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Synthetic abilities of *Euglena* chloroplasts in darkness

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Protein synthesis, normally a light-dependent process in isolated mature chloroplasts of *Euglena gracilis* var. *bacillaris* will take place in darkness if ATP and Mg^{2+} (ATP/Mg) are supplied. Either 5 or 10 mM ATP plus 15 mM $MgCl_2$ are optimal and rates equal to those in the light can be obtained. Since ATP and Mg^{2+} are not stoichiometrically related, and since the optimal Mg^{2+} concentration is similar to that which stabilizes chloroplast ribosomes in vitro, it is suggested that the chloroplast is freely permeable to Mg^{2+} under these conditions. Protein synthesis under these conditions is not inhibited appreciably by DCMU, FCCP, cycloheximide, or by the addition of ribonuclease, but is highly sensitive to chloramphenicol. Carbon dioxide fixation is also a light-dependent process in isolated mature chloroplasts from *Euglena*, but addition of ATP (5 mM) and fructose bisphosphate (5 mM) plus aldolase (1.0 unit/ml) (fructose-1,6-bisphosphate/aldolase) yields CO_2 fixation rates in darkness that are 43% of those normally obtained in the light. Mg^{2+} higher than 1.0 mM (e.g., 16 mM) is somewhat inhibitory. Chlorophyll synthesis from 5-aminolevulinate in 36 h developing chloroplasts from *Euglena* is also light-dependent, but addition of ATP/Mg and fructose-1,6-bisphosphate/aldolase in darkness brings about the accumulation of a compound having the same R_F on chromatography as protochlorophyllide from Barley; a subsequent brief illumination of the chloroplasts converts this compound to a compound with the R_F of chlorophyll. Thus *Euglena* chloroplasts supplied with appropriate additions can carry out protein synthesis, carbon dioxide fixation and most of chlorophyll synthesis in darkness. This versatility is appropriate in photosynthetic organelles isolated from photo-organotrophic cells.

Introduction

Although a great deal is known about the synthetic activities of chloroplasts in the light, less is known about these activities in darkness. Highly purified, intact active chloroplasts from *Euglena* have become available only recently [1,2] and a knowledge of their synthetic capabilities in light and darkness is gradually developing. Energy-requiring synthetic reactions in darkness are of particular interest since *Euglena,* unlike higher plants, is not an obligate phototroph, but is very versatile in using externally supplied substrates in light or darkness. In addition, this organism has a number of light responses which control chloroplast development and other processes in the absence of photosynthesis [3]. If conditions can be found to enable chloroplasts to carry out synthetic reactions related to chloroplast development in darkness, it

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone.

may be possible to study the effects of these low-light responses in the absence of the high-light intensities required for photosynthesis. In this paper we show that conditions can be found which enable highly purified intact *Euglena* chloroplasts to carry out carbon dioxide fixation, protein synthesis and a number of steps of chlorophyll biosynthesis in the absence of light. A brief abstract of this work has appeared previously [4].

Materials and Methods

Cells of *Euglena gracilis* Klebs var. *bacillaris* Cori were grown organotrophically in light or darkness in a medium limited in vitamin B_{12} [1]. Mature chloroplasts, used in protein synthesis and CO_2 fixation, were isolated from 4-day old light-grown cells; developing chloroplasts, used in Chl biosynthesis, were obtained from 3-day old dark-grown cells exposed to light for 36 h [2]. Chloroplast isolation was carried out as previously reported [1], except that the cells were disrupted at 3.45 MPa (500 lb/in^2) in a Yeda pressure cell with N_2, and no trypsin inhibitor was found to be necessary. After purification on Percoll gradients and centrifugation, the pellet of intact plastids was suspended in a solution containing 0.33 M sorbitol/50 mM Tricine-KOH (pH 7.8) (buffer A) for addition to incubation mixtures. Inspection of the plastid suspensions by light microscopy was done continously during the isolation procedure, and additional washings were performed if necessary, to eliminate contaminants such as broken cells and cell debri [2].

