Conversion of Chlorophyll b to Chlorophyll a via 7-Hydroxymethyl Chlorophyll*

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Chlorophyll b is synthesized from chlorophyll a by the oxidation of the methyl group on the ring B of the tetrapyrrole ring to the formyl group. Previously, we reported that chlorophyllide b could be converted to chlorophyll a in isolated cucumber etioplasts indicating the conversion of chlorophyll b to chlorophyll a. To identify the intermediate molecule, we used barley etioplasts instead of cucumber. Chlorophyll a and an additional pigment were found after incubation of chlorophyllide a with isolated barley etioplasts. The pigment has the same retention time and absorption spectrum as 7-hydroxymethyl chlorophyll, which has the hydroxymethyl group on ring B instead of the formyl group of chlorophyllide b. Authentic 7-hydroxymethyl chlorophyll was prepared by reduction of chlorophyll b by NaBH₄. Chlorophyll a accumulated during the incubation of 7-hydroxymethyl chlorophyllide with etioplasts. These findings indicate that chlorophyll b is converted to chlorophyll a via 7-hydroxymethyl chlorophyll. Chlorophyll b and 7-hydroxymethyl chlorophyll accumulated within a short period of incubation of chlorophyllide b with etioplasts. However, chlorophyll a accumulated with a concomitant decrease of chlorophyll b and 7-hydroxymethyl chlorophyll. These observations also suggest that chlorophyll b is converted to 7-hydroxymethyl chlorophyll and then to chlorophyll a. Both steps required ATP.

The earliest precursor for chlorophyll (Chl), heme, and bilin synthesis is 5-aminolevulinic acid in higher plants, and the Chl branch of the pathway begins with insertion of magnesium into the protoporphyrin. The biosynthetic pathway to Chl is fairly well understood. Although the activities of many enzymes involved in the pathway have been demonstrated in crude tissue extracts or intact organelles, very few have been purified and characterized (1). On the other hand, much progress in characterizing bacteriochlorophyll synthesis has been made especially in genetic analysis (2). A 46-kilobase region of the rhodobacter capsulatus contains most of the genetic loci involved in the magnesium branch of the bacteriochlorophyll biosynthetic pathway (3, 4). This region has been sequenced, revealing the existence of 23 open reading frames (5). Bullivar et al. (6) have undertaken a systematic directed mutational analysis of 12 open reading frames to evaluate the role in photopigment biosynthesis of individual open reading frames and identified some genes required for synthesis of bacteriochlorophyll. Chl and bacteriochlorophyll are produced from the same precursors by a pathway which is conserved until its later stages. Therefore, these findings with Rhodobacter are useful for the study of plant Chl synthesis.

Higher plants and green algae have Chl a as an accessory pigment of light-harvesting Chl a/b-protein complexes for photosystems I and II. Chl b differs from Chl a only by the presence of a 7-formyl group in place of a methyl on ring B of the tetrapyrrole ring. Chl b is believed to be synthesized from Chl a by the following observations. 1) Chl b is synthesized after Chl a accumulation (7). 2) Many Chl b-less mutants have been reported, but there are no mutants containing only Chl b (8). 3) Chl b was induced by calcium treatment of the tissues which have only Chl a (7). The methyl group on pyrrole ring B in Chl a has been reported to be oxidized to an aldehyde group by molecular oxygen and 7-hydroxymethyl chlorophyll (HMChl) was postulated as an intermediate molecule from Chl a to Chl b (9). However, conversion of Chl a to Chl b has not been demonstrated in isolated plastids, and the regulation and the mechanism of the reaction are not known.

On the other hand, conversion of Chl b to Chl a was not considered to occur, because reduction of a formyl group to a methyl group is a difficult reaction. However, Chl b to Chl a conversion has been suggested by in vivo experiments which showed the increase in Chl a with the concomitant decrease in Chl b in etiolated seedlings illuminated for a short period and then returned to darkness (10). Recently, we reported the accumulation of Chl a during the incubation of chlorophyllide (Chlide) b with cucumber etioplasts in the dark indicating the conversion of Chl b to Chl a (11, 12).

