CYTOPLASMIC SITE OF SYNTHESIS OF CHLOROPLAST AMINOACYL-tRNA SYNTHETASES IN *EUGLENA GRACILIS*

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1. Introduction

A number of aminoacyl-tRNA synthetase (aa-RS) species are found associated with the chloroplasts of algae [1–4] and higher plants [5–8]. Recently we could report distinct differences between organelle and cytoplasmic leu-RS of *Euglena gracilis* when comparing the chromatographic elution pattern, charging specificity to prokaryotic and eukaryotic $tRNA^{leu}$, molecular weight, stimulation of ATP–pyrophosphate exchange by tRNA, sensitivity to monovalent cations, heat-stability and the effects of purine nucleotides on this thermic behaviour [9]. The enzyme species of the chloroplasts show a light-stimulated increase of activity which is reduced by pretreatment of the cells with chloramphenicol or nalidixic acid [2, 4, 10].

Although these results conclusively demonstrate the *de novo* formation of unique aa-RS during chloroplast biogenesis, the intracellular site of their synthesis is still unclear. This paper presents results of kinetic experiments on the accumulation of aa-RS during the light-induced proplastid-to-chloroplast transformation in photoheterotrophically-grown *E. gracilis* and how it is influenced by chloramphenicol (CAP) and cycloheximide (CHI) as selective inhibitors of the protein synthesis on 70 S and 80 S ribosomes, respectively.

Our results suggest that most, if not all, of the chloroplast-specific aa-RS are formed on cytoplasmic ribosomes. Two categories of enzyme species are discriminated in relation to the control of their synthesis. One group (aspartic acid, leucine, lysine, phenylalanine, tyrosine and valine activating enzymes) seems to be directly controlled by a light-stimulated derepression mechanism whereas for the enzymes of a second group (arginine, isoleucine, serine, threonine) illumination may indirectly exert a stimulatory influence on the synthetase formation along with other light-activated processes of the chloroplast biogenesis.

2. Materials and methods

Growth conditions of the *Euglena* cultures, the preparation of tRNA and enzymes, the determination of chlorophyll and the counting of cell numbers are described earlier [2, 11].

Bleached *E. gracilis* cells are inoculated into fresh medium to grow in the dark for 24 hr in the presence or in the absence of 2 mg/ml CAP. CHI is added concomitantly with illumination. Cells are allowed to grow further until harvested at the times indicated, are washed and concentrated to a fixed cell number of 2×10^8/ml. Aliquots are used for chlorophyll estimation, and enzyme activities are determined after 100% breakage of the cells by sonication in a fixed volume (3 ml). The crude enzyme preparations are centrifuged at 120 000 *g* for 90 min, aliquots are used after being thoroughly dialyzed for the determination of RuDP carboxylase activity and [^{14}C]leucine binding to *Anularia nidulans* tRNA (test for plastid enzyme activity) or plastid mutant tRNA of *E. gracilis* (test for cytoplasmic enzyme activity [9, 11].

The RuDP carboxylase assay contains in 125 µl (in mM): Tris–HCl, pH 8.0, 100; $MgCl_2$, 10; β-mercaptoethanol, 2.5; RuDP, 2.5; $Na^{14}CO_3$, 12.5; enzyme solution of 25 µg protein. Incubation for 10 min at

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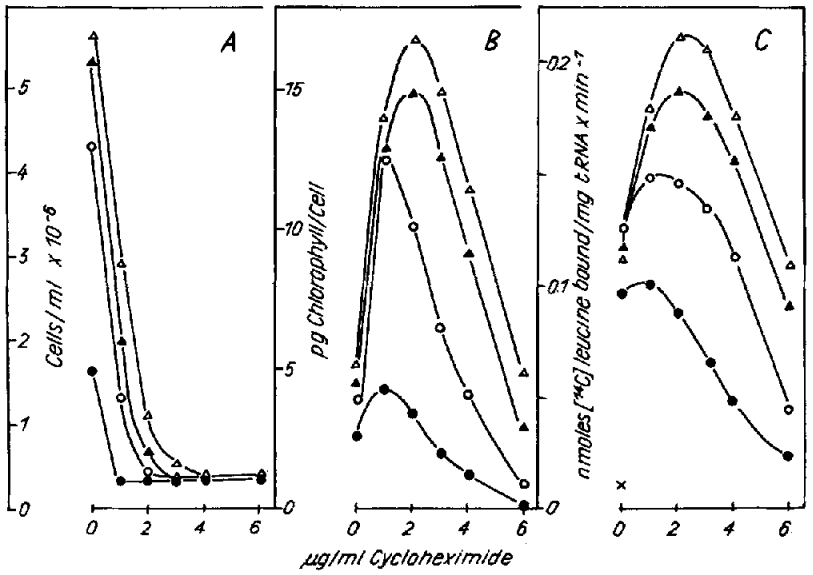