Plastid suspensions (30–100 μg Chl/ml) were incubated at 25°C in the dark or under 70 W/m^2 of red light from Sylvania spot lamps filtered by Rohm and Haas plexiglass (No 2444) transmitting above 650 nm. Protein synthesis was assayed in buffer A with 20–30 μCi of L-[^{35}S]methionine (over 800 Ci/mmol; N.E. Nuclear), and measured as cpm incorporated into washed, hot trichloroacetic acid-insoluble material [2,5]. $^{14}CO_2$ fixation was measured as acid stable radioactivity [2,6] in chloroplast suspensions incubated for 15 min in a solution containing 0.33 M mannitol/2.0 mM NaH_2PO_4/1.0 mM $MgCl_2$/7.0 mM $NaHCO_3$/40 mM Tricine-KOH (pH 7.8) and 5–10 μCi of $NaH^{14}CO_3$ (57.5 mCi/mmol; ICN). Chlorophyll biosynthesis was assayed by incubating chloroplasts with 4–10μCi/ml of 5-amino[4-^{14}C]levulinic acid (53.2 mCi/mmol; N.E. Nuclear) in buffer A, and measured as incorporation of ^{14}C-5-amino[4-^{14}C]levulinic acid into Chl *a* [2,4]; 5-amino[4-^{14}C]levulinic acid was previously shown to be specifically incorporated into the tetrapyrrole moiety of Chl *a* and derivatives [2]. In some cases, each of the assays described above was conducted in darkness in the presence of 5 mM Na_2-ATP (99–100%, Sigma), 15 mM $MgCl_2$, 1.0 unit of aldolase/ml, and/or 5 mM fructose-1,6-bisphosphate.

Total Chl content was measured in 80% (v/v) acetone according to Bruinsma [7].

Photochlorophyllide was extracted from 9-day-old, dark-grown seedlings of Barley. The top 5–10 cm of the seedlings were cut, frozen in liquid nitrogen, powdered in a mortar and extracted three times with ice-cold 80% (v/v) acetone. The combined extracts were mixed with one volume of anhydrous diethyl ether. The ether phase was washed with 1% (w/v) NaCl, dried with anhydrous Na_2SO_4, concentrated under nitrogen, and the pigments were then separated chromatographically in a system described by Seliskar [8].

Results and Discussion

Protein systhesis

Protein synthesis in *Euglena* chloroplasts is a light-dependent process [1,9]. Since protein synthesis requires ATP for amino acid activation and the formation of GTP [10], it seemed reasonable to begin by determining whether the addition of ATP to isolated *Euglena* chloroplasts affects protein synthesis in darkness. The addition of ATP produces a modest rate of protein synthesis in the dark which saturates at about 5 mM ATP (Fig. 1). Addition of Mg^{2+} together with ATP, however, greatly increases the rate of translation in the dark and brings it close to the rate achieved in chloroplasts in the light. Unlike studies with higher plant chloroplasts [11], the relationship between ATP and magnesium in producing this stimulation of protein synthesis in the dark is not stoichiometric, since 15 mM $MgCl_2$ is optimal at either 5 or 10 mM ATP (Fig. 1). As shown in Fig. 2, these two ATP concentrations are optimal for protein

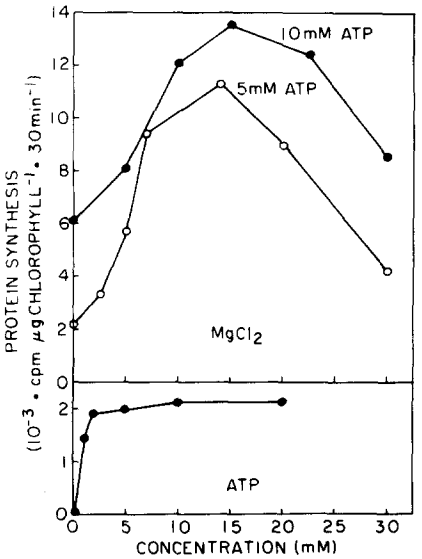

Fig. 1. The influence of ATP and Mg^{2+} on protein synthesis by *Euglena* chloroplasts in darkness. All incubation mixtures contained 0.33 M sorbitol/50 mM Tricine-KOH (pH 7.8)/30 µCi L-[^{35}S]methionine/chloroplasts containing 44 µg of Chl and ATP and/or $MgCl_2$ at the final concentrations shown in the figure. Incubation was at 25°C for 30 min in complete darkness. Lower part: protein synthesis at various ATP concentrations; upper part: protein synthesis at two concentrations of ATP as a function of Mg^{2+} concentration. The two curves are derived from separate experiments.

