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Influence of Photosynthesis and Chlorophyll Synthesis on Polypeptide Accumulation in Greening *Euglena*[1]

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ABSTRACT

Two-dimensional gel electrophoresis resolves total cellular protein from *Euglena gracilis* klebs var *bacillaris* Cori into 640 polypeptides, 79 of which are induced by light exposure. The inhibition of chloroplast translation by streptomycin, the direct inhibition of photosynthesis as well as the indirect inhibition of chlorophyll synthesis by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and the specific inhibition of photosynthesis but not chlorophyll synthesis by DCMU in the presence of 17 millimolar ethanol failed to inhibit the accumulation of 40 polypeptides. These polypeptides appear to be synthesized on cytoplasmic ribosomes and their accumulation is independent of the developmental status of the chloroplast. Streptomycin but not DCMU completely inhibited the accumulation of six polypeptides which are undetectable in mutants lacking chloroplast DNA suggesting that these polypeptides are translated on chloroplast ribosomes. The accumulation of seven polypeptides which are detectable in mutants lacking chloroplast DNA was also inhibited by streptomycin but not by DCMU suggesting that the accumulation of these polypeptides is dependent upon stabilization by a chloroplast translation product. The accumulation of 12 polypeptides was inhibited by streptomycin and by DCMU under conditions in which chlorophyll synthesis was inhibited, but not under conditions in which chlorophyll synthesis was unaffected by DCMU. The inhibition by DCMU of the accumulation of these polypeptides appears to be due to the inhibition of chlorophyll synthesis suggesting that they are components of pigment protein complexes. The accumulation of six polypeptides was inhibited under all conditions in which photosynthesis was inhibited suggesting that the accumulation of these polypeptides is dependent upon a product of photosynthesis.

Chloroplast biogenesis can be operationally divided into two processes; the synthesis of macromolecules and the assembly of these macromolecules into supramolecular complexes which form the photosynthetic apparatus. In *Euglena*, as in most photosynthetic eukaryotes, light regulates the transcription of nuclear and chloroplast genes (6-8). In addition to being regulated at the transcriptional level, recent evidence suggests that, in *Euglena*, translational controls and posttranslational events may play a role in regulating the synthesis and accumulation of chloroplast localized proteins (9, 10, 16, 17, 22). In many organisms, the accumulation in contrast to the synthesis of a number of chloroplast localized proteins is in fact dependent upon their assembly into the supramolecular complexes which constitute the photosynthetic apparatus. Thus, in *Chlamydomonas*, the accumulation of the small subunit of RUBPCase[3] is dependent upon its assembly with the large subunit (27) and the accumulation of the Chl *a/b* binding protein in higher plants appears dependent upon Chl synthesis (1, 2). On the other hand, the assembly of the photosynthetic reaction centers in *Euglena* appears to be a stepwise process which utilizes pools of polypeptides which can be and possibly are synthesized at different times during development (10). This indicates that the accumulation of some components of multipolypeptide complexes is not linked to the assembly of these complexes.

The respective roles of cytoplasmic protein synthesis, chloroplast protein synthesis, photosynthesis, and Chl synthesis in the synthesis and assembly of the photosynthetic apparatus have been assessed in *Euglena* through the judicious use of specific inhibitors such as CHI, Sm, DCMU, and levulinic acid (reviewed in 30). By following the light-dependent increase in the activities of specific enzymes, it has been possible to show that the accumulation of cytoplasmically synthesized enzymes such as NADP glyceraldehyde-3-P dehydrogenase is independent of both photosynthetic CO_2 fixation and protein synthesis on chloroplast ribosomes (4, 14). Other cytoplasmically synthesized enzymes such as the microbody localized isozyme of glycolate dehydrogenase appear to be induced by a product of photosynthetic CO_2 fixation (14). Using two-dimensional gel electrophoresis, the relative levels of approximately 650 polypeptides have been followed during light-induced chloroplast development in *Euglena* (18, 19). Of these polypeptides, the relative amounts of 79 polypeptides increase and the amounts of 72 decrease as a result of light exposure (18). All of these changes are catabolite sensitive (19). Most but not all of the light-induced polypeptides are localized in the chloroplast, whereas many but not all of the peptides which decreased are mitochondrial (21). Studies with bleached mutants have allowed four of the polypeptides which increase as a result of light exposure to be tentatively identified as chloroplast translation products (18). In this paper, we identify, through the judicious use of inhibitors, subsets of polypeptides whose levels increase upon light exposure: those which are synthesized on chloroplast ribosomes, and those which are synthesized on cytoplasmic ribosomes but whose accumulation is dependent upon either chloroplast protein synthesis, photosynthesis, or Chl synthesis.

