Plant Physiol. (1979) 63, 227–231
0032-0889/79/63/0227/05/$00.50/0

Chloroplast Biogenesis

XXIV. INTRACHLOROPLASTIC LOCALIZATION OF THE BIOSYNTHESIS AND ACCUMULATION OF PROTOPORPHYRIN IX, MAGNESIUM-PROTOPORPHYRIN MONOESTER, AND LONGER WAVELENGTH METALLOPORPHYRINS DURING GREENING[1]

Received for publication July 7, 1978 and in revised form September 22, 1978

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ABSTRACT

The intraplastidic localization of the endogenous metabolic pools from protoporphyrin to protochlorophyll was determined in *Cucumis sativus*. The endogenous protoporphyrin, Mg-protoporphyrin monoester + longer wavelength metalloporphyrins, protochlorophyllide and protochlorophyllide ester were membrane-bound. Protoporphyrin was synthesized in the stroma and subsequently became associated with the membranes. The membrane-associated protoporphyrin was then converted into Mg-protoporphyrin monoester + longer wavelength metalloporphyrins by membrane-bound enzymes. Although lysed plastids were capable of converting exogenous δ-aminolevulinic acid to protochlorophyllide, the net synthesis of protochlorophyllide from exogenous δ-aminolevulinic acid was lost upon segregating the lysed plastids into stromal and membrane fractions and then recombining the stromal and membrane fraction prior to incubation. The segregated membrane fraction was still capable of converting protoporphyrin into Mg-protoporphyrin monoester + longer wavelength metalloporphyrins in the presence or absence of the stromal fraction. These results indicated that although the reactions from protoporphyrin to Mg-protoporphyrin monoester and longer wavelength metalloporphyrins could survive a considerable degree of plastid disruption, the reactions from Mg-protoporphyrin monoester and longer wavelength metalloporphyrins to protochlorophyllide were more sensitive to structural disorganization.

Several biosynthetic reactions from protoporphyrin IX to protochlorophyllide were recently demonstrated in a cell-free system (1–6, 10). However, the intrachloroplastic localization of these reactions has still not been demonstrated unambiguously.

In this work it is demonstrated that the endogenous pools of Mg-protoporphyrin monoester and longer wavelength metalloporphyrins (*i.e.* the MPE-equivalent),[3] protochlorophyllide, and protochlorophyllide ester are all membrane-bound. Although the exact chemical structure of the longer wavelength metalloporphyrin component of the MPE-equivalent remains to be established, it is presumed, on the basis of previously reported evidence (8, 10), that they are indeed the putative intermediates between Mg-protoporphyrin monoester and protochlorophyllide. We have also shown that although protoporphyrin IX is synthesized in the stroma, the newly formed protoporphyrin becomes associated with the plastid membranes and is converted to MPE-equivalent by membrane-bound enzymes.

MATERIALS AND METHODS

Plant Material. Etiolated cucumber seedlings were grown for 4 days in the dark at 28 C from *Cucumis sativus* L. cv. Beit Alpha MR seeds which were purchased from the Niagara Chemical Division, FMC Corporation, Modesto, Calif.

Preparation of Protoporphyrin. Protoporphyrin was prepared from protoporphyrin IX dimethyl ester as previously described (10). Just prior to use, the dried protoporphyrin was dissolved in absolute methanol.

Preparation of Lysed Plastid Suspension. Three types of etiochloroplast[4] pellets were prepared as follows. Five g of cotyledons were ground in 7.5 ml of a cofactor-enriched homogenization buffer (8) in which the 200 mM Tris-HCl was adjusted to pH 7.5 at room temperature. The plastid pellet was subsequently obtained as described in more detail elsewhere (8). Fifteen-min etiochloroplast pellets were prepared from etiolated cotyledons which were exposed to about 50 μw cm^{-2} of cool-white fluorescent light for about 15 min during harvesting. Two- or 4-hr etiochloroplast pellets were prepared from excised greening cotyledons that were previously irradiated for 2 or 4 hr with 320 μw cm^{-2} of cool-white fluorescent light at 28 C (8). The various etiochloroplast pellets were lysed in 5 ml of a cofactor-enriched lysing buffer (7) in which the 25 mM Tris-HCl was adjusted to pH 7.25 at room temperature.