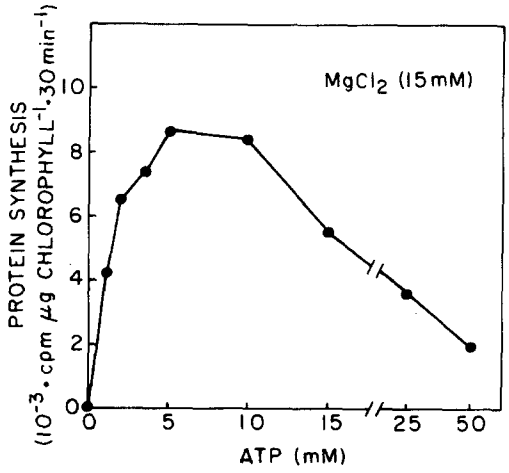

Fig. 2. The influence of ATP concentration on chloroplast protein synthesis in darkness in the presence of 15 mM $MgCl_2$. The incubation mixture contained 0.33 M sorbitol/15 mM $MgCl_2$/50 mM Tricine-KOH (pH 7.8)/20 µCi L-[^{35}S]methionine/chloroplasts containing 32 µg of Chl and ATP at the final concentrations shown in the figure. Incubation was at 25°C for 30 min in complete darkness.

synthesis in the presence of 15 mM $MgCl_2$. Magnesium added in the absence of ATP does not stimulate protein synthesis (Fig. 2), indicating that ATP is limiting in these chloroplasts in the dark. The lack of stoichiometry between Mg^{2+} and ATP suggests that the concentration of the Mg-ATP complex is not the important consideration. Indeed, 15 mM Mg^{2+} is very close to the optimal concentration for stabilizing ribosome preparations from *Euglena* chloroplasts [12]; this suggests that these chloroplasts are freely permeable to magnesium and that magnesium is required for the stability of internal plastid ribosomes, especially if a significant fraction of the internal Mg^{2+} is bound to the thylakoid membranes [13] under these conditions. Others have concluded that chloroplasts from higher plants are freely permeable to Mg^{2+} [14]. During illumination of higher plant chloroplasts Mg^{2+} is released into the stromal compartment from the thylakoid membranes in exchange for protons taken into the intrathylakoid space. The light-induced increase in stromal Mg^{2+} concentration may be involved in the activation of the enzymes of the Calvin cycle [13] and would also be available for other biosynthetic events such as protein and chlorophyll biosynthesis. In darkness, the situation is reversed and the Mg^{2+} would be predominantly bound to the inner and outer surfaces of the thylakoid membranes [13]. Under these conditions, the chloroplast protein synthesis machinery may be severely hampered by a decrease in the available magnesium. Assuming that these considerations are applicable to *Euglena* chloroplasts, a relatively high concentration of external Mg^{2+} would be required to satisfy the magnesium requirement for chloroplast protein synthesis in darkness.

The kinetics of protein synthesis in *Euglena* chloroplasts in light (without ATP plus magnesium) and darkness (with ATP plus magnesium) are quite similar (Fig. 3), and incorporation of methionine into protein continues for at least 30 min. Therefore, ATP and Mg^{2+} at optimal concentrations replace light effectively in these chloroplasts. The limitation beyond 30 min must lie elsewhere; for example, in decreasing organellar integrity, lack of transcripts to prime translation, etc.

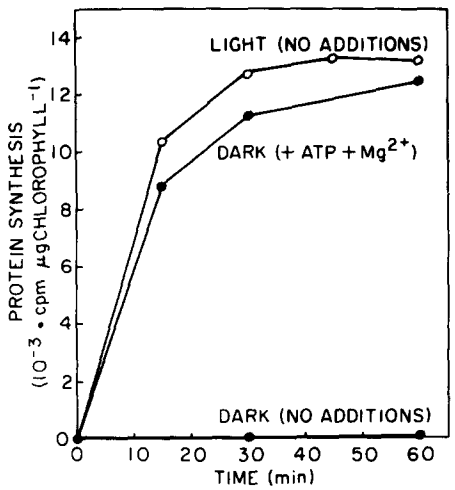

Fig. 3. Comparison of kinetics of protein synthesis in the light and in the dark with or without ATP and Mg^{2+}. The reaction mixture contained 0.33 M sorbitol/50 mM Tricine-KOH (pH 7.8)/30 µCi L-[^{35}S]methionine/chloroplasts containing 50 µg of Chl, and 5 mM ATP and 15 mM $MgCl_2$ where shown. The reaction mixtures were incubated in red light or darkness as indicated in the figure, at 25°C for various times.

