil EL (1987) Electron transport in purified glyoxysomal membranes from castor-bean endosperm. Planta **172:** 1–13

Feierabend J (1975) Developmental studies on microbodies in wheat leaves. III. On the photocontrol of microbody development. Planta **123:** 63–77

Halpin C, Conder MJ, Lord JM (1989) Different routes for integral protein insertion into *Ricinus communis* protein-body and glyoxysome membranes. Planta **179:** 331–339

Heukeshoven J, Dernick R (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis **6:** 103–112

Hicks DB, Donaldson RP (1982) Electron transport in glyoxysomal membranes. Arch Biochem Biophys **215:** 280–288

Hondred D, Wadle DM, Titus DE, Becker WM (1987) Light-stimulated accumulation of the peroxisomal enzymes hydroxypyruvate reductase and serine:glyoxylate aminotransferase and their translatable mRNAs in cotyledons of cucumber seedlings. Plant Mol Biol **9:** 259–275

Hong UN, Schopfer P (1981) Control by phytochrome of urate oxidase and allantoinase activities during peroxisome development in the cotyledons of mustard (*Sinapis alba* L.) seedlings. Planta **152:** 325–335

Huang AHC, Trelease RN, Moore TS (1983) Plant Peroxisomes, American Society of Plant Physiologists Monograph Series. Academic Press, New York, pp 87–155

Johnson KD, Herman EM, Chrispeels MJ (1989) An abundant, highly conserved tonoplast protein in seeds. Plant Physiol **91:** 1006–1013

Kagawa T, Beevers H (1975) The development of microbodies (glyoxysomes and leaf peroxisomes) in cotyledons of germinating seedlings. Plant Physiol **55:** 258–264

Kagawa T, Lord JM, Beevers H (1973) Development of enzymes in the cotyledons of watermelon seedlings. Plant Physiol **51:** 66–71

Kunce CM, Trelease RN, Doman DC (1984) Ontogeny of glyoxysomes in maturing and germinated cotton seeds—a morphometric analysis. Planta **161:** 156–164

Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **227:** 680–685

Luster DG, Bowditch MI, Eldridge KM, Donaldson RP (1988) Characterization of membrane-bound electron transport enzymes from castor bean glyoxysomes and endoplasmic reticulum. Arch Biochem Biophys **265:** 50–61

Ni W, Trelease RN, Eising R (1990) Two temporally synthesized charge subunits interact to form the five isoforms of cottonseed (*Gossypium hirsutum*) catalase. Biochem J **269:** 233–238

Sandalio LM, Del Rio LA (1987) Localization of superoxide dismutase in glyoxysomes from *Citrullus vulgaris* functional implications in cellular metabolism. J Plant Physiol **127:** 395–409

Sandalio LM, Del Rio LA (1988) Intraorganellar distribution of superoxide dismutase in plant peroxisomes (glyoxysomes and leaf peroxisomes). Plant Physiol **88:** 1215–1218

Schägger H, Cramer WA, von Jagow G (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. Anal Biochem **217:** 220–230

Schägger H, von Jagow G (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem **199:** 223–231

Schnarrenberger C, Oeser A, Tolbert NE (1971) Development of microbodies in sunflower cotyledons and castor bean endosperm during germination. Plant Physiol **48:** 566–574

Schopfer P, Bajracharya D, Bergfeld R, Falk H (1976) Phytochrome-mediated transformation of glyoxysomes into peroxisomes in the cotyledons of mustard (*Sinais alba* L.) seedlings. Planta **133:** 73–80

Struglics A, Fredlund KM, Rasmusson AG, Moller IM (1993) The presence of a short redox chain in the membrane of intact potato tuber peroxisomes and the association of malate dehydrogenase with the peroxisomal membrane. Physiol Plant **88:** 19–28

Subramani S (1993) Protein import into peroxisomes and biogenesis of the organelle. Annu Rev Cell Biol **9:** 445–478

Sulter GJ, Harder W, Veenhuis M (1993) Structural and functional aspects of peroxisomal membranes in yeasts. FEMS Microbiol Rev **11:** 285–296

Sulter GJ, Looyenga L, Veenhuis M, Harder W (1990) Occurrence of peroxisomal membrane proteins in methylotrophic yeasts grown under different conditions. Yeast **6:** 35–43

Tchang F, Lecharny A, Mazliak P (1984a) Photostimulation of hydroxypyruvate reductase activity in peroxisomes of *Pharbitis nil* seedlings. I. Action spectrum. Plant Cell Physiol **25:** 1033–1037

Tchang F, Lecharny A, Mazliak P (1984b) Photostimulation of hydroxypyruvate reductase activity in peroxisomes of *Pharbitis nil* seedlings. II. Photoreceptors in blue light. Plant Cell Physiol **25:** 1039–1043

Titus DE, Hondred D, Becker WM (1983) Purification and characterization of hydroxypyruvate reductase from cucumber cotyledons. Plant Physiol **72:** 402–408

Tolbert NE, Oeser A, Kisaki T, Hageman RH, Yamazaki RK (1968) Peroxisomes from spinach leaves containing enzymes related to glycolate metabolism. J Biol Chem **243:** 5179–5184

Trelease RN, Becker WM, Gruber PJ, Newcomb EH (1971) Microbodies (glyoxysomes and peroxisomes) in cucumber cotyledons. Correlative biochemical and ultrastructural study in light- and dark-grown seedlings. Plant Physiol **48:** 461–475

Trelease RN, Doman DC (1984) Mobilization of oil and wax reserves. *In* DR Murray, eds, Seed Physiology. Academic Press, New York, pp 201–245

Trelease RN, Hermerath CA, Turley RB, Kunce CM (1987) Cottonseed malate synthase. Purification and immunochemical characterization. Plant Physiol **84:** 1343–1349

van den Bosch H, Schutgens RBH, Wanders RJA, Tager JM (1992) Biochemistry of peroxisomes. Annu Rev Biochem **61:** 157–197

van Poucke M, Barthe F (1970) Induction of glycolate oxidase activity in mustard seedlings under the influence of continuous irradiation with red and far-red light. Planta **94:** 308–318

Vernon LP (1960) Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. Anal Chem **32:** 1144–1150

Vigil EL (1983) Microbodies. *In* JL Hall, AL Moore, eds, Isolation of Membranes and Organelles from Plant Cells. Academic Press, London, pp 211–236

Vigil EL, Fahimi HD (1990) A quantitative freeze-fracture investigation of intramembranous particles during the transformation of glyoxysomes to peroxisomes in watermelon cotyledons. Eur J Cell Biol **51:** 1–8