Preparation of Subplastidic Fractions. The lysed plastid suspension was centrifuged at 48,000*g* for 30 min in a Beckman No. J-20 rotor. This centrifugation separated the lysed plastids into a soluble protein fraction (designated as the stromal fraction) and a compact green particulate fraction (designated as the membrane fraction). The stromal fraction was decanted and was adjusted to 180 mM with respect to Tris-HCl by the addition of H_2O and 1 M Tris-HCl (pH 7.25) at room temperature. The membrane fraction was suspended in 1 to 2 ml of a cofactor-enriched lysing buffer that had been adjusted to 180 mM with respect to Tris-HCl by the addition of H_2O and 1 M Tris-HCl (pH 7.25) at room temperature.

Incubation of Lysed and Subplastidic Fractions. The reaction mixture which contained the lysed plastids or subplastidic fractions was incubated on a metabolic shaker operated at 10 shakes/min. All incubations were performed at 28 C in the dark for 1 or 2 hr. Each reaction mixture contained 0.97 ml of H_2O, 0.03 ml methanol, and either: (*a*) 2 ml of the lysed plastid

[1] This work was supported by National Science Foundation Research Grants PCM 730-1117-A01 and PCM 76-81682 and by funds from the Illinois Agricultural Experiment Station.

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[3] Abbreviations: ALA: δ-aminolevulinic acid; MPE-equivalent: magnesium-protoporphyrin IX monomethyl ester + longer wavelength metalloporphyrins; Zn-Proto(ester): a mixture of Zn-protoporphyrin IX and Zn-protoporphyrin IX monoester.

[4] As it is now commonly used in the chloroplast literature, the term "etiochloroplast" refers to plastids that have been exposed to light and are developmentally between an etioplast and a fully differentiated chloroplast.

Table I. Intraplastidic Localization of the Endogenous Pools of Protoporphyrin, MPE - Equivalent, Protochlorophyllide and Protochlorophyllide Ester

Stromal and membrane fractions were prepared from 15 min, 2 h and 4 h etiochloroplasts and the amounts of endogenous protoporphyrin, MPE-equivalent, protochlorophyllide and protochlorophyllide ester were determined spectrofluorometrically.[a]

Type of etiochloroplast	pmoles per 100 mg plastid protein							
	Protoporphyrin ±px'[b]		MPE-equivalent ± px[c]		Protochlorophyllide ± px'[b]		Protochlorophyllide ester ± px'[b]	
	stroma	membranes	stroma	membranes	stroma	membranes	stroma	membranes
15 min	0	0	0	0	0	392 ± 16	0	3226 ± 305
15 min	0	0	0	0	0	166 ± 7	0	2133 ± 201
2 h	0	213 ± 75	0	435 ± 122	0	372 ± 15	0	2162 ± 201
2 h	0	284 ± 7	0	345 ± 108	0	485 ± 19	0	1066 ± 101
4 h	0	398 ± 9	0	2207 ± 166	0	2394 ± 95	0	337 ± 32
4 h	0	667 ± 16	0	1811 ± 80	0	2916 ± 116	0	729 ± 69

[a] The endogenous pools of chlorophyllide and chlorophyll were also examined spectrofluorometrically and were always observed to be in the membrane fractions.

[b] p_x' = probable error = (0.67450) x [standard deviation of the mean percent error (9:Table I)]. The probable error is expressed per 100 mg plastid protein.

[c] p_x = probable error = (0.6745) x [standard deviation of the mean discrepancy (11:Table I, column 7)]. The probable error is expressed per 100 mg plastid protein.

suspension, (*b*) 1 ml of the stromal fraction + 1 ml of cofactor-enriched buffer, (*c*) 1 ml of the membrane fraction + 1 ml of cofactor-enriched buffer, or (*d*) 1 ml of the stromal fraction + 1 ml of the membrane fraction.