Chl b stabilizes Chl a/b-binding protein together with Chl a by making a folded structure, resulting in regulation of the accumulation of Chl a/b-binding protein (13, 14). Accumulation of Chl b must be controlled also in plants growing under different light conditions. Plants grown under low intensity light have a low Chl a/b ratio, and the Chl a/b ratio increases after transfer of these plants to a high intensity light condition (15).

As well as conversion of Chl a to Chl b, conversion of Chl b to Chl a would play an important role in adjusting the Chl a/b ratio. To determine the mechanism of the regulation of Chl b to Chl a conversion, the metabolic pathway from Chl b to Chl a must be elucidated. Previously, we could not find a candidate for the intermediate molecule from Chl b to Chl a on high performance liquid chromatography (HPLC) profiles (12). Herein, we found that HMChl was accumulated during the incubation of Chlide b with barley etioplasts and also showed that 7-hydroxymethyl chlorophyllide (HMChlide) was converted to Chl a.
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MATERIALS AND METHODS

Plant Materials—Barley seeds (Hordeum vulgare L. cv. Amaginio) were soaked for 4 h and then surface-sterilized with Antiformin (NaClO solution, 1% active Cl) for 20 min. The seeds were germinated on moist vermiculite in the dark for 6 days at 28 °C. Spinach leaves and gland chrysanthemum leaves for preparation of Chl b and chlorophyllase, respectively, were purchased from a local market.

Preparation of Chl b—Chl b was extracted with acetone from spinach leaves and precipitated as a Chl-dioxane complex by the addition of dioxane and water to the acetone extract. Carotenoid was removed from the precipitate by a DEAE-Sepharose CL-6B column (16). Chl was redisolved in 10 ml of methanol containing 10 mM NaBH₄ and incubated for 10 min at room temperature. After incubation, the reaction mixture was diluted with 10 ml of NaCl-saturated water and Chls were transferred into 5 ml of diethyl ether and dried. Chl b has a C-7 formyl group on ring B and C-13 keto group on ring E. Both of them are readily reduced to alcohol groups by NaBH₄ in methanol, but the formyl group is reduced more reduced compared to the keto group. HPLC analysis of the Chls revealed that very little Chl b remained and that most of Chl b was converted to HMChl after treatment with NaBH₄. A small amount of 7-hydroxymethyl 13-hydroxychlorophyll b and its epimer type was found after treatment. We used this preparation as HMChl for the assay of Chl a synthesis, because 7-hydroxymethyl 13-hydroxychlorophyll b type did not interfere with Chl a synthesis in isolated plastids. HMChl was prepared from HMChl by chlorophyllase as described for Chl preparation.

Preparation of HMChl and HMChl—HMChl was prepared by reducing Chl b with NaBH₄ (18). One μmol of Chl b purified by HPLC was dissolved in 10 ml of methanol containing 10 mM NaBH₄ and incubated for 10 min at room temperature. After incubation, the reaction mixture was diluted with 10 ml of NaCl-saturated water and Chls were transferred into 5 ml of diethyl ether and dried. Chl b has a C-7 formyl group on ring B and C-13 keto group on ring E. Both of them are readily reduced to alcohol groups by NaBH₄ in methanol, but the formyl group is reduced more reduced compared to the keto group. HPLC analysis of the Chls revealed that very little Chl b remained and that most of Chl b was converted to HMChl after treatment with NaBH₄. A small amount of 7-hydroxymethyl 13-hydroxychlorophyll b and its epimer type was found after treatment. We used this preparation as HMChl for the assay of Chl a synthesis, because 7-hydroxymethyl 13-hydroxychlorophyll b type did not interfere with Chl a synthesis in isolated plastids. HMChl was prepared from HMChl by chlorophyllase as described for Chl preparation.

Isolation of Etioplasts and Chloroplasts—Thirty g of 6-day-old etiolated, 12-h illuminated barley leaves were homogenized in a 200 ml isolation buffer containing 50 mM Heps-NaOH (pH 8.0), 500 mM sorbitol, 5 mM cysteine, 2 mM EDTA, and 0.2% bovine serum albumin. The homogenate was filtrated through a nylon mesh and centrifuged at 2,000 × g for 7 min. The pellet was suspended in 4 ml of a reaction buffer containing 50 mM Heps-KOH (pH 7.5), 500 mM sorbitol, 10 mM MgCl₂, 2 mM ATP, 0.2% bovine serum albumin (etiochloroplast or chloroplast suspension).

