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Chloroplast biogenesis 82: Development of a cell-free system capable of the net synthesis of chlorophyll(ide) *b*

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Abstract

Recent experimental evidence (see part 80) suggests that the chlorophyll (Chl) *b* pathway is biosynthetically heterogeneous. The dissection of this biosynthetic heterogeneity requires the availability of a cell-free system capable of the net synthesis of Chl(ide) *a* and *b*. The development of such a system is described.

Additional key words: 5-aminolevulinic acid; *Cucumis*; etiochloroplast; tetrapyrroles.

Introduction

The conversion of chlorophyll (Chl) *a* and/or chlorophyllide (Chlide) *a* [Chl(ide) *a*] to Chl(ide) *b* involves conversion of the methyl group at position 3 of the Chl macrocycle (Fisher nomenclature) to a formyl group. Mass spectra of [3-hydroxymethyl]-Chl *b* extracted from leaves greened in the presence of either $^{18}O_2$ or $H_2{}^{18}O$ revealed that ^{18}O was incorporated only from molecular oxygen into the 3-formyl group of Chl *b* (Porra *et al.* 1993, 1994). The high enrichment using $^{18}O_2$ and the absence of labeling by $H_2{}^{18}O$ indicated that molecular oxygen is the sole precursor of the 3-formyl oxygen of Chl *b* in greening maize leaves. This in turn suggested that a mono-oxygenase, that converts a methyl to a hydroxymethyl group, and a hydroxymethyl dehydrogenase, that converts hydroxymethyl to a formyl group, might be involved in the oxidation of the methyl group to formyl. These putative enzymes have so far eluded detection and purification from higher or lower plants.

Recent results suggest that the Chl *b* pathway, like the Chl *a* biosynthetic pathway, is biosynthetically heterogeneous (Rebeiz *et al.* 1999). The fine dissection of this

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Abbreviations: ALA, 5-aminolevulinic acid; Chl, chlorophyll; Chlide, chlorophyllide; Chl(ide), Chl, Chlide, or a mixture of both. Unless preceded by MV or DV, tetrapyrrole names are used generically to designate metabolic pools that may consist of MV and DV components.

putative biosynthetic heterogeneity will require the availability of a cell-free system capable of the net synthesis of Chl(ide) *a* and *b* from early and late precursors. To our knowledge such a cell-free system is not presently available. The first cell-free system from higher plants, capable of ^{14}C-5-aminolevulinic acid (ALA) conversion to ^{14}C-Chl *b in organello* was described in 1971 (Rebeiz and Castelfranco 1971). This work was confirmed by Huang and Hoffman (1990). A qualitative study of the conversion of Chl(ide) *a* to Chl(ide) *b* in a *Scenedesmus obliquus* mutant *in vitro* was made by Kotzabasis and Senger (1989). In this work the development of an *in organello* system from higher plants capable of the net synthesis of Chl(ide) *a* and *b* from exogenous ALA is described.

Materials and methods

Plants: Cucumber seeds (*Cucumis sativus* L. cv. Beit Alpha MR) were germinated in moist vermiculite at 28 °C for 4-5 d in darkness. The seedlings were pretreated either with a 2.5 ms flash of "white actinic light" followed by 60 min of darkness or with metal halide irradiation (120 μmol m^{-2} s^{-1}) for 4 or 24 h.

Preparation of etiochloroplasts: After removing the hypocotyl hooks of the pretreated cotyledons, 85-90 g batches of tissue were homogenized in a *Waring blender* (2 bursts, 5 s each) under subdued cool white fluorescent laboratory light (4.0 μmol m^{-2} s^{-1}) in 230 cm^3 of a homogenization medium consisting of 0.5 M sucrose, 15 mM Hepes, 30 mM Tes, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM cysteine, and 0.2 % bovine serum albumin (m/m) at a room temperature, pH 7.7 (Daniell and Rebeiz 1982, Rebeiz *et al.* 1984). The homogenate was passed through four layers of cheese cloth and one layer of miracloth. The plastids were pelleted by centrifuging the homogenate at 200×*g* for 3 min followed by centrifuging the resulting supernatant for 10 min at 1500×*g*. The plastid pellet was gently resuspended in 10 cm^3 of homogenization medium. The resuspended plastids were further purified by layering 6 cm^3 of the suspension over 25 cm^3 of homogenization medium containing 35 % *Percoll*, in a 50 cm^3 centrifuge tube and centrifugation at 6 000×*g* for 5 min in a *Beckman JS-13* swinging bucket rotor at 1 °C. Intact plastids recovered as a pellet were gently resuspended in 5 cm^3 of medium consisting of 0.5 M sucrose, 0.2 M Tris-HCl, 20 mM $MgCl_2$, 2.5 mM EDTA, 1.25 mM methanol, 20 mM ATP, 40 mM NAD, 8 mM methionine, and 5 μM phytol at a room temperature, pH 7.7 (Daniell and Rebeiz 1982, Rebeiz *et al.* 1984).