Fig. 1. Effects on cell multiplication, chlorophyll content and plastid leucyl-tRNA synthetase activity of different CHI concentrations during the greening process and in dependence on the period of drug action. Dark-grown *E. gracilis* cells are inoculated to a final concentration of 4×10^5 cells/ml and illuminated with 1200 lux. CHI in the concentrations indicated in the abscissa are added concomitantly. The cultures are harvested 24 hr (●-●-●), 48 hr (○-○-○), 72 hr (▲-▲-▲) and 96 hr (△-△-△) after illumination and aliquots are used for the determination of cell number, chlorophyll content and aminoacylation activity.

28°C. The reaction is stopped by addition of 25 µl 20% trichloroacetic acid, 50 µl aliquots are measured on filter paper discs in a Tricarb scintillation spectrometer.

The aminoacylation assay contains in 250 µl (in mM): Tris–HCl, pH 7.5, 100; ATP, 2.5; $MgCl_2$, 10; KCl, 12; β-mercaptoethanol, 2.5; deacylated tRNA, 0.02; [^{14}C] leucine, 0.01. Aliquots of the enzyme preparations with 50 µg protein for the plastid Leu-RS and 10 µg protein for the cytoplasmic Leu-RS assay are used. Incubation was for 10 min at 28°C. The reaction is stopped with acid at 0°C and 100 µl aliquots are transferred to filter paper discs, washed and counted according to [11].

Amino acids, uniformly labelled with ^{14}C, with specific activities between 45 and 180 mCi/mmole, were obtained from UVVVR, Prague, Czechoslovakia; ATP, dinatrium salt, from Boehringer and Soehne, Mannheim; cycloheximide from Fluka AG, Buchs, Switzerland; D-*threo* chloramphenicol from VEB Berlin-Chemie. All reagents used had the p.a. grade.

3. Results and discussion

Although successfully used as a specific inhibitor of the 80 S ribosomal protein synthesis, CHI is a drug that can cause complex effects in plant cells or tissues [12–14]. As demonstrated in fig. 1., CHI produces quite different results in *E. gracilis* depending on the concentration and period of action of the antibiotic. All concentrations used inhibit cell multiplication completely (fig. 1, A), but a recovery is observed 2 days after the treatment of the cells with low concentrations (1–3 µg/ml) of CHI. In the same concentrations the drug remarkable stimulates the accumulations of chlorophyll (fig. 1, B), ribulosediphosphate (RuDP) carboxylase (not demonstrated) and the chloroplast-specific Leu-RS (fig. 1, C). In any case it is assured that the plastid enzyme concentration is the rate-limiting factor in the assay.

Using slightly higher CHI concentrations (4–6 µg/ml) the synthesis of chlorophyll, RuDP carboxylase and organelle Leu-RS is also strongly reduced (fig. 1) whereas the activity of the cytoplasmic Leu-R remains nearly unchanged (table 1, last line): The

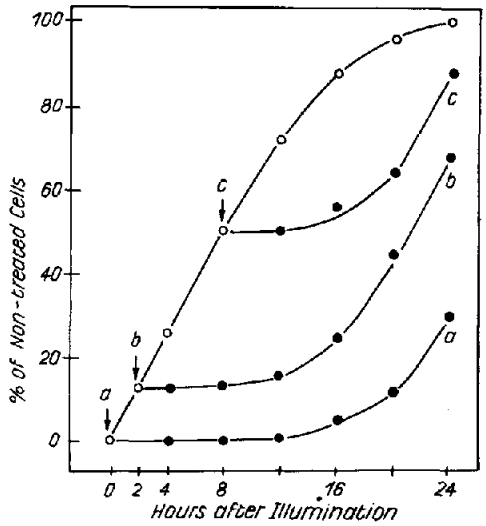

Fig. 2. Inhibition of plastid Leu-tRNA synthetase by CHI added at various times after illumination of dark-grown cells. Cells are treated with 6 μg/ml CHI 0 (a), 2 (b), or 8 (c) hr after illumination (•-•-•); non-treated cells (o-o-o). Enzyme preparations and determination of activity as indicated in Materials and methods. The activity measured after 24 hr of illumination of non-treated cells is the 100% reference.

enzyme activity appears maintained at the level measured prior to CHI additions, since the turnover of the cytoplasmic proteins is arrested under the influence of CHI.

