Organelle Formation in the Presence of a Protease Inhibitor

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SUMMARY: Light-dependent chlorophyll formation and growth of Euglena is blocked by the protease inhibitor phenylmethane sulfonyl fluoride (PMSF). Inhibition of chlorophyll formation can be completely relieved by addition of ammonium phosphate, and to a limited extent by some amino acids. Growth is not resumed upon addition of nitrogen sources. PMSF inhibits protease activity from Euglena extracts. It is proposed that light stimulates a protease dependent break-down of protein to supply nitrogen for organelle biosyntheses.

Introduction

The formation of cellular organelles is characterized by dramatic changes in form and size as well as extensive syntheses of organelle specific proteins, pigments, and other constituents (7, 9, 12). While progress has been made in understanding the biosynthetic machinery involved in the synthesis of organelle components such as proteins (7), the regulatory features of such synthesis remain unclear. One aspect of regulation includes extensive cooperation between organelle and cell in providing a supply of both nutrients and informational macromolecules (4,6,8). Such cooperation would be important when a cell, such as Euglena gracilis, is induced to form chloroplasts while in a medium devoid of sufficient nutrients to support growth. This paper is concerned with the question of how nitrogen-containing compounds such as amino acids are made available by the cell for organelle biosyntheses.

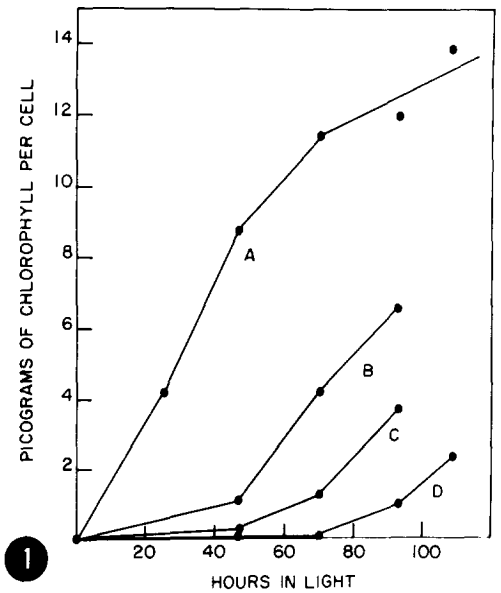
One means of supplying amino acids could include light stimulation of a

protease dependent degradation of cellular protein during chloroplast development. In this connection, it is known that phenylmethane sulfonyl fluoride (PMSF) is a specific inhibitor of serine type proteases *in vitro* (2) and the compound can block protein degradation of a procaryote, *E. coli* (5). We decided to take advantage of the ability of *Euglena* to survive treatment with the compound and examine the formation of chloroplasts by this eucaryote in the presence of PMSF.

Methods: Methods for the growth and maintenance of *Euglena* under non-growing conditions, as well as conditions for light-induced chloroplast development and extraction of chlorophyll have been described elsewhere (11). Stock solutions of PMSF were freshly prepared in 95% ethanol, and sufficient ethanol was added to control cultures to a concentration not exceeding 0.08%.

RESULTS AND DISCUSSION: Figure 1 shows the effects of various concentrations of PMSF on chlorophyll formation by dark-grown, resting *Euglena* incubated in the light. Chlorophyll formation, a good index of chloroplast formation, is inhibited for 70 hours by 40 ug/ml of PMSF, while control cells complete chlorophyll formation by 70 hours. PMSF treated cells recover, however, to form expected levels of chlorophyll after a total of 8-9 days of further illumination.

Figure 2 shows the results of an attempt to increase the extent of PMSF inhibition by incubating *Euglena* with PMSF in darkness, prior to chloroplast development. In the case described here, cells were incubated in darkness for 16 hours with PMSF. The cells were then exposed to light. The data obtained indicates that pre-incubation in darkness with PMSF reduced inhibition. The extent of inhibition using 30 ug/ml under these conditions is roughly equivalent to using about 20 ug/ml without pre-incubation. Thus, dark-grown *Euglena* may detoxify the PMSF. The possibility that PMSF is destroyed by incubating with the medium was checked. Dark grown resting cells were removed from the medium by centrifugation; PMSF was added to the medium and incubation allowed to proceed in darkness for 16 hours. Dark-grown resting cells were added to the PMSF containing medium and the culture was exposed to light. The extent of inhibition obtained with 30 ug/ml was not diminished.



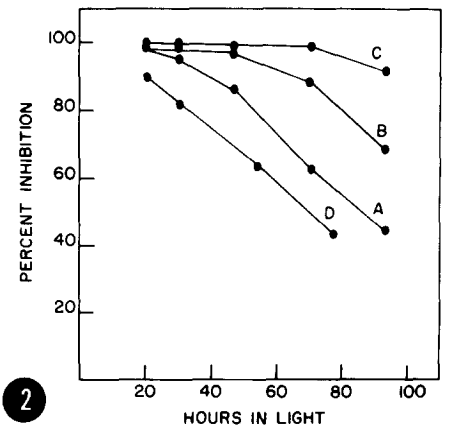


Figure 1. Effect of various concentrations of PMSF on chlorophyll formation by Euglena. Curve A) chlorophyll content of cells without PMSF; B) chlorophyll content with 20 ug/ml PMSF; C) 30 ug/ml PMSF; and D) 40 ug/ml PMSF. These data represent average values from 5 separate experiments.