Incubation of Chl with Etioplasts—Chl b (100 pmol) or HMChl (100 pmol) were incubated with 100 μl of etioplast suspension in the presence of 60 nmol of phytyl phosphatase for 30 min at 28 °C. Phytyl phosphatase was prepared according to Joo et al. (19). All procedures were done under dim green light.

Incubation of Chl by HPLC—After incubation of Chl b or HMChl with etioplasts, 400 μl of acetone was added and the mixture was centrifuged at 5,000 × g for 3 min. Five hundred μl of hexane was added to the supernatant, and esterified Chls were transferred to the hexane phase. The hexane phase was dried under reduced pressure, and Chls were analyzed by HPLC on an octadecyl silica column (6 × 150 mm) with methanol as the elution agent at a flow rate of 1.5 ml/min at 40 °C. The profiles were monitored by absorbance at 450 nm. Chls were quantified from the chromatographic peak areas after calibration of the chromatographic response with known quantities of the relevant pigment.

Preparation of [14C]Chl b—[14C]Chl b was prepared from the cucumber cotyledons fed with 5-[14C]aminolevulinic acid as described previously (17).

Determination of Radioactivity of Chl—After incubation of 10,000 cpm of [14C]Chl b with 500 μl of chloroplast suspension for 30 min, Chls were analyzed by HPLC at room temperature and fractions were collected every 30 s. The radioactivity of each fraction was measured in a liquid scintillation counter.

RESULTS

In Vitro Conversion of Chl b to Chl a—One possible mechanism for the conversion of the formyl group of Chl b to the methyl group of Chl a is the oxidation of the formyl group to a carboxyl group followed by decarboxylation and methylation. To examine the possibility, Chl b was incubated with cucumber etioplasts in the presence of S-methyl-[14C]adenosylmethionine as a methyl group donor. Radioactivity was incorporated into protochlorophyll but not into Chl a in the dark (data not shown). Protochlorophyll has a methyl group which comes from S-adenosylmethionine. This indicates that transmethylation does not occur in the conversion of Chl b to Chl a. Therefore, we examined the next possible mechanism in which the formyl group is reduced to a methyl group via a hydroxymethyl group.

Previously, we could not detect HMChl after incubation of Chl b with cucumber etioplasts, although we observed an accumulation of Chl a and Chl b (12). If HMChl is an intermediate molecule from Chl b to Chl a, it should accumulate in the reaction mixture. In the present study, we used barley etioplasts and, as described below, observed HMChl accumulation. Unhydrogenated Chl (esterified with geranylgeraniol, dihydrogeranylgeraniol, tetrahydrogeranylgeraniol) accumulated as well as Chl esterified with phytol because Chl b was esterified with endogenous geranylgeraniol and the side chain was hydrogenated to a phytol group successively. These Chl derivatives interfered with the detection of the intermediate molecule from Chl b to Chl a. To reduce the accumulation of these Chl derivatives, Chl b was incubated in the presence of phytyl pyrophosphatase because Chl b was preferentially esterified with phytyl pyrophosphatase.

Fig. 1 shows the HPLC elution profile of Chls accumulated after incubation of Chl b with barley etioplasts in the presence of phytyl pyrophosphatase. Chl a and Chl b accumulated indicating that Chl b was converted to Chl a (Fig. 1, A and B). In addition to Chl a and Chl b, we found a new pigment with a retention time on HPLC different from those of unhydrogenated Chls (Fig. 1B, peak 1). We compared the retention time of the pigment with authentic HMChl to show that the pigment corresponding to peak 1 is HMChl. Both chemically synthesized HMChl and peak 1 have a retention time of 5.67 min (Fig. 1, B and C).

Peak 1 was collected, and its absorption spectrum in diethyl ether was determined (Fig. 2). The absorption spectrum was identical with that of authentic HMChl (Fig. 5). We concluded from these experiments that the pigment corresponding to peak 1 is HMChl.

