THE ASSOCIATION OF PROTEIN SYNTHESIS WITH PROTOCHLOROPHYLLIDE HOLOCHROME REGENERATION IN DARK-GROWN BARLEY LEAVES[1]

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SUMMARY: A light-stimulated increase in incorporation of radioactive amino acids into protein associated with protochlorophyllide holochrome occurs concomitantly with the regeneration of phototransformable protochlorophyllide in dark-grown barley leaves. This increase in radioactivity and the protochlorophyllide regeneration process are both abolished by incubation of the leaves with inhibitors of cytoplasmic protein synthesis. Prelimiary data implicate protein in the molecular weight range of 45,000-60,000 daltons in this process.

It is generally accepted that both cytoplasmic and chloroplast ribosomes are involved in the transition from etioplast to chloroplast (1,2), but relatively little is known concerning either the sequence in which the various membrane protein components are produced or the mechanism or mechanisms which control this coordination in higher plants.

The first discernible change which occurs in etiolated tissue on illumination is the reduction of protochlorophyllide to chlorophyllide. The reduction or phototransformation takes place only when the protochlorophyllide is complexed to a particular membrane-bound protein. The entire complex has been named protochlorophyllide holochrome (3). Protochlorophyllide holochrome

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has been extracted in an active form (i.e., capable of undergoing phototransformation in vitro) from several plant species (4,5,6,7).

Since this early phototransformation event might be involved in the triggering of the subsequent stages of chloroplast differentiation, information is needed concerning the fate of the holochrome complex after phototransformation. Several workers have reported that the chlorophyllide which is formed in the phototransformation reaction is bound to a protein of lower molecular weight than that of the holochrome protein (7,8,9). On the basis of these data, it was proposed that the holochrome complex undergoes dissociation after the phototransformation reaction. Other evidence, however, points to a "recycling" of at least a portion of the holochrome material (10,11).

It is possible that this chlorophyllide-binding subunit is generated de novo to reconstitute the holochrome complex; this would then constitute one of the initial events of protein synthesis associated with chloroplast biogenesis. Evidence for this possibility is provided by the data of other workers (12,13) which demonstrate a link between the phototransformation reaction and synthesis of a particular cytoplasmically produced polypeptide constituent of chloroplast membranes on the y-1 mutant of Chlamydomonas.

In this report we present evidence that the incubation of dark-grown barley leaves under conditions which bring about the synthesis of phototransformable protochlorophyllide holochrome also results in increased incorporation of radioactive amino acids into an acetone insoluble fraction. Exposure of the leaves to inhibitors of cytoplasmic protein synthesis results in a decrease in both this incorporation and in the synthesis of phototransformable protochlorophyllide holochrome.

METHODS: Plant Material: Barley seeds (Hordeum vulgare var. Numar) were germinated in vermiculite moistened with full strength Hoagland's solution in total darkness for 7 days.

Extraction of Protochlorophyllide and Chlorophyllide Holochrome: The following method was developed for extraction of photoactive protochlorophyllide holochrome from dark-grown barley leaves. The etiolated leaves were excised, minced with a sharp razor blade and frozen in a stainless steel con-

tainer surrounded by a jacket of dry ice-acetone. They were then transferred to a chilled mortar surrounded by dry ice and ground into a fine powder. The powder was transferred to a Waring blender and ground in 100% glycerol (2 ml glycerol/g fresh leaves) for 2 min. The homogenate was filtered through Miracloth and the filtrate was diluted 1:1 with 200 mM tricine pH 8.5, 20 mM DTT, 3% saponin[4]. It was then made 20% (w/v) with respect to ammonium sulfate and centrifuged at 12,000 x g for 60 min. The supernatant was removed, made up to 38% ammonium sulfate, and centrifuged as before. The resulting precipitate was resuspended in 100 mM tricine, pH 8.5, 10 mM DTT, 1.5% saponin, frozen to -18°C, thawed, and further centrifuged at 84,000 x g for 20 min.

Absorption spectra of the supernatant were measured using a Cary 14 spectrophotometer with a Model 1462 scattered-transmission accessory.