The 3 ml of cofactor-enriched reaction mixture contained: 360 μmol of Tris-HCl (pH 7.25 at room temperature), 50 μmol of K-phosphate, 1 μmol $MgCl_2$, 10 μmol glutathione (reduced form), 0.6 μmol coenzyme A, 0.8 mmol methanol, 0.9 μmol ATP, and 0.15 μmol NAD. Two ml of lysed plastid suspension contained between 2 and 5 mg of plastid protein. One ml of the stromal fraction contained between 0.5 and 1 mg protein and 1 ml of the membrane fraction contained between 2 and 5 mg protein.

When the reaction mixture contained exogenously added δ-amino-levulinic acid (ALA), the 0.97 ml of H_2O contained 100 nmol ALA. When the reaction mixture contained exogenously added protoporphyrin, between 0.3 and 1.7 nmol of protoporphyrin were present in the 0.8 mmol of methanol. This represented between 20 and 210 nmol of protoporphyrin/100 mg plastid protein. The concentrations of protoporphyrin added were within the saturation range for this substrate (10).

Recentrifugation of Lysed Plastid and Subplastidic Incubation Mixtures After Dark Incubation. In recentrifugation experiments, a lysed plastid reaction mixture or a stroma + membrane reaction mixture that was preincubated with or without ALA for 2 hr in the dark was centrifuged at 48,000*g* for 30 min. This centrifugation separated the incubated reaction mixtures into a soluble protein fraction (stroma) and a compact yellowish green particulate fraction (membranes). The stromal fraction was brought to a volume of 3 ml with cofactor-enriched buffer and the membrane fraction was suspended in 3 ml of cofactor-enriched buffer.

Extraction of Pigments Present Before and After Incubation. At the beginning or end of incubation, 15 ml of acetone-0.1 N NH_4OH (9:1, v/v) were added to each 3 ml reaction mixture. Preparation of the hexane-extracted acetone fractions which contained protoporphyrin, MPE-equivalent, Zn-protoporphyrin + Zn-protoporphyrin monoester [Zn-Proto(ester)] as well as protochlorophyllide, and preparation of the hexane extract which contained protochlorophyllide ester were previously described (8, 9).

Spectrofluorometry. Fluorescence emission spectra were recorded on a Perkin-Elmer spectrofluorometer model MPE-3, equipped with a corrected spectra accessory as described previously (8). All spectra were recorded at room temperature on aliquots of the hexane-extracted acetone fraction and on aliquots of the hexane extract. The spectra were recorded at an excitation bandwidth of 6 nm and an emission bandwidth of 3 nm. The excitation energies reaching the sample at 400, 420, and 440 nm were about 106, 90, and 76 μw cm^{-2}, respectively.

Determination of Amount of MPE-Equivalent in Presence of Zn-Protoporphyrins. The determination of the amount of MPE-equivalent in mixtures of Zn-Proto(ester) + MPE-equivalent was described elsewhere (11). This determination consisted of calculating the fluorescence integral between 592 and 620 nm which was contributed solely by the MPE-equivalent from the fluorescence emission spectrum of the hexane-extracted acetone fraction which contained the mixture of metalloporphyrins. The MPE-equivalent fluorescence integral between 592 and 620 nm was converted to pmol of MPE-equivalent by reference to a standard curve.

Determination of Amounts of Protoporphyrin and Protochlorophyll.[5] The amounts of protoporphyrin and protochlorophyll (*i.e.* protochlorophyllide and protochlorophyllide ester) were calculated by formulas which were described previously (9).

RESULTS

Intraplastidic Localization of Endogenous Porphyrin, Metalloporphyrin, and Protochlorophyll Pools. Stromal and membrane fractions were prepared from 15 min, 2-hr and 4-hr etiochloroplasts and the pools of protoporphyrin, MPE-equivalent, protochlorophyllide and protochlorophyllide ester in the two fractions were determined spectrofluorometrically. The results are reported in Table I.