Etiochloroplast incubation: Each incubation consisted of 0.95 cm^3 of plastid suspension (3-5 mg protein) and 0.05 cm^3 of 10 mM ALA. Incubation was carried out at 28 °C for 15-60 min on a reciprocating water bath operated at 50 oscillations per min under 4 μmol m^{-2} s^{-1} of cool white fluorescent radiation. Incubations were terminated by precipitation with 10 cm^3 of acetone: 0.1 M NH_4OH (9:1, v/v).

Pigment extraction: The acetone extracts containing the tetrapyrrole pigments were cleared of insoluble lipoproteins by centrifugation at 39 000×*g* for 12 min. Chl *a*, a fully esterified tetrapyrrole, was removed from the aqueous acetone solution by

extraction with 1 volume of hexane followed by a second extraction with 1/3 volume of hexane. The more polar monocarboxylic tetrapyrroles such as Pchlide *a* and Chlide *a* remained in the hexane-extracted aqueous acetone fraction. The amount of Pchlide *a* and Chlide *a* was determined spectrofluorometrically on aliquots of the hexane-extracted acetone fraction as described in Rebeiz *et al.* (1975). One cm^3 aliquot of the hexane extract containing the Chl was dried under N_2 gas and the residue was redissolved in 4 cm^3 of 80 % acetone. The amount of Chl *a* and *b* in the acetone solution was determined spectrofluorometrically as described in Bazzaz and Rebeiz (1979). Fluorescence spectra were recorded as described in Rebeiz *et al.* (1975). The endogenous ALA content of the isolated plastids was determined as described by Mauzerall and Granick (1956).

Results

Plastids were prepared from etiolated cucumber cotyledons at three different stages of greening. Their capacity to convert ALA to Chl(ide) *a* and *b* was monitored as described under Materials and methods.

In the first set of experiments, etioplasts were prepared from etiolated cotyledons that were potentiated for Chl(ide) *b* biosynthesis by pretreatment with one 2.5 ms flash of "actinic white light" followed by 60 min of dark incubation. Such plastids were incapable, however, of Chl(ide) *b* net synthesis *in vitro* (Table 1, A).

In a second set of experiments, the cotyledons were greened for 24 h (120 μmol m^{-2} s^{-1} of metal halide radiation) prior to the preparation of etiochloroplasts. At this stage the cotyledons had accumulated large amounts of Chl *a* and *b*. The evaluation of the Chl *b* biosynthetic activity *in vitro* was rather uncertain because of the high background of accumulated Chl *a* and *b* (Table 1, *B*).

In a third set of experiments, etiolated cotyledons were greened for 4 h before etiochloroplast preparation. At this stage of greening, the lag-phase of Chl *b* biosynthesis had been removed and the tissue had just started active Chl *b* biosynthesis (Rebeiz 1967). As a consequence, although Chl *b* biosynthesis was fully potentiated, the amount of accumulated Chl(ide) *a* and *b* was not large enough to interfere with the detection of Chl(ide) *a* and *b* biosynthesis *in vitro*. As shown in Table 1, *C,D*, net Chl(ide) *a* and *b* biosynthesis was observed after 15, 30, and 60 min of incubation.

Discussion

The demonstration of metabolic pathways is a multistep process. It involves at least three stages: (*a*) the detection and characterization of metabolic intermediates, (*b*) the demonstration of precursor-product relationships between putative intermediates, and (*c*) purification and characterization of enzymes involved in the metabolic interconversions. Recent developments in the understanding of the Chl *a* biosynthetic heterogeneity (Rebeiz *et al.* 1994, Adb El Mageed *et al.* 1997), and the discovery of

putative biosynthetic intermediates and end products of the Chl *b* pathway, such as MV Pchlide *b* (Shedbalkar *et al.* 1991), MV Chlide *b* (Aronoff 1981, Duggan and Rebeiz 1981), DV Chlide *b* (Rebeiz, unpublished), and DV Chl *b* (Brereton *et al.* 1983, Wu and Rebeiz 1985), as well as preliminary precursor-product investigations *in vivo* (Rebeiz *et al.* 1999) suggest that the Chl *b* pathway may also be biosynthetically heterogeneous.