MATERIALS AND METHODS

Euglena gracilis klebs var *bacillaris* Cori maintained in our laboratory in the dark for many years was used throughout this work. Conditions for cell growth (13), the preparation of resting cells (13), light-induced chloroplast development (13), the treatment of cells with inhibitors of translation and electron transport (14), and the determination of Chl content have been described

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[3] Abbreviations: RUBPCase, ribulose bisphosphate carboxylase; CHI, cycloheximide; Sm, streptomycin.

(14). Total cellular protein was extracted with phenol and separated by two-dimensional gel electrophoresis. Gels to be compared were loaded with equal amounts of protein, stained with silver nitrate, and analyzed as described previously (18). Polypeptides whose relative amounts were altered in three independent experiments are reported. The figures represent a set of gels run and stained at the same time.

Polypeptides are referred to by an alphanumeric system consisting of a letter (A–D, A being the most acidic) corresponding to the isoelectric focusing sector of the gel in which the polypeptide is found and a number corresponding to the apparent mol wt of the polypeptide rounded to the nearest 1000. For proteins of the same mol wt, a decimal is added with the most acidic protein corresponding to 0.1. A composite map indicating the relative positions of all of the *Euglena* polypeptides resolved under our conditions has been published (17).

RESULTS

Light-Dependent Changes in Relative Polypeptide Levels in the Presence of Inhibitors of Chloroplast and Cytoplasmic Protein Synthesis. Two-dimensional gel electrophoresis reproducibly resolves total cellular protein extracted from dark-grown resting *Euglena* into 640 ± 45 polypeptides which are detectable by silver staining (18). Sm is a specific inhibitor of translation on *Euglena* chloroplast ribosomes (28). Of the 79 polypeptides whose relative amount increases as a result of light exposure (Fig. 1, a and b, □), the accumulation of 41 polypeptides was not inhibited by the addition of Sm 12 h prior to light exposure (Fig. 1c, □), a time ensuring maximum permeation of this inhibitor (3). These polypeptides are translated on cytoplasmic ribosomes and their accumulation is independent of chloroplast translation. Sm addition completely inhibited the accumulation of 20 polypeptides (Fig. 1c, △; Table I), three of which, polypeptides A17, B51, and D54, were previously identified as chloroplast gene products based on their presence in dark-grown resting cells and their absence from the bleached mutant W_3BUL (18) which has lost most if not all of its chloroplast genome (30). The accumulation of an additional 18 polypeptides (Fig. 1c, ◇; Table I) was only partially inhibited by Sm. A majority of the polypeptides whose accumulation was partially or fully inhibited by Sm are localized in the chloroplast (21) and detectable in mutants which have lost most if not all of their chloroplast DNA and chloroplast translational machinery (18). This suggests that they are synthesized on cytoplasmic ribosomes, but their accumulation and/or photoinduction is dependent on a product of chloroplast translation. Sm had no effect on the light-dependent decreases in the amounts of 72 polypeptides (these polypeptides are not indicated on Fig. 1). Many of these polypeptides are found in mitochondria (21). Polypeptide levels were unaltered in Sm-treated resting cells maintained in the dark suggesting that the degradation through turnover of proteins synthesized on chloroplast ribosomes is negligible in the dark. When CHI, a specific inhibitor of translation on cytoplasmic ribosomes (29), was added to dark-grown resting cells and the cells were maintained in the dark or exposed to light for 72 h to complete chloroplast development, there was no change in the relative amount of any of the 640 polypeptides resolved by two-dimensional gel electrophoresis (data not shown). The results reported in this paper indicate that at least six of the polypeptides whose levels were unaltered by light exposure in cells treated with CHI, polypeptides A51, A17, B105, B51, C47, and D54, are coded by chloroplast DNA and synthesized on chloroplast ribosomes (Table I). Cytoplasmic protein synthesis thus appears to be required either directly (nuclear coded cytoplasmically synthesized polypeptides) or indirectly for all of the light-dependent changes in polypeptide levels. This finding is not unexpected in light of the recent report of a rapidly turning over cytoplasmically synthesized protein which is required for chloroplast translation in *Euglena* (22).