At the three stages of greening that were investigated, all of the endogenous pools from protoporphyrin to protochlorophyll were membrane-bound. This was shown by the complete absence of these tetrapyrrole intermediates from the stromal fraction. The membrane fraction of 15-min etiochloroplasts was enriched in protochlorophyllide ester but lacked any protoporphyrin or MPE-equivalent. At times this fraction also lacked protochlorophyllide. These results are compatible with previous protochlorophyll determinations performed on freshly isolated 15-min etiochloroplasts (9). In 2-hr etiochloroplasts, the endogenous tetrapyrrole pools consisted of membrane-bound protoporphyrin, MPE-equivalent, and protochlorophyll. The membrane-bound protochlorophyll pool was composed of both protochlorophyllide and protochlorophyllide ester. After 2 additional hr of illumination (*i.e.* in 4-hr etiochloroplasts), the amount of membrane-bound protochlorophyllide and MPE-equivalent increased sharply while that of protochlorophyllide ester decreased. The membrane-bound MPE-equivalent exhibited the same spectrofluorometric properties as the MPE-equivalent of isolated chloroplasts which was shown previously to consist of Mg-protoporphyrin monoester and longer wavelength metalloporphyrins (8).

[5] Protochlorophyll refers to a mixture of protochlorophyllide and protochlorophyllide ester.

The data in Table I indicated that the endogenous pigment pools from protoporphyrin to protochlorophyll were membrane-bound.

Biosynthetic Competence of Lysed Plastids and Subplastidic Fractions in Presence of ALA. The biosynthetic competence of lysed plastids and of subplastidic fractions was determined by incubation in the presence of added ALA.

Lysed plastids prepared from both 15-min and 4-hr etiochloroplasts were capable of synthesizing and accumulating large amounts of protoporphyrin and lesser amounts of MPE-equivalent (Table II). Small amounts of protochlorophyllide were also synthesized. These results indicated that the protochlorophyllide biosynthetic chain did retain some of its activity after structural disorganization of the plastids.

Stromal and membrane fractions were then prepared from lysed plastids and were incubated with ALA. The results of four experiments are reported in Table III. Although the stromal fraction was capable of synthesizing large amounts of protoporphyrin from ALA, it did not catalyze the synthesis of MPE-equivalent or protochlorophyllide. The membrane fraction was essentially unable to synthesize either Mg-porphyrins or phorbins from ALA. However, when stromal and membrane fractions were recombined, the capacity to synthesize MPE-equivalent was restored, but the protochlorophyllide biosynthetic activity was not. Difference fluorescence spectra of the synthesized MPE-equivalent exhibited a Mg-protoporphyrin emission maximum at about 597 nm and a longer wavelength metalloporphyrin emission band between 600 and 615 nm. They were similar to those reported earlier for isolated etiochloroplasts (10).

Interaction of Plastid Stroma and Membranes during Synthesis and Accumulation of Protoporphyrin from ALA. Comparison of the results in Tables I and III suggested that protoporphyrin may be formed by the stromal enzymes and then becomes associated with the plastid membranes. In order to test this hypothesis, lysed pastids as well as stromal + membrane fractions were incubated with ALA in order to induce the biosynthesis and accumulation of protoporphyrin. After incubation, the amount of newly formed protoporphyrin was determined, and the lysed plastid reaction mixtures were centrifuged in order to separate the stromal fraction from the membrane fraction. The preincubated stromal + membrane reaction mixtures were recentrifuged in order to reseparate the stromal fraction from the membrane fraction. The distribution of protoporphyrin in the (re)segregated fractions was then determined (Table IV).

Table II. Biosynthesis of Protoporphyrin, MPE-Equivalent and Protochlorophyllide by Lysed Plastids in the Presence of Exogenous ALA

Lysed plastids were prepared from both 15 min and 4 h etiochloroplasts and were incubated with 100 nmoles of ALA for 1 or 2 h. The amounts of protoporphyrin, MPE-equivalent and protochlorophyllide that were biosynthesized during incubation were determined spectrofluorometrically.