Table 1. Conversion of exogenous 5-aminolevulinic acid (ALA) to Chl(ide) *a* and *b* [nmol per 100 mg plastid protein] by isolated etiochloroplasts. Etiochloroplasts were prepared from etiolated cucumber cotyledons pretreated with "white light" as follows. *A*: 2.5 ms actinic flash followed by 60 min of darkness. *B*: 120 μmol m^{-2} s^{-1} for 24 h. *C* and *D*: 120 μmol m^{-2} s^{-1} for 4 h. Each incubation contained 0.05 cm^3 of 10 mM ALA (about 10.4 to 13.8 μmol, depending on the protein content of the plastids). The endogenous ALA content amounted to an insignificant amount of about 0.003 μmol per 100 mg plastid protein. All values ± standard deviation are means of two replicates.

Exp.	Irradiation	Tetrapyrrole	Incubation time [min] 0	15	30	60
A	2.5 ms	MV Chlide *a*	3.1 ± 0.4	-	-	3.1 ± 0.3
		MV Chl *a*	21.8 ± 6.1	-	-	14.7 ± 2.1
		MV Chlide *b*	0.3 ± 0.0	-	-	0.4 ± 0.2
		MV Chl *b*	0.7 ± 0.2	-	-	0.8 ± 0.5
B	24 h	MV Chlide *a*	45.1 ± 0.6	-	-	180.3 ± 13.7
		MV Chl *a*	11849.5 ± 1137.7	-	-	12325.0 ± 205.1
		MV Chlide *b*	12.5 ± 3.9	-	-	24.1 ± 0.8
		MV Chl *b*	4380.4 ± 177.2	-	-	4577.4 ± 316.2
C	4 h	MV Chlide *a*	14.3 ± 5.3	-	43.0 ± 8.3	64.7 ± 12.7
		MV Chl *a*	97.3 ± 19.6	-	181.7 ± 11.7	108.6 ± 4.0
		MV Chlide *b*	1.9 ± 0.3	-	4.3 ± 0.6	6.9 ± 1.3
		MV Chl *b*	18.5 ± 4.4	-	33.2 ± 2.1	22.7 ± 0.1
D	4 h	MV Chlide *a*	12.1 ± 0.7	25.3 ± 2.4	52.3 ± 1.2	55.0 ±3.0
		MV Chl *a*	138.0 ± 15.7	272.9 ± 8.8	244.7 ± 14.4	200.8 ± 4.5
		MV Chlide *b*	1.8 ± 0.1	2.8 ± 0.1	5.9 ± 1.0	7.0 ± 0.2
		MV Chl *b*	35.0 ± 1.9	59.4 ± 0.9	70.9 ± 2.0	62.1 ± 2.3

Because of the highly branched nature of the proposed Chl *b* biosynthetic pathway, dissection and demonstration of the fine details of the various Chl *b* biosynthetic routes cannot rely solely on precursor-product relationship studies *in vivo* (Rebeiz *et al.* 1988, 1999). Demonstration of precursor-product relationships among putative Chl *b* biosynthetic intermediates, requires the availability of *in organello* or cell-free systems capable of the net conversion of early and late intermediates of the pathway into Chl *b*. The *in organello* systems so far described are only capable of very low conversion rates of ^{14}C-ALA to ^{14}C-Chl *b* (Rebeiz and Castelfranco 1971, Huang and Hoffman 1990). The only study describing the apparent net synthesis of Chl *b* from Chl(ide) *a in vitro* in a *S. obliquus* mutant was qualitative in nature (Kotzabasis and Senger 1989).

The *in organello* system described in this work is capable of the net conversion of exogenous ALA into Chl(ide) *a* and *b* (Table 1, *C, D*). A useful *in organello* system capable of the net synthesis of Chl *b* should also be capable of using exogenous tetrapyrroles as substrates. On many occasions, we have demonstrated that isolated plastids are indeed capable of using exogenous tetrapyrroles as substrates in tetrapyrrole biosynthetic reactions (Mattheis and Rebeiz 1977, Tripathy and Rebeiz 1986). As such we believe that the *in organello* system described in this paper should be extremely useful in the elucidation of the precursor-product details of various Chl *b* biosynthetic routes.

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