Relationship between Photosynthesis, Chl Synthesis, and Light-Dependent Changes in the Relative Levels of Specific Polypeptides. Sm inhibits Chl synthesis and the development of the capacity to photosynthetically fix CO_2 (3). Photosynthesis is known to be required for the induction of at least one enzyme in *Euglena*, the microbody isozyme of glycolate dehydrogenase (14), while the synthesis of Chl is known to be required for the accumulation of the Chl binding proteins (1, 2). To distinguish these secondary effects of Sm on polypeptide accumulation from the direct action of this inhibitor on chloroplast translation, polypeptide accumulation was followed in cells exposed to light under conditions in which photosynthesis and Chl synthesis were inhibited as well as under conditions in which only photosynthesis was inhibited.

If dark-grown resting cells are suspended in fresh resting medium containing 10 μM DCMU and exposed to light, photosynthetic CO_2 fixation is completely inhibited and, after 72 h of light exposure, the cells contain 30% of the Chl found in untreated cells (14). The light-induced synthesis of NADP-glyceraldehyde-3-P dehydrogenase is not inhibited (14). Of the 79 polypeptides whose levels increase after light exposure, 52 polypeptides, including five polypeptides (A51, A17, B105, B51, C47) whose accumulation was completely inhibited by Sm, were found at the same level in untreated cells and in DCMU-treated cells (Fig. 1d; Table I). The light-dependent accumulation of 14 polypeptides including the large subunit of RUBPCase, polypeptide D54 (18), was partially inhibited (Fig. 1d, ◇; Table I), and the accumulation of an additional 13 polypeptides was fully inhibited by DCMU (Fig. 1d, △; Table I). Polypeptide levels were unaffected in resting cells maintained in the dark for 72 h with 10 μM DCMU. The light-dependent decrease in the levels of 72 polypeptides, many of which are found in mitochondria (21), was unaffected by DCMU (polypeptides not indicated on Fig. 1). The inhibition of the accumulation of some but not all light-induced polypeptides by DCMU indicates that this inhibition is a direct effect of the inhibition of photosynthesis and/or Chl synthesis rather than an indirect effect resulting from an inhibition of mitochondrial energy production and thus, a lack of an adequate supply of ATP for translation.

If DCMU is added to dark-grown resting cells at the time of light exposure and low levels of a utilizable carbon source are also added, CO_2 fixation is completely inhibited (24, 25). Chloroplast development is, however, relatively unaffected as evidenced by the amount of Chl synthesized (14, 24) and the rate of photosynthetic CO_2 fixation measured after the cells are washed free of DCMU (24). To distinguish an inhibition of polypeptide accumulation by DCMU which is due to an inhibition of Chl synthesis from an inhibition due to an inhibition of photosynthesis, polypeptide levels were determined in DCMU-treated cells supplemented with 17 mM ethanol at the time of light exposure. Light-dependent polypeptide accumulation, however, is catabolite sensitive (19). In contrast to 84 mM ethanol which inhibits Chl synthesis (12), the induction of a number of chloroplast-localized enzymes (12, 30), the induction of a peroxisomal enzyme (14), and all of the light-dependent changes in polypeptide levels detectable by two dimensional gel electrophoresis (19), the addition of 17 mM ethanol has little effect on Chl synthesis (14, 24), and the light-dependent accumulation of 57 polypeptides (Fig. 1e; Table I). The accumulation of only 22 polypeptides was partially inhibited by 17 mM ethanol (Fig. 1e; ◇) indicating that different genes show differing levels of sensitivity to catabolite repression. In contrast to the concentration dependence of catabolite repression, the polypeptides which are induced by 84 mM ethanol, A35, B57, A34.1, A34.2, C68, and D87 (19) were also fully induced by 17 mM ethanol (polypeptides not indicated on Fig. 1). The light-dependent accumulation of