Type of etio-chloro-plast	Length of incubation	pmoles per 100 mg plastid protein: Protoporphyrin Net synthesis[a] ± P_x'[b]	MPE-equivalent Net synthesis[a] ± P_x[c]	Protochlorophyllide Net synthesis[a] ± P_x'[b]
15 min	1 hr	9,474 ± 224	232 ± 105	224 ± 9
15 min	2 hr	28,004 ± 661	479 ± 74	123 ± 5
4 h	1 h	6,740 ± 159	453 ± 137	367 ± 98[d]

[a] Net synthesis is the difference between the amount of pigment observed before and after incubation.

[b] P_x' = absolute uncertainty of the Net synthesis and is calculated by the following formula: $P_x' = \sqrt{p_{x_1}'^2 + p_{x_2}'^2}$ where P_{x_1}' is the probable error of the amount of pigment before incubation and p_{x_2}' is the probable error of the amount of pigment after incubation. Probable error (p_x') is defined in Table I.

[c] P_x = absolute uncertainty of the Net synthesis and is calculated by the following formula: $P_x = \sqrt{p_{x_1}^2 + p_{x_2}^2}$ where p_{x_1} is the probable error of the amount of pigment before incubation and p_{x_2} is the probable error of the amount of pigment after incubation. The absolute uncertainty is expressed per 100 mg of plastid protein. Probable error (p_x) is defined in Table I.

[d] This figure may be lower than the actual amount of protochlorophyllide net synthesis since after incubation, the reaction mixture was extracted with acetone-NH_4OH under low light intensity. Thus some of the protochlorophyllide may have been photo-transformed.

Table III. Biosynthesis of Protoporphyrin, MPE-Equivalent and Protochlorophyllide by Subplastidic Fractions in the Presence of Exogenous ALA

Stromal, membrane, and stromal + membrane fractions were prepared from 15 min and 4 h etiochloroplasts and incubated with 100 nmoles of ALA for 2 h. The amounts of protoporphyrin, MPE-equivalent and protochlorophyllide that were biosynthesized during incubation were determined spectrofluorometrically.

Type of etio-chloro-plast	Type of sub-plastidic fraction	pmoles per 100 mg plastid protein: Protoporphyrin Net synthesis ± P_x'	MPE-equivalent Total synthesis[a] ± P_x	Protochlorophyllide Net synthesis ± P_x'
15 min	stroma	4,170 ± 98	32 ± 300	0
	membranes	260 ± 6	85 ± 96	-3,930 ± 280
	stroma + membranes	3,853 ± 96	322 ± 73	-702 ± 127
15 min	stroma	10,491 ± 248	33 ± 337	0
	membranes	68 ± 2	0	129 ± 5
	stroma + membranes	393 ± 16	264 ± 65	41 ± 2
4 h	stroma	8,926 ± 211	0	0
	membranes	-667 ± 16	17 ± 150	-904 ± 141
	stroma + membranes	1,561 ± 42	593 ± 124	-557 ± 99
4 h	stroma	3,011 ± 71	0	0
	membranes	0	12 ± 105	-1,857 ± 357
	stroma + membranes	1,189 ± 28	111 ± 86	-1,994 ± 269

[a] Total synthesis is the sum of Net synthesis and Consumption (10). Net synthesis is the difference between the amount of pigment observed before and after incubation with substrate. Consumption is the difference between the amount of pigment observed before and after incubation without substrate. The absolute uncertainty (P_x) of the Total synthesis is calculated by the following formula: $P_x = \sqrt{p_{x_1}^2 + p_{x_2}^2}$ where P_{x_1} is the absolute uncertainty of the Net synthesis and P_{x_2} is the absolute uncertainty of the Consumption.

Table IV. Intraplastidic Localization of Protoporphyrin Accumulation

Lysed plastids as well as stromal + membrane reaction mixtures were prepared from 15 min and 4 h etiochloroplasts and incubated for 2 h with 100 nmoles of ALA. After incubation, the reaction mixtures were centrifuged at 48,000xg for 30 min to (re)separate the stromal fraction from the membrane fraction, and the intraplastidic distribution of the newly synthesized protoporphyrin was determined spectrofluorometrically. The absolute uncertainty is defined in Table II.