If HMChl is an intermediate molecule from Chl b to Chl a, HMChl would be converted to Chl a in isolated etioplasts. To show whether HMChl can be converted to Chl a, HMChl was prepared from HMChl by chlorophyllase treatment and incubated with etioplasts. After incubation, HMChl and Chl a accumulated (Fig. 1D), indicating that HMChl was esterified and converted to Chl a. From these observations, we conclude that Chl b is converted to Chl a via HMChl.

Next we investigated whether Chl b is converted to Chl a by chloroplasts as well as etioplasts. [14C]Chl b was incubated with chloroplasts prepared from 12-h illuminated seed-
The radioactive isotope of chlorophyll b was incubated with isolated barley etioplasts for 30 min. The pigment was extracted and eluted from the plate, and the absorption spectrum of the pigment was determined in diethyl ether.

The fraction was dried and the pigments were separated by thin layer chromatography on a cellulose plate with hexane/2-propanol (20:1) to remove carotenoid which co-eluted with chlorophyll. The pigment was eluted from the plate, and the absorption spectrum of the pigment was determined by absorption at 663 nm.

To carry out the quantitative analysis, we determined the molar extinction coefficient of HMChl. Chl b was reduced to HMChl by NaBH₄ treatment, and HMChl was purified by HPLC. Purified HMChl, 355 (± 5) µg was dissolved in 30 ml of diethyl ether, and the absorbance at 655.5 nm was measured (0.795). The molar extinction coefficient of HMChl at 655.5 nm was determined from these values, and the molecular weight of HMChl (909.5). The molar extinction coefficient of HMChl in diethyl ether was 6.11 × 10⁴ (m⁻¹ cm⁻¹), and the amount of HMChl in diethyl ether was determined by the following equation:

\[ \text{HMChl (nmol/ml)} = 16.4A_{655.5} \]

Fig. 5 shows the absorption spectra of Chl a, Chl b, and HMChl in diethyl ether.

Time Course of Accumulation of Chls—Fig. 6 shows the time course of Chls accumulated during incubation of Chl b or HMChl with etioplasts. The first 20 min of incubation of Chl b with etioplasts, a large amount of Chl b was accumulated and the amount of Chl b reached a maximum at 20 min. After 20 min of incubation, Chl b gradually decreased. HMChl increased during the first 30 min and then gradually decreased. The level of Chl a was lower than that of HMChl during the first 20 min. However, it increased linearly during incubation. HMChl rapidly accumulated during the incubation of HMChl with etioplasts and reached a maximum at 20 min. The accumulation of Chl a was much greater when HMChl was used as a substrate instead of Chl b. These changes in Chl during incubation were consistent with the finding that HMChl is an intermediate molecule from Chl b to Chl a.

The Effect of ATP on the Conversion of Chl b to Chl a—Previously, we could not determine whether ATP is necessary for the reduction of Chl b to Chl a because phytyl pyrophosphate was not included in the reaction mixture. ATP was necessary for the phosphorylation of geranylgeraniol to geranylgeranyl pyrophosphate. Esterification of Chl b with geranylgeraniol did not proceed without exogenous ATP, because the endogenous ATP pool is very small in isolated etioplasts. In the pres-
ent experiments, we used phytol pyrophosphate because ester-
ification of Chlide could proceed without addition of ATP. When
Chlide was incubated without ATP, Chl was accumulated
with a small amount of HMChl and Chl (Table I). In the
reaction mixture containing ATP, HMChl and Chl increased
with a concomitant decrease in Chl. This indicates that con-
version of Chl to HMChl did not proceed without ATP. A
small amount of Chl accumulated during the incubation of
HMChl without ATP. A large amount of Chl accumulated
during the incubation of HMChl in the presence of ATP
indicating that conversion of HMChl to Chl also required
ATP.

