Plant Physiol. (1972) 50, 185–186

Short Communication

The Biosynthesis of Metal Porphyrins by Subchloroplastic Fractions[1]

Received for publication September 28, 1971

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The biosynthesis of Mg protoporphyrin monoester, protochlorophyll and chlorophyll *a* and *b* by crude homogenates, isolated etioplasts, and differentiating chloroplasts has recently been described by Rebeiz *et al.* (6–9). In the present communication we wish to report briefly on the capacity of subchloroplastic fractions to form free porphyrins and metal porphyrins.

MATERIALS AND METHODS

Cucumber seeds (*Cucumis sativus* L. cv. Alpha Green) were germinated in vermiculite (Terra Lite) at 24 C in complete darkness for 4 days (7, 8). Cotyledons were harvested with full hypocotyl hook as previously described (2). They were placed in beakers with enough distilled H_2O to keep them moist and were irradiated with white fluorescent light for 4 hr (8). Developing chloroplasts were isolated from the greening cotyledonary tissues. Five grams of cotyledons were ground in 7.5 ml of fortified 0.2 M tris-HCl, 0.5 M sucrose, pH 8.0, containing 37.5 μmoles of GSH, 2.25 μmoles of CoA, 3.75 μmoles of $MgCl_2$, 375 μmoles of potassium phosphate, 180 μmoles of methanol, 3 μmoles of ATP, and 0.45 μmole of NAD. The slurry was filtered through four layers of cheesecloth, and the resulting crude homogenate was centrifuged at 0 C for 3 min at 200*g*. The supernatant was centrifuged for 7 min at 1500*g*, and the resulting chloroplast pellet was lysed in 21 ml of fortified 25 mM tris-HCl, 25 mM potassium phosphate buffer, pH 7.7, lacking sucrose but containing the same concentration of GSH, CoA, $MgCl_2$, methanol, ATP, and NAD as the homogenization buffer. The suspension was kept on ice in the dark for 15 min then centrifuged at 105,000*g* for 60 min in a Spinco No. 40 rotor. This centrifugation separated the lysed plastids into a water-clear soluble protein fraction (S_3) and a compact, green, particulate fraction (P_3). S_3 was withdrawn with an eye dropper, and P_3 was suspended in 21 ml of the fortified lysing buffer. Two milliliters of S_3, P_3 and $S_3 + P_3$ (1:1 v/v) were incubated in glass tubes on a metabolic shaker with 25 μmoles of ALA.[3] The incubation was carried out at 28 C under 5 ft-c of white fluorescent light with moderate shaking speed for 16 hr. Three milliliters of the reaction mixture at pH 7.7 contained: 150 μmoles of tris-HCl, 100 μmoles of sucrose, 100 μmoles of potassium phosphate buffer, 1 μmole of $MgCl_2$, 10 μmoles of GSH, 0.6 μmole of CoA, 1.2 mmoles of methyl alcohol, 1 μmole of ATP, and 0.15 μmole of NAD.

The reaction was stopped by the addition of 15.0 ml of acetone:0.1 N NH_4OH (9:1 v/v) and centrifuged at 39,000*g* for 10 min. The pellet was saved for the extraction of free porphyrins and the acetone:NH_4OH supernatant containing metal porphyrins and free porphyrins was extracted with an equal volume of hexane. The hexane extracted most of the β-carotene and chlorophyll. The metal porphyrins remaining in the hexane-extracted acetone:NH_4OH supernatant were transferred to ether as previously described (6). The proteinaceous pellet and the aqueous acetone phase that was left after metal porphyrins extraction were pooled and used for the extraction of free porphyrins as described previously (6).

Free porphyrins were separated by ascending paper chromatography on Whatman No. 3 MM paper in 2,6-lutidine:0.05 N NH_4OH (5:3.5 v/v). Metal porphyrins, in ether, were separated on thin layers of Silica Gel H in benzene-ethyl acetate-ethanol (8:2:2 v/v). All fractions were routinely monitored by their absorption spectra and recorded with a Beckman double beam spectrophotometer Model DK-2A. All absorbancies used in quantitative determinations were derived from the appropriate spectra.

The amount of uroporphyrin in acidified, saturated sodium acetate (pH 2.5) was estimated from absorbancy at 402 nm using the molar extinction coefficient (5.41×10^5) and the correction factors reported by Rimington and Sveinsson (10) for uroporphyrin in 0.5 N HCl.

The copro + protoporphyrin fraction in ether containing mostly coproporphyrin was estimated from its absorbancy at 401 nm and a molar extinction coefficient of 1.8×10^5 (6). When protoporphyrin was predominant in this fraction, its absorbancy at 404 nm and a molar extinction coefficient of 1.5×10^5 were used instead (6).