Type of etio-chloro-plast	Treatment	Protoporphyrin (pmoles per assay): Net synthesis[a] ± P_x'	Distribution after (re)centrifugation ± P_x': stroma	membranes
15 min	Lysed plastids	1,260 ± 30	145 ± 3	1,079 ± 25
4 h	Lysed plastids	1,652 ± 40	41 ± 1	1,537 ± 36
15 min	Stroma + Membranes	34 ± 1	0.5 ± 0.01	30 ± 1
4 h	Stroma + Membranes	150 ± 4	1 ± 0.02	110 ± 3

[a] In addition to the Net synthesis of protoporphyrin, the reaction mixtures also accumulated uroporphyrin and coproporphyrin. After (re) centrifugation, the uroporphyrin and coproporphyrin remained in the stromal fraction.

As expected, significant amounts of protoporphyrin were formed from ALA by the lysed plastids and by the stromal + membrane fractions. After (re)centrifugation, most of the protoporphyrin was found in the membrane fraction of the lysed plastid and the stromal + membrane incubations. Both the lysed plastid and stromal + membrane incubation mixtures synthesized and accumulated significant amounts of uroporphyrin and coproporphyrin, but after (re)centrifugation, these tetrapyrroles remained in the stromal fraction.

Intraplastidic Localization of Conversion of Protoporphyrin into MPE-Equivalent. The intraplastidic localization of the conversion of protoporphyrin into MPE-equivalent was determined by comparing the MPE-equivalent biosynthetic activity of lysed plastids to the stromal and membrane fractions upon incubation with exogenously added protoporphyrin.

Lysed plastids prepared from 15-min and 4-hr etiochloroplasts were capable of converting exogenously added protoporphyrin into MPE-equivalent (Table V). The reaction failed to take place in the absence of lysed plastids or upon heat inactivation of the

lysed plastids (Table V), thus indicating its enzymic nature.

The stromal fractions prepared from 15-min and 4-hr etiochloroplasts were usually unable to catalyze the conversion of exogenously added protoporphyrin into MPE-equivalent, while the membrane fractions were as active as the lysed preparations (Tables V and VI). This was interpreted as an indication that the enzymes that catalyzed the conversion of protoporphyrin into MPE-equivalent were membrane-bound. It was also observed that the stromal fraction prepared from 15-min etiochloroplasts had only a slight inhibitory effect on the activity of the membrane fraction, while the stromal fraction prepared from 4-hr etiochloroplasts caused a more pronounced inhibition of the 4-hr etiochloroplast membrane fraction (Table VI). The nature of this inhibition is presently unknown. Difference fluorescence spectra, of the MPE-equivalent that was synthesized by the membrane fraction from exogenous protoporphyrin, were similar to those reported earlier for isolated etiochloroplasts (10).

DISCUSSION

From the data reported in this paper, it appears that the biosynthesis of MPE-equivalent from ALA is a compartmentalized process. Protoporphyrin appears to be synthesized from ALA in the stroma of the plastids (Table III). The newly formed protoporphyrin becomes associated with the membrane fraction (Table IV), and the chelation of Mg by protoporphyrin appears to be catalyzed by a membrane-bound enzyme (Table VI). The enzymic nature of the chelation reaction is shown by the data of Table V.

Table V. Conversion of Exogenous Protoporphyrin into MPE-Equivalent by Lysed Plastids

Lysed plastids were prepared from 15 min and 4 h etiochloroplasts and incubated with protoporphyrin for 1 h. The amount of MPE-equivalent that was formed during incubation was determined spectrofluorometrically. The total synthesis and absolute uncertainty were defined in Table III.

Type of etiochloroplast	p moles per 100 mg plastid protein: Exogenous protoporphyrin added	p moles per 100 mg plastid protein: MPE-equivalent Total synthesis ± P_x
15 min	32,453	519 ± 94
15	210,000	2,274 ± 282
15 min Heated[a]	1,125[c]	0
None[b]	344[c]	0
4 h	20,068	251 ± 110
4 h	38,930	289 ± 132

[a] The lysed 15 min etiochloroplasts were heated at 100°C for 2 min.

[b] This reaction mixture contained only the cofactor-enriched buffer.

[c] These figures refer to the total amount of exogenous protoporphyrin added per assay.

