Communication

Evidence that Isolated Developing Chloroplasts Are Capable of Synthesizing Chlorophyll b from 5-Aminolevulinic Acid

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ABSTRACT

Developing chloroplasts isolated from cucumber (Cucumis sativus L. var Beit Alpha) cotyledons are capable of incorporating [14C]5-aminolevulinic acid into chlorophyll (Chl) b and Chl a when incubated under photosynthetic illumination. Thin layer chromatography and high pressure liquid chromatography were employed to analyze the pigments. The specific radioactivity in Chl a was over three times higher than that found in Chl b. Both Chl a and b synthesizing activities in organello decayed rapidly at approximately the same rate. We conclude that concomitant synthesis of Chl a/b-binding apoprotein is not required for Chl b synthesis.

Chl in higher plants is synthesized from ALA2 which is derived from glutamate (2). Conversion of glutamate into ALA is the rate-limiting step for the synthesis of Pchlide (11) and Chl (4). Chl synthesis requires SAM for the methylation of Mg-protoporphyrin and light is required for the photoreduction of Pchlide and for the photosynthetic production of ATP and reducing power needed for Chl synthesis (6). The two predominant forms of Chl found in higher plants are Chl a and b which differ only in the presence of a methyl group or formyl group at position 3 in Chl a and b, respectively. The early steps in Chl biosynthesis have been elucidated; but the later steps are still controversial, including the reaction sequence and mechanism of Chl b formation (3, 6). One of the prevailing views is that Chl b is derived directly from the oxidation of Chl a (3, 6).

Chl is synthesized within the chloroplast and is attached noncovalently onto protein; free Chl does not accumulate (13, 14). Chl b is associated exclusively with nuclear encoded CAB proteins whereas Chl a is associated with both the nuclear encoded CAB and chloroplast encoded polypeptides. All the enzymes required for Chl synthesis are nuclear encoded and imported into the chloroplast (3). Therefore, isolated plastids have been used quite widely in studies of Chl biosynthesis, particularly the earlier steps of the pathway (3, 6). Developing cucumber plastids, isolated through Percoll, have been previously shown to be competent to synthesize Pchlide from glutamate (9) and Pchlide (10), Chlide (8), and Chl a (4) from ALA. Although isolated developing chloroplasts are capable of synthesizing Chl a from ALA, our knowledge Chl b synthesis in isolated chloroplasts has not been reported (4). In this communication we report that intact chloroplasts isolated from greening cucumber seedlings are active in synthesizing Chl b from ALA in the absence of extrachloroplast factors.

MATERIALS AND METHODS

Materials

Cucumber (Cucumis sativus L. var Beit Alpha) seed was a gift from Dr. Paul Castelfranco, University of California, Davis. Percoll was from Pharmacia; 5-amino[4-14C]levulinic acid (57.3 mCi/mmol), from New England Nuclear; SAM and silica gel TLC plate (250 µm layer thickness, 2–25 µm mean particle size, 60 Å mean pore diameter) were from Sigma.

Plant Growth and Chloroplast Isolation

Cucumber seeds were germinated in the dark on moist vermiculite, lined with filter paper. After 6 d the seedlings were illuminated continuously for 20 h by incandescent light, 60 μE·m⁻²·S⁻¹, at room temperature. Cotyledons were harvested and intact chloroplasts were isolated as reported previously (8, 9) except that the grinding buffer consisted of 50 mM Hepes-KOH (pH 7.7), 0.34 M sorbitol, 1 mM EDTA, 1 mM MgCl₂, 5 mM cysteine, and 0.2% BSA.

Incubation for Chl Synthesis

Incubations were for 1 h, at 30°C, in a shaking incubator under white light, 60 μE·m⁻²·S⁻¹. The mixture (200 µL) contained: 50 mM Hepes-KOH (pH 7.7), 0.34 M sorbitol, 1 mM EDTA, 1 mM MgCl₂, 34.9 µM [14C]ALA (57.3 µCi/mmol), 1 mM SAM, and plastids equivalent to 1 to 1.5 mg protein (40–60 µg Chl) as determined by the Bradford method (5). The reactions were initiated by addition of the chloroplasts, and terminated by rapid cooling and addition of 800 µL acetone.

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2 Abbreviations: ALA, 5-aminolevulinic acid; SAM, S-adenosyl-L-methionine; CAB, Chl a/b binding polypeptide.