The effect of various compounds on translation in *Euglena* chloroplasts incubated in the light without additions or in darkness with ATP plus Mg^{2+} is shown in Table I. EDTA (15 mM) inhibits protein synthesis in darkness as might be expected from the requirement of the system for Mg^{2+} (Figs. 1 and 2). FCCP, an uncoupler of phosphorylation, strongly inhibits protein synthesis in the light, as would be expected if light provided ATP from photosynthetic phosphorylation. For some unexplained reason FCCP also shows a small amount of inhibition in darkness; however, it might be affecting the stromal pH as indicated by Werdan et al. [15]. DCMU (1.0 µM) strongly inhibits protein synthesis in the light, but has no effect in darkness. In other experiments, 10 µM DCMU inhibited light-driven protein synthesis completely. Thus, added ATP (in the presence of magnesium) in darkness replaces ATP normally supplied by non-cyclic photophosphorylation in the light. (There is some evidence, however, that DCMU may indirectly cause inhibition of cyclic phosphorylation [16]). Cycloheximide, an inhibitor of translation on the 87 S cytoplasmic ribosomes of *Euglena* [17] has no effect on protein synthesis in either light or darkness as expected; lack of inhibition by ribonuclease in light or darkness similarly rules out the contribution of any adherent external RNA to protein synthesis under either set of conditions and testifies to the intactness of the chloroplasts. Inhibition by chloramphenicol in light or darkness verifies that protein synthesis takes place on internal 70 S-type ribosomes of the *Euglena* chloroplasts.

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON PROTEIN SYNTHESIS IN *EUGLENA* CHLOROPLASTS IN LIGHT OR DARKNESS

The incubation mixture contained 0.33 M sorbitol/20 µCi L-[^{35}S]methionine/chloroplasts containing 20 µg of Chl and 50 mM Tricine-KOH (pH 7.8). For the incubations in the dark, it also included 5 mM Na_2-ATP plus 15 mM $MgCl_2$.

Added to incubation mixture	Protein synthesis (% inhibition)	
	light	dark
Nothing [a]	0.0	0.0
EDTA (15 mM)	–	72.0
FCCP (1.0 µM) [b]	70.0	28.0
DCMU (1.0 µM) [b]	60.0	0.0
Cycloheximide (5.0 µg/ml)	0.0	0.0
D-threo chloramphenicol (80 µg/ml)	95.0	93.0
Ribonuclease A (25 µg/ml)	0.0	0.0

[a] The rates of protein synthesis in light and darkness, without additions, were 15394 and 16617 cpm/µg Chl per 30 min, respectively.

[b] DCMU and FCCP were added in ethanol; controls showed no effect of the solvent on chloroplast activity.

The results obtained for protein synthesis in *Euglena* chloroplasts in darkness incubated in optimal concentrations of Mg^{2+} and ATP indicate that this process is similar in all respects to chloroplast protein synthesis in the light and that light probably supplies ATP from non-cyclic photophosphorylation.

Carbon dioxide fixation

The requirements for carbon dioxide assimilation in darkness are shown in Table II. In the absence of additions, CO_2 fixation is negligible. The addition of Mg^{2+}/ATP and fructose-1,6-bisphosphate/aldolase is stimulatory and yields rates that are about 30% of those achieved in the light without additions. The dark rate is increased to 43% of the light rate if 15 mM $MgCl_2$ is omitted indicating that magnesium in excess of the 1.0 mM

TABLE II

EFFECT OF ATP, Mg^{2+}, FRUCTOSE BISPHOSPHATE AND ALDOLASE ON CO_2 FIXATION BY ISOLATED CHLOROPLASTS OF *EUGLENA* IN DARKNESS

ATP, $MgCl_2$, fructose-1,6-bisphosphate (FBP) and aldolase were added at final concentrations of 5 mM, 15 mM, 5 mM and 1.0 unit/ml, respectively. The activity of the chloroplasts in the light with no additions was 21.7 μmoles $^{14}CO_2$ fixed/mg Chl per h.