Illumination of Leaves, Exposure to Radioactivity and to Inhibitors: Excised leaves were placed with their cut ends in 1/5 strength Hoagland's solution with 30 μC of a (^{14}C)-amino acid mixture (New England Nuclear #445). The leaves were incubated for 60 min in darkness, exposed to 15 min of illumination from a bank of fluorescent lights to allow for phototransformation of pre-existing protochlorophyllide, and returned to darkness for 90 min to allow for regeneration of pigment. In cases where chloramphenicol, cycloheximide or emetine were used, the excised leaves were incubated with the inhibitors for 1 hr prior to exposure to radioactivity. Protein was determined by the method of Lowry (14). 200 μl aliquots of the various samples were counted in a Parkard Tri Carb liquid scintillation spectrometer, using Aquasol as a counting fluid.

RESULTS AND DISCUSSION: Extraction of phototransformable protochlorophyllide holochrome from dark-grown barley leaves - absorption spectra of glycerol extracts and resuspended ammonium sulfate precipitates: Absorption spectra of glycerol extracts obtained from dark-grown barley leaves are shown in Figures 1a and 1b. Figure 1a is the spectrum obtained from dark-grown leaves ("D" sample) unilluminated before extraction, while Figure 1b was obtained from leaves which had been briefly illuminated *in vivo* ("L" sample) and returned to darkness to allow for the regeneration of protochlorophyllide holochrome as described in "Methods". In the D sample, before illumination, peaks at 648 and 635 nm are apparent (Fig. 1a). After a 2 min *in vitro* exposure to light a peak at 672-3 nm appears, owing to the reduction of the protochlorophyllide to chlorophyllide. In the region of absorption of protochlorophyllide, the 635 nm peak disappears, while a peak at 628 nm persists. The latter peak is presumably due to non-phototransformable protochlorophyllide.

In the "L" sample (Fig. 1b), prior to illumination, the 648 nm peak is not

[4]Abbreviations: DTT, dithiothreitol; CAP, chloramphenicol; Eme, Emetine; SDS, sodium dodecyl sulfate