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TLC

Aliquots of the hexane phase (see above) were dried under N₂, dissolved in CHCl₃, and separated on silica gel plates according to (7). Plates were exposed to Kodak x-ray film (XAR-5) at -70°C. After autoradiography, radioactive zones were eluted with 80% acetone and quantitated by scintillation counting. The absorption spectrum of each eluted band was also recorded using a Beckman DU-70 spectrophotometer and used to calculate the amount and purity of Chl (1, 12).

HPLC

Portions of the hexane phase (see above) were dried, taken into 80% acetone, and filtered through a 0.45 µm syringe filter into amber HPLC vials. The pigments were separated with a Hewlett Packard 1082B Liquid Chromatograph essentially as described by Thayer and Björkman (15). The samples were eluted at a flow rate of 1.1 mL·min⁻¹ using 100% solvent A (acetoni-trile:methanol 85:15 v/v) for the first 10 min followed by a 15 min linear gradient, starting with 25% and ending with 45% of solvent B (ethyl acetate), the rest being solvent A. The column was allowed to reequilibrate in solvent A for 5 min prior to the next injection. Chls were detected by their λ₄₃₅, and other pigments by their λ₄₄₅. The peaks were identified by their absorption spectra which were obtained by scanning the peaks during a run using the built-in scan mode.

Eluates corresponding to Chl a and b peaks were collected into microfuge tubes, each collection lasting 0.5 min. The collections were dried, redissolved in 80% acetone, and the absorption spectrum of each was measured. With this measurement, the identity of the pigment was confirmed, and its amount and purity were estimated. Radioactivity was measured by counting aliquots of the acetone solution.

RESULTS

Isolated chloroplasts from greenning cucumber cotyledons incorporated [¹⁴C]ALA into Chl a and Chl b when incubated in the light in the presence of SAM. The incorporation ranged from 1.5 to 2.9 nmol of [¹⁴C]ALA per mg protein, or 30 to 70 pmol of [¹⁴C]ALA per µg Chl, in 1 h. The ratio of Chl a to b in these plastids was between 3 and 3.2.

To address the question of whether Chl b was synthesized in organello, extracted pigments were separated by TLC or HPLC and further analyzed. Figure 1 shows a TLC chromatogram and its autoradiograph. Seven pigment bands were resolved, including Chl a and b, pheophytin, and the carotenoids: β-carotene, lutein, violaxanthin, and neoxanthin (Fig. 1, track 1). Chl a, pheophytin, and Chl b were radioactively labeled, although Chl b had less radioactivity than Chl a (track 2). No radioactivity was associated with the carotenoids. The identity of individual bands was confirmed by their absorption spectrum taken after elution into 80% acetone. Based on the measurements of absorbance and radioactivity, the specific radioactivity was determined to be 8760 dpm per nmol.

Extraction and Quantitation of Chl

Plastids were extracted into 1 mL of 80% acetone (v/v) and then centrifuged in a microfuge at 4°C for 5 min. The pellet was reextracted with 100 µL of 0.225 M NH₄OH and 400 µL of acetone and centrifuged as before, and the supernatants were pooled. An aliquot of the pooled supernatants was taken to measure the total Chl content spectrophotometrically (1). To the pooled supernatants, 150 µL of saturated NaCl solution was added and the Chl extracted into 2 mL of hexane. The aqueous phase was reextracted with 1 mL of hexane. The combined hexane extracts were washed twice with equal volumes of a solution prepared by diluting saturated NaCl fivefold. An aliquot of the hexane phase was mixed with 5 mL of scintillation fluid (Budget solve, Research Products International) and the radioactivity was measured on a LKB 1214 Rackbeta Liquid Scintillation Counter. Counting efficiency for ¹⁴C was 90 to 95%. To measure specific radioactivity, an aliquot of the hexane extract was dried under a stream of N₂, redissolved in 80% acetone, and its Chl content and radioactivity were measured as above. Amounts of ALA incorporated into Chl a and Chl b were estimated from the specific radioactivity and recoveries based upon endogenous levels of Chl a and Chl b in the original extract and purified sample.