Table VI. Conversion of Exogenous Protoporphyrin into MPE-Equivalent by Subplastidic Fractions

Stromal, membrane, and stromal + membrane fractions were prepared from 15 min and 4 h etiochloroplasts and were incubated with protoporphyrin for 2 h. The amount of MPE-equivalent that was biosynthesized during incubation was determined spectrofluorometrically. The total synthesis and absolute uncertainty were defined in Table III.

Type of etiochloroplast	Type of subplastidic fraction	Exogenous Protoporphyrin Added (pmoles per assay)	MPE-equivalent Total synthesis ± P_x (pmoles per 100 mg plastid protein)
15 min	stroma	1,197	67 ± 417
	membranes	1,197	311 ± 104
	stroma + membranes	1,197	205 ± 83
4 h	stroma	1,089	114 ± 370
	membranes	1,089	586 ± 277
	stroma + membranes	1,089	194 ± 198

Although the stromal enzymes were capable of converting ALA to protoporphyrin (Table III), they were usually incapable of converting protoporphyrin to MPE-equivalent (Table VI). The membrane-bound enzymes were considerably less active than the stromal enzymes in producing protoporphyrin from ALA (Table III) but were capable of catalyzing the conversion of protoporphyrin to MPE-equivalent (Table VI). Therefore, the cooperation of the stromal and membrane fractions during MPE-equivalent biosynthesis appears to be confined to the association with the membranes of the protoporphyrin that is synthesized in the stroma. This association seems to be specific since the newly formed uroporphyrin and coproporphyrin did not associate with the membranes. Of course, at the pH of the incubation medium (~7.25), protoporphyrin would be expected to be less soluble in the stromal fraction than uroporphyrin or coproporphyrin. The higher solubility of protoporphyrin in the hydrophobic environment of the membranes may be one mechanism by which the plastid membranes acquire the correct porphyrin substrate for further processing by membrane-bound enzymes.

From Table I, it appears that all of the endogenous metabolic pools from protoporphyrin to Chl are membrane-bound. As mentioned above, the enzyme responsible for the insertion of Mg into protoporphyrin also appears to be membrane-bound (Table VI). Griffiths (2) has demonstrated that the conversion of exogenous protochlorophyllide to chlorophyllide and/or Chl is also catalyzed by the etiochloroplast membrane fraction. Thus, all of the reactions between protoporphyrin and Chl have been demonstrated to be membrane-bound except for the reactions between MPE-equivalent and protochlorophyllide whose intraplastidic localization remains to be determined. At present, there is no reason to believe that the localization of these reactions is any different than that of the other reactions between protoporphyrin and Chl. Therefore, the transition of the Chl biosynthetic chain from the soluble state to the membrane-bound state seems to occur at the level of protoporphyrin.

When lysed plastids and subplastidic reaction mixtures were prepared from 4-hr etiochloroplasts and incubated with ALA or protoporphyrin, a net synthesis of MPE-equivalent was not always observed (*i.e.* the difference between the amount of pigment measured before and after incubation was not always a positive value). As previously discussed (10), this phenomenon was observed when the amount of MPE-equivalent biosynthesis from ALA or protoporphyrin was lower than the rate of consumption of the endogenous and nascent metalloporphyrins. Under these conditions, the total extent of MPE-equivalent formation could be determined by summing the amount of MPE-equivalent net synthesis in the presence of substrate and the amount of MPE-equivalent consumption in the absence of substrate. This summation gives a value for the total MPE-equivalent synthesis (10), and it is this value which is reported in Tables III, V, and VI for MPE-equivalent.

The synthesis of MPE-equivalent was always accompanied by the synthesis of Zn-Proto(ester). The amount of Zn-Proto(ester) varied from 70 to 100% of the total metalloporphyrins that accumulated at the end of incubation. For example, the stromal fractions prepared from 15-min and 4-hr etiochloroplasts accumulated only Zn-Proto(ester), whereas the corresponding membrane fractions accumulated between 70 and 90% Zn-Proto(ester) in addition to the MPE-equivalent which was reported in the tables.

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