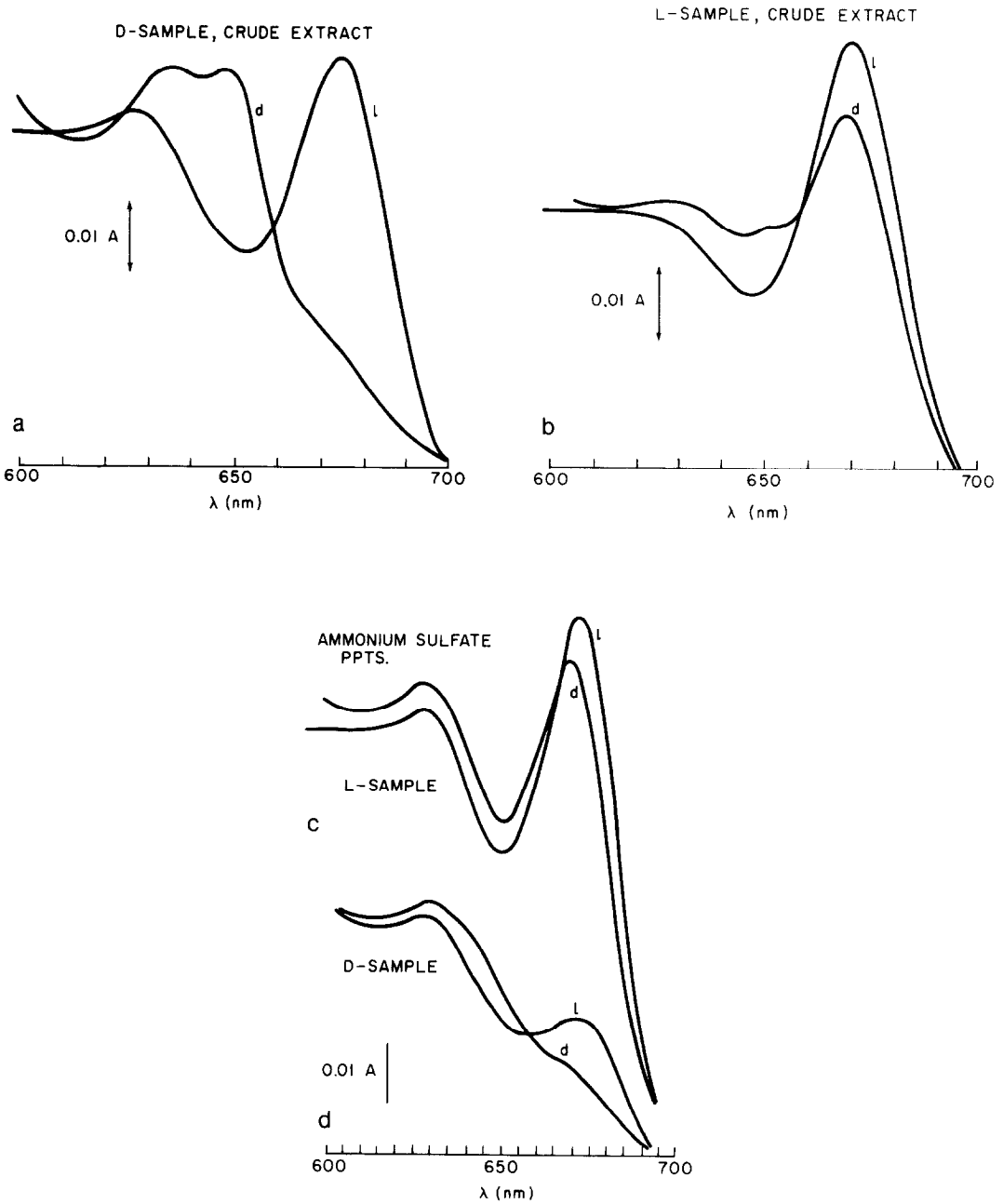

Figure 1a Absorption spectrum of "D" sample - crude extract. d - before illumination; 1 - after 2 min illumination.

Figure 1b Absorption spectrum of "L" sample - crude extract. d - before illumination; 1 - after 2 min illumination.

Figure 1c,d Absorption spectra of D and L samples - resuspended ammonium sulfate precipitates. d - before illumination; 1 - after 2 min illumination.

Table 1

Incorporation of radioactive amino acids into protein and pigment-containing fractions of extracts from dark-grown barley leaves

Experiment	Sample	ΔA_{670} on illumination	cpm/mg protein	total cpm: Acetone soluble	total cpm: Acetone insoluble
(1) Crude extract	D	0.017	428		
	L	0.0035	2,291		
(2) $(NH_4)_2SO_4$ ppt. resuspended	D	0.011	2,460	5,915	48,860
	L	0.009	6,000	10,955	136,535
(3) $(NH_4)_2SO_4$ ppt. resuspended	LOD	---	729		
	L90D	0.005	2,398		

present, and there is a larger absorption in the 630 nm range that at 650 nm. A similar absorption spectrum was obtained by Griffiths (15,16) from isolated etioplast preparations obtained from dark-grown, briefly illuminated barley leaves. A peak at 670 nm is due to chlorophyllide formed as a result of the 15 min *in vivo* illumination. After a subsequent *in vitro* illumination of the sample, there are decreases in absorption at both 650 nm and 630 nm, and a corresponding increase in 670 nm absorption due to newly formed chlorophyllide. The crude extracts of Figs. 1a and 1b were diluted with tricine-saponin buffer and fractionated with ammonium sulfate, as described in "Methods". The absorption spectra (before and after illumination) of the resuspended, clarified precipitates of the D and L samples are shown in Figs. 1c and 1d. Both samples have retained some phototransforming acitivity, as witnessed by the increase in absorption in the chlorophyllide region (670 nm) upon illumination.