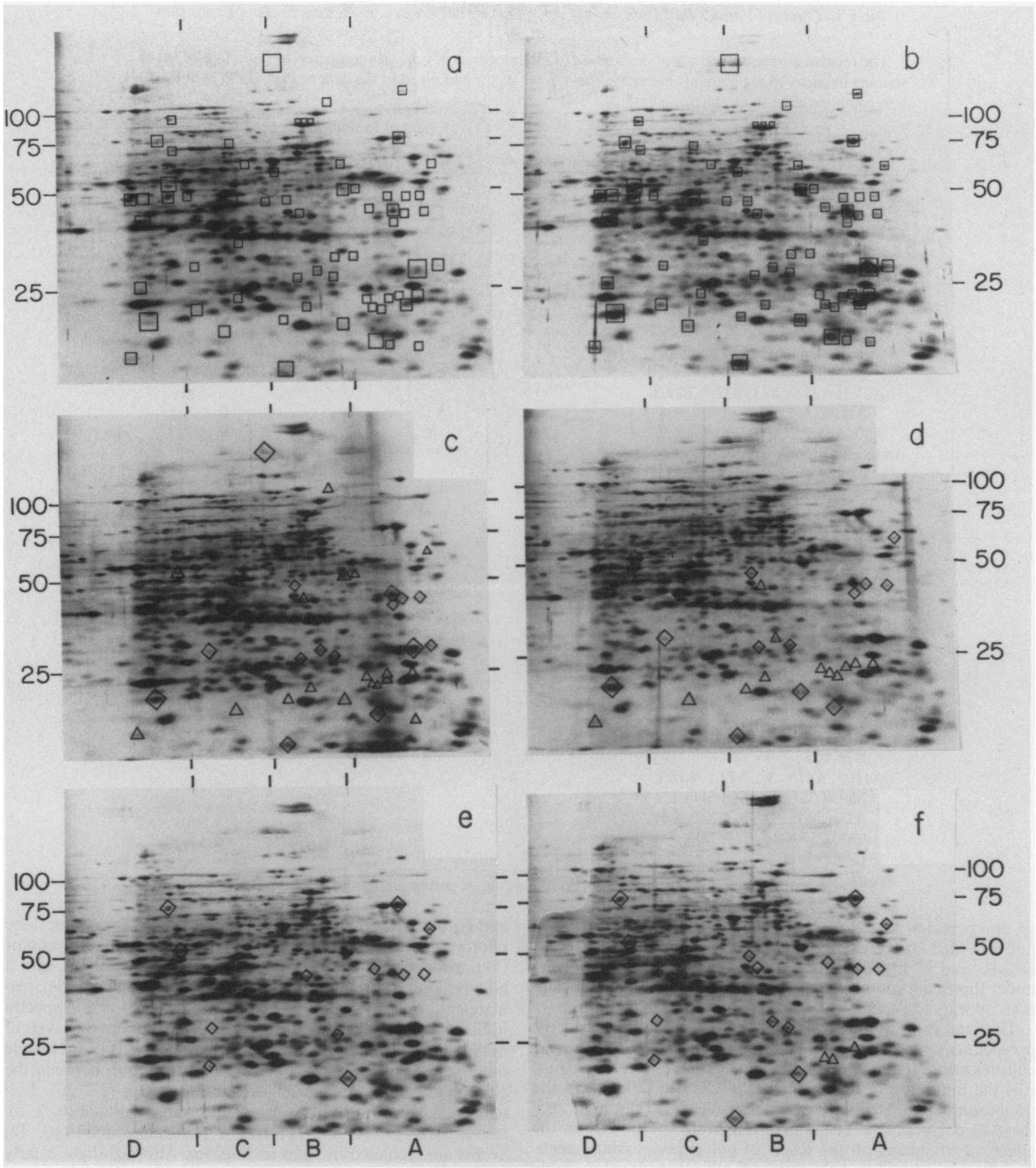

FIG. 1. Streptomycin, DCMU, and ethanol inhibition of the light-dependent changes in the polypeptide composition of *Euglena*. Silver-stained two-dimensional gels of 60 μg total cellular protein extracted from dark-grown resting *Euglena* maintained in the dark for 72 h (a), or exposed to light for 72 h with no additions (b), the addition of 0.05% streptomycin 12 h prior to light exposure (c), the addition of 10 μM DCMU (d), the addition of 17 mM ethanol (e), or the addition of 17 mM ethanol and 10 μM DCMU (f) at the time of light exposure. Polypeptides whose relative amounts increase upon light exposure are enclosed in squares. Polypeptides whose light-dependent increase is partially or fully inhibited are enclosed by diamonds or triangles, respectively. The letters on the bottom edge of the electrophoretograms indicate the sectors used to identify polypeptides by alphanumeric nomenclature. The numbers along the vertical edge indicate approximate mol wt ($\times 10^{-3}$).

Table I. *Effects of Various Inhibitors on Polypeptide Levels in Dark-Grown Resting Euglena Exposed to Light*

The relative amounts of polypeptides present in inhibitor-treated cells was compared by a visual analysis of staining intensity to the amounts present in dark-grown cells maintained in the dark or exposed to light for 72 h to complete chloroplast development.