Additions (+) to intact chloroplasts				Carbon dioxide fixation (% of rate in light)
ATP	Mg^{2+}	FBP	aldolase	
–	–	–	–	0.0
+	+	+	+	30.0
+	–	+	+	43.0
–	+	+	+	11.0
–	–	+	+	14.0
+	+	–	+	4.0
+	+	+	–	1.0

normally present in the incubation mixture is inhibitory for CO_2 fixation. This concentration of Mg^{2+} (15 mM) is not completely unphysiological, however, since it provides optimal rates of translation in these chloroplasts in the dark (see above). While addition of ATP is stimulatory, fructose-1,6-bisphosphate/aldolase is most effective in stimulating dark fixation in the absence of added ATP. These results can be explained as follows. Exogenous ATP can be used for CO_2 fixation just as it is used for protein synthesis in darkness. The addition of fructose-1,6-bisphosphate/aldolase, however, yields triose phosphate which on entering the chloroplasts, provides additional ATP as well as reducing power in the form of NADPH by serving as a substrate for the chloroplast triose phosphate dehydrogenase(s). Fructose-1,6-bisphosphate itself does not appear to be metabolized by the chloroplasts, since it is inactive in the absence of aldolase (Table II). Thus, the combination of ATP and fructose-1,6-bisphosphate plus aldolase in darkness provides enough internal ATP and NADPH to replace at least 40% of the ATP and reducing power normally generated in the light. Since 10 μM DCMU inhibits CO_2 fixation completely in the light in these chloroplasts [9], what is replaced is the ATP and reducing power provided, probably, by non-cyclic electron flow. The same concentration of DCMU showed only a 10–20% inhibition of optimal CO_2 fixation in darkness in the present experiments. DBMIB (10 μM), an inhibitor of electron flow at plastoquinone [18], inhibited CO_2 fixation essentially completely in light and in darkness. The inhibition in light undoubtably results from blockage of non-cyclic electron flow at plastoquinone; the inhibition of dark CO_2 fixation in the presence of ATP and fructose-1,6-bisphosphate/aldolase is unexplained especially, since addition of oxaloacetate (1.0 or 5.0 mM) did not relieve the inhibition [15]. Carbon dioxide fixation in darkness is linear for at least 15 min and shows no lag (data not shown). Our previous studies indicated that CO_2 fixation by these chloroplasts in the light showed an initial lag of about 5 min which could be eliminated by the addition of fructose-1,6-bisphosphate/aldolase as in other systems [4,9,19].

A number of photosynthetic enzymes in higher plant chloroplasts requires activation by electrons from Photosystem I via ferredoxin and thioredoxin or other systems [23] to show good activity. If such systems in *Euglena* chloroplasts are similar, there may be alternative routes for mobilizing reducing power for enzyme activation in darkness. For example, the NADPH produced from triose phosphate via triose phosphate dehydrogenase in darkness might be used to reduce ferredoxin via ferredoxin-NADP reductase, a feasible but uphill route [24]. Since *Euglena* is known to contain NADPH-thioredoxin reductase [25], the NADPH formed from triose phosphate in darkness might be used for the reduction of thioredoxin if the reductase and the appropriate thioredoxin were accessible to each other.

Chlorophyll synthesis from 5-amino[4-^{14}C]levulinic acid

We have previously demonstrated a light-dependent incorporation of label from glutamate or 5-amino[4-^{14}C]levulinic acid into the tetrapyrrole portion of Chl *a* by isolated *Euglena* chloroplasts [2]. Label from [2-^{14}C]glycine was not incorporated, indicating that the synthesis occurs via the C-5 pathway of 5-aminolevulinic acid formation [2]. When these chloroplasts are incubated in darkness, there is little incorporation of 5-amino[4-^{14}C]levulinic acid into acetone-ether soluble com-