The amount of metal porphyrins in ether and in the presence of some chlorophyll and protochlorophyll contamination was estimated as "Mg protoporphyrin equivalent" by the following equation: nmoles per ml = $56.61\ A_{583} - 13.24\ A_{624} - 7.79\ A_{648}$ where A_{583}, A_{624}, and A_{648} represent the absorbancies of the ether extract at 583, 624, and 648 nm respectively. The above equation was derived from the molar extinction coefficients of Mg protoporphyrin, protochlorophyll and chlorophyll *a* + *b* in ether at 583, 624, and 648 nm (Table I).

RESULTS

Incubation of the soluble protein fraction (S_3) with ALA and cofactors led to the biosynthesis of free porphyrins with

[1] This work was supported by Research Grant GB8546 from the National Science Foundation.

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[3] Abbreviation: ALA: δ-aminolevulinic acid.

Table I. *Molar Extinction Coefficients of Mg Protoporphyrin, Protochlorophyll and Chlorophyll a + b in Ether Used in Deriving the Equation Given in the Text*

Tetrapyrrole	Wavelength	Molar Extinction Coefficient[1]	Reference no. in "Literature Cited"
	nm		
Mg protophorphyrin	583	18.2×10^3	1
	624	0.47×10^3	1
	648	0.31×10^3	1
Protochlorophyll	583	8.36×10^3	4
	624	35.6×10^3	4
	648	0.24×10^3	4
Chlorophyll *a* + *b*	583	6.72×10^3	11
	624	8.97×10^3	11
	648	33.6×10^3	11

[1] These values were derived from data presented in the references indicated.

Table II. *Biosynthetic Capacity of Subchloroplastic Fractions Isolated from Cucumber Cotyledons*

Tetrapyrrole	Pigment Accumulation		
	Soluble protein fraction	Green particulate fraction	Soluble protein fraction + green particulate fraction
	nmoles/100 mg protein		
Uroporphyrin	323.4	11.4	297
Copro + protoporphyrin	31.9	3.6	90
Metal porphyrins[1]	none	1.0	9.0

[1] Not corrected for recovery losses.

typical "aetio" spectra. This activity was gradually lost upon storing S_3 at −18 C in the dark.

Chromatography in 2,6-lutidine:0.05 N NH_4OH (5:3.5 v/v) separated the porphyrins into three main bands with R_F values of 0.07, 0.52, and 0.71. These values correspond closely to the respective chromatographic mobilities of uroporphyrin, coproporphyrin, and protoporphyrin in this solvent system (5, 6). No free protoporphyrin monoester or metal porphyrins were detected by chromatography in acetone-petroleum ether-acetic acid (3:7:0.01, v/v) (6) or by spectrophotometry of the metal porphyrin ether fraction. The amounts of free porphyrins produced are given in Table II.

The green particulate fraction (P_3) was capable of a very limited biosynthetic activity when incubated with ALA and cofactors (Table II). However, upon incubating the soluble protein fraction with the green particulate fraction in the presence of ALA and cofactors, substantial amounts of free porphyrins and metal porphyrins were formed (Table II). Although incubations were routinely performed under 5 ft-c of white fluorescent light, the reaction proceeded as well in the dark. Only 7 to 10% of the metal porphyrins present in the initial acetone:NH_4OH supernatant were recovered in the ether extract. The copro + protoporphyrin fraction in ether consisted mainly of protoporphyrin (R_F = 0.74) and minor amounts of coproporphyrin (R_F = 0.52), as evident by chromatography in 2,6-lutidine:0.05 N NH_4OH (5:3.5 v/v). It exhibited a protoporphyrin absorption spectrum in ether, with maxima at 633, 576, 536, 502, and 404 nm (3, 6). The metal porphyrin fraction in ether exhibited a metal porphyrin spectrum with two absorption bands of equal intensity at 587 and 542 nm and a Soret absorption maximum at 417 nm. Chromatography on Silica Gel H in benzene-ethyl acetate-ethanol (8:2:2 v/v) revealed the presence of two (sometimes one) slow moving bands and a faster moving one. The fast moving band cochromatographed with standard Mg protoporphyrin monoester ahead of standard protochlorophyllide (6). Silica gel eluates of the fast moving band in methanol-acetone (4:1 v/v) exhibited typical Mg porphyrin spectra with absorption maxima at 587 to 590, 547 to 550, and 416 to 418 nm. These values compare favorably with the maxima at 589, 551, and 418 nm reported by Granick (1) for Mg protoporphyrin dimethyl ester in ether and for Mg protoporphyrin in 50% ethanol containing 0.02 N KOH.

Preliminary investigations of the slow moving metal porphyrins indicate that they might be the metal chelates of proto and coproporphyrin. A more detailed study of the specific nature of the ligand and metal(s) of these metal porphyrins is now in progress.

These results suggest that (*a*) the biosynthesis of protoporphyrin from ALA is catalyzed by a soluble enzyme system extractable from chloroplastic preparations, (*b*) the biosynthesis of metal porphyrins from ALA requires the interaction of both the soluble enzyme system and the particulate fraction.

The significance of these findings to the intrachloroplastic localization of the protochlorophyll and chlorophyll biosynthetic pathway is now being evaluated.

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