Polypeptides and the Presumptive Control of Their Accumulation	Extent of Inhibition[a]			
	Streptomycin	DCMU	Ethanol	DCMU + Ethanol
Chloroplast translation products				
A51, A17, B105, B51, C47	++	0	0	0
D54 (LS RUBPcase)	++	+	+	+
Cytoplasmic translation products, accumulation dependent on chloroplast translation				
A43, A28.1, A28.2, B150, B145, C41, C29	+	0	0	0
Accumulation dependent on chlorophyll synthesis				
A24.1, A24.3, A23, B22.2, B22.3, B21, C19, D17	++	++	0	0
A40, A18.2, B27.2, D21	+	+	0	0
Induced by a product of photosynthetic CO_2 fixation				
A24.2, A22.2, A22.3	++	++	0	++
B44.1, B15	+	+	0	+
B29	+	++	0	+
Ambiguous				
A59, B20.1	++	+	+	+
B42	++	++	+	+
A42.1, A42.2, B27.1, C29	+	+	+	+
B22.1	0	+	0	0
Cytoplasmic translation products, accumulation independent of the developmental status of the chloroplast				
A76, A44, C22.2, D78.1-.6, D76.1-.5	0	0	+	+
A110, , A47.1-.3, , A22.1, A18.1, B89.1-.3, B62.1, B60, B44.2, B32.1, B32.2, C75, C72.2, C69, C63, C50, C49, C36, D93, D49.1-.3, D26	0	0	0	0

[a] The extent of inhibition is expressed as: 0, no inhibition; +, partial inhibition; ++, complete inhibition.

six polypeptides, A24.2, A22.2, A22.3, B44.1, B29, and B15, was not inhibited by 17 mM ethanol but was inhibited by 10 μM DCMU and 17 mM ethanol (Fig. 1f). As Chl levels are normal under these conditions, the accumulation of at least six polypeptides appears to depend upon photosynthetic CO_2 fixation.