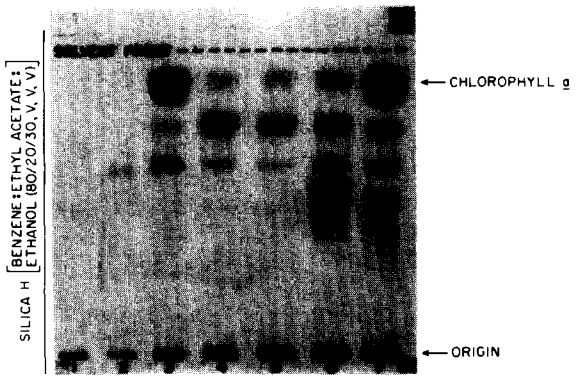

Fig. 4. Autoradiogram of a chromatographic separation of ether-soluble pigments from *Euglena* chloroplasts incubated with [4-^{14}C]5-amino[4-^{14}C]levulinic acid in darkness. The incubation mixtures contained 0.33 M sorbitol/50 mM Tricine-KOH (pH 7.8)/4.0 μCi 5-amino[4-^{14}C]levulinic acid and chloroplasts containing 80 μg of Chl. In addition, certain incubations contained 5 mM ATP, 15 mM $MgCl_2$, 5 mM fructose-1,6-bisphosphate or 1.0 unit/ml aldolase in various combinations as shown in the figure. Incubations were conducted in red light or darkness as indicated in the figure for 45 min at 25°C. At the end of the incubation, one half of each dark-incubated sample was illuminated for 2 min with red light (70 W/m^2). All samples were then extracted with 80% (v/v) acetone and the pigments were then extracted into diethyl ether. The ether extracts were used for thin layer chromatography on silica H [8] and the dried plate was exposed to Kodak SB-5 X-ray film.

pounds (Fig. 4), although, as we have previously shown, 5-amino[4-^{14}C]levulinic acid uptake by the plastids is light-independent [2]. A brief exposure to light (2 min) after the dark incubation has no effect (lane 2 in Fig. 4). In continuous light Chl *a* is formed as previously shown [2]. At least two other compounds are formed as well. Addition of ATP/Mg^{2+} allows the labeling of the faster moving of these two compounds more heavily in darkness (lane 4), but 2 min of light at the end of the dark incubation does not change the pattern significantly (lane 5). The addition of ATP/$MgCl_2$ plus fructose-1,6-bisphosphate/aldolase in darkness (lane 6) results in a similar pattern of labeling, but a new slower-running spot appears, which has the same R_F as authentic protochlorophyllide from etiolated barley chromatographed in the same system (data not shown). Illumination for 2 min at the end of the dark incubation results in the loss of most of the slow-moving material and an increase in the Chl *a* spot (lane 7). The small amount of radioactivity moving to the Chl *a* position in darkness (lanes 4, 5 and 6) can be attributed to a small amount of protochlorophyll which is known to be formed in *Euglena* [20] and is expected to move to the same position as Chl *a* in this chromatographic system [8]. Taken together, the evidence indicates that at least two unknown compounds and perhaps some protochlorophyll are formed from 5-amino[4-^{14}C]levulinic acid by *Euglena* chloroplasts in darkness with added ATP/Mg. On addition of fructose-1,6-bisphosphate/aldolase, however, a compound with the R_F of protochlorophyllide is formed as well. When ATP/Mg is deleted, fructose-1,6-bisphosphate/aldolase causes the accumulation of this material (data not shown), probably because the conversion of triose phosphates to phosphoglycerate within the chloroplasts results in the concomitant formation of ATP [15,19]. On brief illumination, the protochlorophyllide appears to be photoconverted to Chl as is well documented for *Euglena* cells [20,21]. The small amount of this slow-moving spot that remains after illumination indicates that some of the protochlorophyllide is non-photoconvertible as it is in whole cells [21], that chlorophyllide is formed as an intermediate, that small amounts of another compound also move to this position, or that the illumination was not sufficient to convert all the protochlorophyllide, although this last possibility is unlikely, since the protochlorophyll(ide) phototransformation in *Euglena* requires very little light.

That the formation of protochlorophyllide should require NADPH generated internally from the oxidation of triose phosphate produced from exogenously-added fructose-1,6-bisphosphate/aldolase is not unexpected. Several steps in the biosynthetic pathway of Chl from 5-aminolevulinic acid require reducing power in the form of NADPH including reduction of one of the vinyl groups of the tetrapyrrole nucleus, the protochlorophyll(ide) to chlorophyll(ide) phototransformation and the reduction of geranylgeraniol on the tetrapyrrole to phytol [22]. Perhaps the reason fructose-1,6-bisphosphate addition leads to more radioactivity in the Chl position in darkness (lane

6 compared with lanes 4 and 5) is that the formation of protochlorophyll may require reduction of a long side chain such as geranylgeraniol.

Acknowledgments

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