**DISCUSSION**

We used Chlide instead of Chl as the precursor of Chl.
Chl is lipophilic due to the presence of a prenyl side chain and
does not dissolve in the incubation mixture, but Chlide is
soluble and can be incubated with intact plastids without de-
tergent (21). We also used etioplasts instead of chloroplasts
because etioplasts have no Chls, and a small amount of Chls
(below 1 pmol) which accumulated during incubation can be
detected by HPLC. Chl synthesis starts after onset of illumi-
nation in angiosperms, and some enzymes responsible for Chl
synthesis are induced by light treatment. However, our preli-
mary experiments showed that high conversion activity existed
in etiolated tissues and that the activity did not change during
greening (data not shown). It is reasonable for the plants
to have a high conversion activity before the accumulation of
Chl.

Chl and HMChl accumulated during the incubation of
Chlide with barley etioplasts. HMChl was also converted
to Chl by isolated etioplasts. From these observations, we
concluded that Chl was converted to Chl via HMChl. Pre-
viously, HMChl could not be detected by HPLC in the reaction
mixture after incubation of Chlide with cucumber etioplasts.

To examine whether cucumber has the same pathway from Chl
to Chl, HMChl was used as a substrate. Incubation of
HMChl with cucumber etioplasts resulted in the accumula-
tion of Chl indicating that Chl is converted to Chl via
HMChl in cucumber cotyledons. HMChl did not accumulate
probably due to the high activity of HMChl reductase in cu-
cumber etioplasts.

The values of the equilibrium constant of alcohol dehydroge-
nases are, in general, negative, and these enzymes do not
require ATP. However, conversion of Chl to HMChl occurred
only in the presence of ATP. These findings suggest that Chl
reductase required ATP for its activation. Activation of the
enzymes responsible for Chl synthesis was reported for mag-
nesium chelatase (22). Magnesium insertion, which is the first step unique to Chl synthesis, is a two-step reaction activated by ATP.

Chl b is considered to be synthesized from Chl a, but the mechanism of formation of the formyl group of Chl b is not known. Experiments using $^{18}$O$_2$ showed that molecular oxygen is the precursor of 7-formyl oxygen of Chl b in higher plants (23, 24), and HMChl was proposed as a hypothetical intermediate molecule from Chl a to Chl b (9, 25). However, we could not detect HMChl or Chl b by HPLC after incubation of Chloride a with etioplasts or chloroplasts (data not shown). This indicates that conversion from Chl a to HMChl does not occur in our system. We also incubated HMChl with etioplasts or chloroplasts under various conditions, but HMChl was preferentially converted to Chl a, and Chl b could not be detected. One possible explanation is that the value of the equilibrium constant of HMChl reductase (enzyme responsible for the conversion from HMChl to Chl b) is very negative similar to other alcohol dehydrogenases, and Chl b is not accumulated without removing Chl b from the reaction mixture by some mechanism. However, at present, we cannot exclude the possibility that another molecule is an intermediate from Chl a to Chl b.

Plants respond to various intensities of light under which they are grown by adjusting the composition and structure of their photosynthetic apparatus (15, 26). Growth under low intensity illumination induces an increase in the number of light-harvesting complexes associated with each photosystem to absorb enough light, whereas growth under high intensity light reduces the number of light-harvesting complexes and increases the number of core complexes. During adaptation to high light intensity, Chl b which is released from light-harvesting complexes would be converted to Chl a by the Chl cycle and used for the formation of core complexes of the photosystems. This hypothesis is supported by the observation that increases in Chl a and core complexes were accompanied by a decrease in Chl b and light-harvesting Chl a/b-protein complexes (15). The Chl cycle would also play an important role in constructing photosystems during greening. If more Chl b is synthesized than required, Chl b would be converted to Chl a and would bind to Chl a-protein complexes. Previously, we reported that light-harvesting Chl a/b-protein complexes act as a temporary pool of Chl when Chl synthesis is raised (27). Chl b which is pooled in light-harvesting Chl a/b-protein complexes would be converted to Chl a and used for the formation of other Chl-protein complexes. As described above, the Chl cycle enables flexible regulation of the accumulation of Chls and Chl protein complexes and plays an important role in the formation and reorganization of photosystems.