The inhibition by 10 μM DCMU of the accumulation of 12 polypeptides was completely reversed when the DCMU-treated cultures were supplemented with 17 mM ethanol (Fig. 1f). Thus, it is the inhibition of Chl synthesis by DCMU rather than the inhibition of photosynthesis which prevents the accumulation of these 12 polypeptides. The conclusions from the studies on the effects of inhibitors on the levels of polypeptides whose accumulation is induced by light exposure are summarized in Table I.

DISCUSSION

The *Euglena* chloroplast is a highly organized structure which contains many supramolecular complexes such as the photosynthetic reaction centers. The assembly of these complexes require stoichiometric amounts of specific polypeptides, some of which are coded by nuclear DNA and synthesized on cytoplasmic ribosomes and some of which are coded by chloroplast DNA and synthesized on chloroplast ribosomes (10, 30). At least three photoreceptors, a blue-absorbing nonchloroplast photoreceptor (11), a red-absorbing non-chloroplast photoreceptor (5), and a blue-red-absorbing chloroplast photoreceptor (11), Pchl(ide), regulate chloroplast biogenesis in *Euglena*. Detailed action spectra indicate that the chloroplast photoreceptor regulates the levels of both chloroplast and nuclear coded gene products (7, 11) while other studies have shown a synergistic interaction between the chloroplast and non-chloroplast photoreceptors (23, 26). Evidence has accumulated to suggest that transcriptional (6, 7, 8), translational (9, 16, 17, 22) and/or posttranslational (10, 22) events are regulated by light in *Euglena*. Although light clearly regulates the abundance of nuclear and chloroplast gene transcripts (6, 8), changes in the abundant *in vivo* translation products (20) do not correlate with changes in the abundant *in vitro* translation products (16). In some cases, stoichiometric amounts of specific polypeptides found within the chloroplast results from posttranslational events such as the instability of an unassembled polypeptide, as seen for the small subunit of RUBPCase (27), rather than from a direct coupling between the level of transcription of nuclear and chloroplast genes coding for the polypeptides comprising a specific chloroplast component. Through the judi-

cious use of inhibitors, it has been possible to group the light-induced polypeptides resolved by two-dimensional gel electrophoresis into specific response classes which represent a number of translational and posttranslational interactions.

The accumulation of five polypeptides, A51, A17, B105, B51, and C47, was completely inhibited by Sm. The accumulation of these polypeptides was unaffected by DCMU under conditions in which Chl synthesis was inhibited by 70% (14) and photosynthesis was fully inhibited (24, 25), indicating that the accumulation of these polypeptides is not linked to photosynthetic electron transport or the accumulation of Chl. Based on our inability to detect two of these polypeptides, A17 and B51, in the bleached mutant W_3BUL (18), we tentatively concluded that these polypeptides were chloroplast gene products; a conclusion supported by the present study. The remaining three polypeptides, A51, C47, and B105, which appear to be translated on chloroplast ribosomes, are undetectable in dark-grown wild type cells. Since their basal level is below the limits of detection by silver staining (18), their absence from W_3BUL can not be used to provide evidence confirming their identification as chloroplast translated and coded polypeptides. The partial inhibition by Sm but not by DCMU of the accumulation of seven other polypeptides is probably not a direct result of the inhibition of chloroplast translation and does not represent a secondary effect arising from an inhibition of Chl synthesis or CO_2 fixation. This inhibition is probably an example of the failure of cytoplasmically synthesized proteins to accumulate in the absence of the chloroplast translation products which are required for the formation of a functional, stable, supramolecular complex. In the absence of assembly, these polypeptides are rapidly degraded.