The effect of illumination on incorporation of radioactive amino acids into extracts of dark-grown barley leaves: Table I shows the results of three separate experiments in which the incorporation of radioactive amino acids into extracts of D and L samples was measured. In experiments (1) and (2)

the leaves were exposed to light or dark incubation, as previously described. In experiment (3) all leaves were illuminated for 15 min; half were harvested immediately (L0D), while the other half were returned to darkness for a further 90 min (L90D) to allow time for the regeneration of protochlorophyllide holochrome. Approximately 2.5 times more ^{14}C was associated with the holochrome containing fraction in the L than in the D sample (experiments (1) and (2)). In addition, for both the L and D samples, approximately ten times more ^{14}C was incorporated into the acetone insoluble fraction of the extracts than was incorporated into the acetone soluble fraction (experiment (2)). This is evidence that little of the ^{14}C which was incorporated into the holochrome-containing fraction could have been in the pigment. Three times more ^{14}C was associated with the L90D than the L0D sample in experiment (3).

Effect of inhibitors on the light-stimulated incorporation of radioactive amino acids into extracts of dark-grown barley leaves and on regeneration of protochlorophyllide holochrome: The effect of different concentrations of cycloheximide on the regeneration of phototransformable protochlorophyllide holochrome in dark-grown barley leaves was investigated, and the results are shown in Fig. 2. Regeneration was increasingly inhibited, with only 23% of the control value being attained in leaves exposed to the highest cycloheximide concentration. Table 2 shows the results of another experiment in which the leaves were exposed to emetine at 50 μg/ml (also an inhibitor of cytoplasmic protein synthesis) and to chloramphenicol. The emetine treatment resulted in a complete abolishment of the regeneration of active protochlorophyllide holochrome and also in a decrease in specific activity of protein in the holochrome-containing preparation. Exposure to emetine plus chloramphenicol resulted in a further decrease in specific activity. SDS-polyacrylamide gel electrophoresis was carried out on D, L and L+ inhibitors. Preliminary results indicate an increase in radioactivity of protein in the molecular weight range of 45,000-60,000 daltons in L as compared with D and with L+ inhibitors (data not shown here).

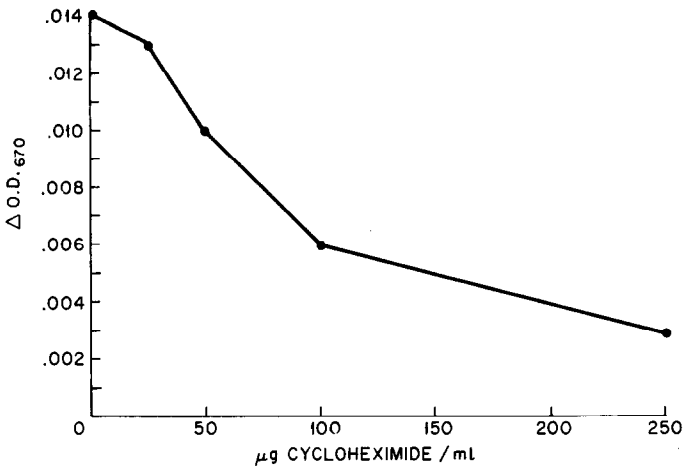

Figure 2 Effect of increasing cycloheximide concentration on the regeneration of phototransformable protochlorophyllide. Leaves were exposed to cycloheximide and to *in vivo* illumination as described in "Materials and Methods".

Table 2

Effect of inhibitors of protein synthesis on the regeneration of active protochlorophyllide holochrome

	ΔA_{670} on illumination	(cpm/mg protein) $(NH_4)_2SO_4$ ppt.
L	.005	3,000
L + Eme (50μg/ml)	.000	1,800
L + CAP (100μg/ml) + Eme	.000	1,300

From the results presented here, it appears that there is *de novo* protein synthesis associated with the regeneration of phototransformable protochlorophyllide holochrome. The proteins involved would seem to be synthesized on 80S ribosomes. Nadler and Granick (16) also reported an effect of cycloheximide treatment on chlorophyll biosynthesis. They interpreted this effect in terms of light-induced production of protein or proteins needed to bring about ALA synthesis. However, from the results reported here it seems that the re-

generation of protochlorophyllide holochrome itself may be linked to protein synthesis on 80S ribosomes.

It is possible that the effect described here is an expression of light-stimulated synthesis of a protein(s) analogous to the L protein of Eytan and Ohad (12), and the polypeptides b and c of Hoober and Stegemann (11). Further work is needed to determine how many components are involved and whether any of them are actual constituents of the holochrome complex itself.

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