Figure 1. Incorporation of [¹⁴C]ALA into Chl a and b by isolated cucumber plastids analyzed by TLC. Plastids were incubated with [¹⁴C]ALA under standard conditions and Chl was extracted and run on TLC as described in "Materials and Methods." The sample containing 81000 dpm was spotted and developed for 10 min. Track 1, TLC chromatogram of the pigments; track 2, autoradiograph of track 1. Seven pigmented bands were separated as shown in track 1 in descending order from the solvent front: β-carotene, yellow; pheophytin, blue-gray; Chl a, blue-green; Chl b, yellow-green; lutein, yellow; violaxanthin, yellow; and neoxanthin, yellow. Violaxanthin and neoxanthin were seen as faint bands below lutein. In track 2, radioactivity corresponded to the origin, Chl b, Chl a, and pheophytin.
Chl a and 2630 dpm per nmol Chl b, i.e. the total radioactivity in Chl a was about 10 times that in Chl b. From the absorption spectrum of the Chl b sample, we calculated that only 9% of the Chl in the sample could not be attributed to Chl b. Assuming that all the contaminant is due to Chl a, the maximum possible contamination of Chl a in the Chl b sample would be 788 dpm (8760 dpm × 0.09) or 30% of the radioactivity attributed to Chl b.

Similar results were obtained using HPLC as shown in Figure 2A. Chl a and b were well separated; elution times of the two peaks were over 2 min apart. Eluates corresponding to the Chl a and b peaks were collected and transferred into 80% acetone for measurement for the absorption spectrum (Fig. 2, B and C) and radioactivity. The specific radioactivity of the Chl a fraction was 7750 dpm/nmol, and that of Chl b fraction was 2080 dpm/nmol. The calculated upper limit of impurity, for the HPLC sample, in the Chl b fraction was 5%.

The salient features of the Chl a and b absorption spectra (Fig. 2, B and C) were as follows. For Chl b (Fig. 2B), the absorption maxima in the blue and red region were at 459 nm and 646 to 647 nm, respectively, the ratio of the blue maximum to the red maximum being 2.72 to 2.74. There was a shoulder at 433 to 439 nm, and a minor peak at 597 to 598 nm. For Chl a (Fig. 2C), the blue and red maxima were at 431 and 663 nm, respectively, the blue to red ratio being 1.11 to 1.13. There were another two absorption peaks in the blue region at 412 to 413 nm and 378 to 382 nm, and one in the red region at 617 to 618 nm. These absorption spectra are characteristic of Chl b and a, respectively (12).

Since Chl b synthesis had not been previously observed in organello, we tested whether in organello Chl b synthesis is more labile than Chl a synthesis. Isolated chloroplasts were preincubated for various amounts of time prior to the addition of ALA and SAM. The results presented in Figure 3 show that the specific radioactivity of both Chl a and b decrease markedly in response to increasing durations of preincubation. On a percentage of control basis, the specific radioactivity of Chl a and b decreased at roughly the same rate indicating

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**Figure 2.** HPLC separation and absorption spectrum of Chl extracted from isolated plastids after the incubation with [14C]ALA. The experimental procedures are described in "Materials and Methods." A, HPLC chromatogram showing elution pattern of Chl and carotenoids: N, neoxanthin; V, violaxanthin; A, antheraxanthin; L, lutein; Cb, Chl b; Ca, Chl a; and Car, β-carotene. Chl a and b fractions were manually collected. B, Absorption spectrum of the Chl b fraction after transfer to 80% acetone as described in "Materials and Methods." C, Absorption spectrum of the Chl a fraction in 80% acetone.
that the synthesis of both forms of Chl are labile in isolated chloroplasts.

**DISCUSSION**

The separation of pigments by TLC and HPLC was used to show that \(^{14}\text{C}\)ALA is converted into both Chl \(a\) and \(b\) in *organello*. Purity of the isolated fractions was evaluated by spectroscopy. The absorption spectra obtained from samples purified by TLC and HPLC (Fig. 2, B and C) agree well with reported values (12), and calculations based on the spectra revealed little impurity in each pigment fraction. We estimate that, at most, 30% of the radioactivity in the 'Chl \(b\) fraction' can be attributed to Chl \(a\) contamination. These results clearly prove that the enzymes required for converting ALA into Chl \(b\) are present and active in the isolated plastids. Our evidence is also consistent with the notion advanced by Bhaya and Castelfranco (4) that Chl synthesis occurs independently of apoprotein synthesis. Their conclusion was based on the observation that in *organello* Chl \(a\) synthesis is insensitive to concentrations of chloramphenicol that inhibit chloroplast protein synthesis. Our conclusion is based on the fact that isolated chloroplasts can synthesize Chl \(b\) even though Chl \(b\) is associated only with cytoplasmically synthesized CAB protein.