Polypeptide D54 is the large subunit of RUBPCase (18), a polypeptide whose gene has been sequenced and localized on the restriction map of *Euglena* chloroplast DNA (15). As expected, the accumulation of polypeptide D54 is fully inhibited by Sm but unexpectedly, its accumulation is partially inhibited by DCMU. Polypeptides A59 and B20.1 show a similar sensitivity to these inhibitors. The specific activity of RUBPCase is reduced in dark-grown resting cells exposed to light in the presence of DCMU (25). Upon removal of DCMU, there is a rapid increase in RUBPCase activity which is found even when translation on chloroplast or cytoplasmic ribosomes is inhibited (25). Associated with the increase in enzyme specific activity is the formation of the pyrenoid, the structure within the chloroplast whose major structural component is thought to be RUBPCase (25). Based on these findings, it has been proposed that, as a result of photosynthesis, aggregates of RUBPCase are formed giving rise to the pyrenoid and an increase in enzyme specific activity (25). The partial inhibition of the accumulation of polypeptide D54 and possibly polypeptides A59 and B20.1 by DCMU therefore reflects the posttranslational role which photosynthesis may play in determining the conformation and stability of chloroplast components.

In dark-grown resting cells exposed to light, the carbon and energy required for the early stages of Chl synthesis are derived from the breakdown of the storage cabohydrate paramylum (29) while photosynthetic CO_2 fixation appears to provide the carbon and energy required for the latter stages of Chl synthesis. Thus, Chl synthesis is inhibited by 70% in cells exposed to DCMU at the time of light exposure (14), while in DCMU-treated cells provided with a supplemental carbon source, there is no inhibition of Chl synthesis (14, 24). The accumulation of 12 polypeptides was inhibited in both Sm-treated cells and DCMU-treated cells, but not in DCMU-treated cells supplemented with 17 mM ethanol. The accumulation of these polypeptides thus appears to be linked to Chl accumulation as reported for the accumulation of the Chl *a/b* binding proteins (1, 2). In the absence of Chl synthesis, these polypeptides are probably synthesized and then degraded within the chloroplast. The inhibition of the accumulation of these polypeptides by Sm appears to be an indirect consequence of the Sm inhibition of Chl synthesis.

The accumulation of six polypeptides was inhibited by Sm under all conditions in which photosynthesis was inhibited. As previously found for the induction of the microbody-localized isozyme of glycolate dehydrogenase (14), these polypeptides are probably induced by a product of photosynthesis rather than directly by light exposure. Sm inhibits their accumulation by inhibiting the development of the capacity for photosynthetic CO_2 fixation.

Of the 79 polypeptides whose levels increase after light exposure, the accumulation of 41 polypeptides was unaffected by Sm, a specific inhibitor of translation on chloroplast ribosomes (28). In the bleached mutant W_3BUL, which appears to have lost most if not all of its chloroplast DNA (30), these 41 polypeptides are found at levels comparable to their levels in wild type dark-grown resting cells (18) confirming their synthesis on cytoplasmic ribosomes from information contained in nuclear DNA. Light exposure, however, has little effect on the accumulation of most of these polypeptides in W_3BUL (18). In the limited number of cases where light induces the accumulation of a polypeptide in W_3BUL, the amount of polypeptide accumulated by W_3BUL is always less than in wild type cells (18). Since the accumulation of these polypeptides in wild type cells is independent of chloroplast translation and the developmental status of the chloroplast, the induction of these polypeptides is probably controlled, at least in part, by light acting through the chloroplast localized photoreceptor, Pchl(ide), which is not found in the bleached mutant W_3BUL (30). Thus, the role of the chloroplast genome in the photoinduction of these polypeptides appears to be an indirect one, it provides some of the information required for the synthesis of one of the sensory systems controlling induction.

Taken together, studies of mRNA abundance, studies of polypeptide synthesis, and studies of polypeptide accumulation indicate that light controls the polypeptide composition of *Euglena* by regulating transcriptional, translational, and posttranslational events. Transcriptional and possibly translational control provide an on/off switch which controls the maximum levels of polypeptides available for chloroplast biogenesis regardless of light intensity and photosynthetic rate. Posttranslational controls may provide a way to modulate the actual levels of chloroplast constituents in order to adapt to changes in light intensity and CO_2 availability as well as in order to coordinate accumulation of components of supramolecular complexes which are coded by different genomes and synthesized in different cellular compartments.

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