Figure 2. Extent of inhibition of chlorophyll formation by cells incubated with PMSF before and during illumination. A) cells with 20 ug/ml PMSF; B) cells with 30 ug/ml PMSF; C) cells with 40 ug/ml PMSF; D) cells with 30 ug/ml PMSF which had been incubated in the dark for 16 hours before illumination. These data represent average values of 5 separate experiments.

If PMSF is affecting proteases, thus eliminating degradation of proteins as a nitrogen source, addition of an ammonium nitrogen source would be expected to relieve the effects of PMSF. Since Euglena contains ample supplies of carbon containing compounds, including polysaccharide, it is possible to test such a reversal. Figure 3 shows the extent of inhibition of chlorophyll formation by dark-grown resting cells incubated in light with PMSF, and by cells incubated with PMSF and ammonium phosphate. It is evident that cells treated with both PMSF and ammonium phosphate are able to form chlorophyll much more readily than cells given PMSF only. Phenylalanine, glycine, methionine and glutamic acid each have been tested at 1×10^{-3}M as reversal agents but none is as effective in restoring chlorophyll formation as ammonium phosphate. This difference may be due to the inability of Euglena to transport amino acids in sufficient amounts.

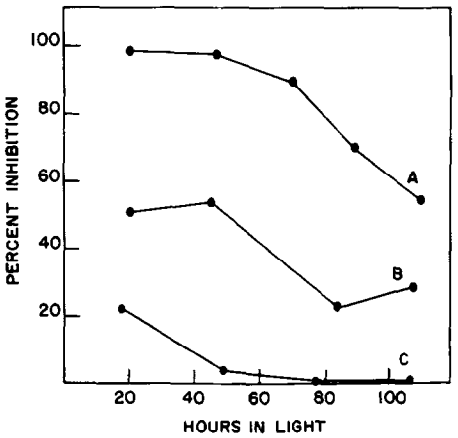


Figure 3. Extent of inhibition of chlorophyll formation by cells treated with PMSF and ammonium phosphate. Dark-grown resting cells were exposed to light in the presence of PMSF (30 ug/ml), Curve A; PMSF (30 ug/ml) and ammonium phosphate (1×10^{-4} M), Curve B; PMSF (30 ug/ml) and ammonium phosphate (1×10^{-3} M), Curve C; dark-grown resting cells which were exposed to light in the presence of ammonium phosphate (1×10^{-3} M) served as a control for this experiment. These data represent average values from 6 separate experiments.

The effect of PMSF on development can be compared with the effects of PMSF on cell division in Euglena. Addition of 30 ug/ml of PMSF to a growing culture blocks further cell division. Since the growth medium contains two nitrogen sources, glutamic acid and ammonium phosphate, it is evident that illumination results in the participation of an ammonium nitrogen source which can support chloroplast development but is not crucial to cell division. The source is probably specific in support of chloroplast development since cells containing fully developed chloroplasts also fail to grow in the presence of PMSF. It should be pointed out that when growing cells are treated with PMSF, diluted, and then plated onto nutrient agar, colony formation readily occurs in the dark or light without diminution of efficiency. In addition, green colony formation in the light is not blocked in these cells.

In view of the in vitro inhibition of protease activity by PMSF, we tested the effect of PMSF on protease activity from Euglena extracts. The data of Table 1 show that protease activity in extracts of Euglena can be inhibited by PMSF. This finding, along with the observation that protease activity cannot be detected

Table 1. Protease Activity in Untreated and PMSF-treated Extracts of Euglena.

Experiment	Nanomoles tyrosine equivalents released per 10^6 cells per hour. Activity in extracts	Activity in extract treated with PMSF (1.9×10^{-4} M)
1	1.5	0.9
2	1.7	0.9
3	1.4	0.7

Extracts were prepared from resting cells exposed to light for 24 hours. Harvested cells were frozen and thawed at 4°C with 0.1M glycine-HCl buffer pH 3.5, and broken by passage through a French pressure cell at 3000 lbs. per square inch. The broken cell homogenate was centrifuged for 10 minutes at 10,000 RPM, and the supernatant retained for protease assay with denatured hemoglobin according to the method of Anson (1) using the phenol reagent (3).
Extracts were treated with PMSF as follows: The extract was dialyzed overnight against 500 volumes of 0.01 M imidazole buffer, pH 6.8. One portion of the dialyzed enzyme was incubated with 1.9×10^{-4} M PMSF for one hour while another portion was incubated with ethanol only for one hour at 37°C. Each portion was then assayed for protease activity. About 10% of the activity was lost during dialysis.

in extracts prepared from PMSF-treated Euglena cells, strongly suggest proteases are among the sites of action of PMSF.

Given the presumed specificity of PMSF for serine type proteases and the finding that ammonium nitrogen can relieve much of the inhibition, a connection between protease activity and chlorophyll formation seems likely. It is also evident from the reduced PMSF inhibition by pre-incubation in the dark and the inhibition of growth that PMSF may have other effects in addition to interfering with nitrogen supply. The results obtained with Euglena differ from those obtained with the procaryote, E. coli in that bacterial growth is not markedly affected by PMSF (5). Protease inhibitors are known to block growth of trans-

formed animal cells while non-transformed cells are not affected (10). In the case of Euglena, dark grown resting cells utilize available endogenous carbon and nitrogen sources for the formation of chloroplasts. Such chloroplasts would permit autotrophic growth and thus the capacity of Euglena to employ its cellular material for the formation of chloroplasts has a distinct adaptive significance. Part of the capacity of resting Euglena to respond to light may involve the mobilization of cellular proteins in which proteases and protein degradation are important in supplying nitrogen-containing precursors for intracellular development.

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