In their earlier attempt to follow \(^{14}\text{C}\)ALA incorporation into Chl, Bhaya and Castelfranco (4) did not find that Chl \(b\) was labeled. Our ability to label Chl \(b\) might be attributed to two methodological differences. We used nearly a threefold higher concentration of \(^{14}\text{C}\)ALA and modified the chloroplast isolation procedure to avoid washing the Percoll-centrifuged pellet. Notably, high activity of Pchlide and Chlhide synthesis has been observed with plastids isolated without the washing step \((8, 9, 11)\). In a preliminary experiment (data not shown), we found the wash step greatly decreased the Chl synthesis activity of the plastids. This observation is consistent with our result that preincubation greatly diminished the ability of the plastids to synthesize Chl \(a\) or \(b\) (Fig. 3). It is likely that one or more enzymes common to the synthesis of both chlorophylls are very labile and, therefore, any delay prior to assaying Chl synthesis significantly reduces incorporation. Since the amount of Chl \(a\) synthesized is greater than Chl \(b\) synthesized, it is conceivable that under suboptimal conditions, synthesis of only Chl \(a\) and not Chl \(b\) will be observed.

The specific radioactivity of Chl \(a\) was over 3 times greater than that of Chl \(b\) (Fig. 3). This difference could reflect the fact that rates of Chl \(b\) synthesis are underestimated due to either dilution effects or the relative instability of Chl \(b\) compared to Chl \(a\), or alternatively the rates of Chl \(b\) synthesis relative to Chl \(a\) synthesis are indeed lower in *organello* than in *vivo*. Dilution is the simplest explanation to account for this difference. If we assume that Chl \(b\) is made from Chl \(a\), as has been suggested \((3, 6)\), the radiolabel metabolized to Chl \(b\) would be diluted by endogenous unlabeled Chl \(a\). We estimate that approximately 0.5 to 1 nmol Chl was synthesized in isolated chloroplasts containing 30 nmol Chl \(a\). If none of this newly synthesized Chl was assembled, a maximum free Chl \(a\) pool of 1 to 2 nmol, or 2 to 4% of the endogenous pool, would be sufficient to dilute the specific radioactivity to the observed level. If the Chl \(a\) were rapidly assembled, an even smaller free Chl \(a\) pool would be required. Estimates of the free Chl pool suggest that it is less than 2 to 3% \((14)\) and possibly within the effective concentration.

Why might Chl \(b\) synthesis be lower in *organello* than in *vivo*? The fact that in *organello* rates of Chl \(b\) synthesis dropped at the same rate as Chl \(a\) synthesis indicates that a common reaction is labile and is not indicative that the Chl \(b\) reaction is particularly sensitive. Conceivably certain factors unique to Chl \(b\) synthesis may be limiting in the isolated plastid. The data of Maloney et al. \((13)\) suggest one possible factor is CAB apoprotein. They found that cycloheximide administered to greening Chlamydomonas cells greatly inhibited the synthesis of transitory Chl \(b\) species, but had little effect on Chl \(a\) species. They suggested that Chl \(b\) accumulated only to the extent that it could be accommodated in light harvesting complexes. While we have demonstrated that extra-plastid apoprotein synthesis is not required for Chl \(b\) synthesis, we have not ruled out the possibility that apoprotein enhances Chl \(b\) synthesis. Synthesis of the apoprotein may not be required because Chl can be assembled onto protein via exchange reactions \((4)\) or onto partially assembled CAB polypeptides that still have available Chl binding sites. There are a number of ways CAB apoprotein might enhance the apparent rate of Chl \(b\) synthesis. In general, Chl might be stabilized by assembly onto apoprotein. Because isolated chloroplasts are unable to synthesize CAB but are able to synthesize Chl \(a\) apoproteins, Chl \(a\) binding sites may be more numerous than Chl \(b\) binding sites and consequently in *organello* synthesized Chl \(b\) may turn over faster than Chl \(a\). Another possibility is that CAB apoprotein is a substrate in the conversion of Chl \(a\) to \(b\). This might occur if Chl \(a\) was
first assembled onto CAB apoprotein prior to conversion to Chl b.

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LITERATURE CITED