Reduction of the hydroxymethyl group to a methyl group is difficult due to the strong bond of carbon and the hydroxyl group. The mechanism of cleavage of the carbon-hydroxyl bond is well known for ribonucleotide reductase, which plays a central role in DNA biosynthesis catalyzing the reduction of ribonucleotides to their corresponding deoxyribonucleotides by replacing the C-2 hydroxyl group with a hydrogen atom (for review, see Ref. 28). This enzyme contains a free tyrosyl radical at its catalytic site. Hydroxuryrea inhibits the ribonucleotide reductase by quenching free radicals, but HMChl reductase was not inhibited by hydroxuryrea (data not shown). Furthermore, HMChl reductase requires ATP for synthesis of Chl a, but ribonucleotide reductase requires it only when the feedback inhibition of dATP is reversed. These observations indicate that HMChl reductase and ribonucleotide reductase replace the hydroxyl group with a hydrogen atom via different mechanisms. Analysis of HMChl reductase would be useful in elucidating the mechanisms of reduction of the hydroxy group as well as chloroplast biogenesis.

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REFERENCES

1. Seng, M. O. (1993) Photosynth. Biol. 57, 189–206
2. Bauer, C. B., Bollivar, D. W., and Suzuki, J. Y. (1993) J. Bacteriol. 175, 3919–3925
3. Yen, H.-C., and Marrs, B. L. (1982) J. Bacteriol. 162, 619–629
4. Marrs, B. L. (1981) J. Bacteriol. 146, 1003–1012
5. Zsebo, K. M., and Hearst, J. E. (1984) Cell 37, 937–947
6. Bollivar, D. W., Suzuki, J. Y., Beatty, J. T., Dobrovolski, J. M., and Bauer, C. E. (1994) J. Mol. Biol. 237, 562–640
7. Tanaka, A., and Tsuji, H. (1981) Plant Physiol. 68, 567–570
8. Chunaeu, A. S., Mirnaya, O. N., Maslov, V. G., and Bachehli, A. (1991) Photosynthetica 25, 291–301
9. Beale, S. I., and Weinsein, J. D. (1990) Biosynthesis of Heme and Chlorophylls (Dailey, H. A., ed) pp. 287–392, McGraw-Hill, New York
10. Kupke, D. W., and Huntington, J. L. (1963) Science 140, 49–51
11. Ito, H., Tanaka, Y., Tsuji, H., and Tanaka, A. (1993) Arch. Biochem. Biophys. 306, 148–151
12. Ito, H., Takaichi, S., Tsuji, H., and Tanaka, A. (1994) J. Biol. Chem. 269, 22034–22038
13. Bellemare, G., Bartlett, S. G., and Chua, N. H. (1982) J. Biol. Chem. 257, 7762–7767
14. Paulsen, H., Finkenzeller, B., and Kühlein, N. (1993) Eur. J. Biochem. 215, 809–816
15. Anderson, J. M. (1996) Annu. Rev. Plant Physiol. 37, 93–136
16. Omata, T., and Murata, N. (1980) Photoschem. Photobiol. 31, 183–185
17. Holdren, M. (1961) Biochem. J. 78, 359–364
18. Holt, A. S. (1959) Plant Physiol. 34, 310–314
19. Jo, C. N., Park, C. E., Kramer, J. K. G., and Kates, M. (1973) Can. J. Biochem. 51, 1527–1536
20. Shiozaki, T., and Sasa, T. (1983) Biochem. Biophys. Acta 756, 127–131
21. Rüdiger, W., Benz, J., and Guthoff, C. (1980) Eur. J. Biochem. 109, 193–200
22. Walker, C. J., and Steinberg, J. D. (1994) Biochem. J. 299, 277–284
23. Schneegurt, M. A., and Beale, S. J. (1992) Biochemistry 31, 11671–11683
24. Porra, R. J., Schäfer, W., Crie, E., Katheider, I., and Scheer, H. (1993) FEBS Lett. 323, 31–34
25. Porra, R. J., Schäfer, W., Crie, E., Katheider, I., and Scheer, H. (1994) Eur. J. Biochem. 219, 671–679
26. Tanaka, A., and Tsuji, H. (1983) Plant Cell Physiol. 24, 101–108
27. Tanaka, A., Tanaka, Y., and Tsuji, H. (1994) Planta 192, 92–97
28. Stubbe, J. (1990) Adv. Enzymol. Relat. Areas Mol. Biol. 63, 349–419