Similar experiments are performed with light-induced cells at earlier stages of chloroplast development and with shorter periods of CHI treatment. Since the antibiotic can penetrate *Euglena* cell membranes very rapidly, the increase of plastid Leu-RS is blocked immediately at any time of CHI addition (fig. 2). After a certain period this inhibition can be overcome giving rise to the various degrees of enzyme inhibition in CHI-treated cells after 24 hr of illumination. This experiment clearly demonstrates that the suppression by CHI of organelle Leu-RS formation is not dependent on the developmental stage of the organelles. Consequently, the light-activated development of a protein synthesizing system in the plastids [15, 16] is no prerequisite for organelle-specific Leu-RS synthesis which most probably takes place on cytoplasmic ribosomes.

Nevertheless, we have observed a remarkable inhibition of the light-stimulated increase of plastid aa-RS after addition of CAP to dark-grown cells [2, 10]. This effect was interpreted either that both chloroplast and cytoplasmic ribosomes contribute to the plastid aa-RS formation or that CAP seemingly inhibits the enzyme synthesis by a reflection of the limited amount of enzyme molecules at the certain stage of CAP-inhibited plastid development. We expect an elucidation with experiments using CHI, CAP and a combination of the two drugs together.

Table 1 shows the effect of 4 and 6 μg/ml CHI on the light-induced synthesis of chlorophyll, RuDP carboxylase and plastid Leu-RS in *E. gracelis* in the absence or presence of 2 mg/ml CAP added 24 hr prior to illumination. While chlorophyll and RuDP carboxylase formation after CAP treatment is nearly 100 % suppressed, the light-stimulated synthesis of plastid Leu-RS is only partially (40%) decreased. The reduced synthesis of Leu-RS caused by CHI alone seems not to be altered in the presence of CHI plus CAP suggesting that the latter does not interfere with the CHI effect. The weak inhibition or even the stimu-

Table 1

Effects of CAP and CHI alone or in combination on the light-dependent increase of chlorophyll, RuDP carboxylase, plastid and cytoplasmic leucyl-tRNA synthetases. The figures are given as percentage of non-treated controls. For details see Materials and methods.

	Antibiotic concn./ml				
	2 mg CAP	no CAP		2 mg CAP	
	no CHI	4 μg CHI	6 μg CHI	+4 μg CHI	+6 μg CHI
Chlorophyll	< 5	62	17	24	6
RuDP carboxylase	< 5	130	55	16	10
Plastid Leu-RS	60	58	28	65	30
Cytoplasmic Leu-RS	110	100	75	115	85

Table 2

Effects of cycloheximide and chloramphenicol on the light-induced increase of enzyme activity of chloroplast-specific aminoacyl-tRNA synthetases.

Amino Acid	Enzyme activity (% of non-treated controls)		
	2 mg CAP/ml	6 μg CHI/ml	2 mg CAP+6 μg CHI/ml
Arginine	97	60	58
Aspartic Acid	71	26	23
Isoleucine	93	77	64
Leucine	60	32	30
Lysine	90	21	18
Phenylalanine	62	40	22
Serine	105	62	87
Threonine	105	93	92
Tyrosine	51	23	20
Valine	66	34	35

Dark-grown *E. gracilis* cultures are illuminated for 24 hr. CAP is added 12 hr prior, CHI at the same time with illumination. Enzyme preparation and assay of ^{14}C-labelled amino acid binding to *Anularia nidulans* tRNA are the same as in table 1 but optimal concentrations for ATP and Mg^{2+} of the individual aa-RS species are used [9].

lation of chlorophyll and RuDP carboxylase formation after CHI addition, however, is strongly depressed in the CAP-pretreated cells. On the other hand, the inhibition by CAP alone of chlorophyll and RuDP carboxylase accumulation (but not Leu-RS synthesis) is abolished to a certain extent after CHI addition. Similar results are interpreted as the formation of a cytoplasmic derepressor that in combination with a repressor of plastid origin regulates chlorophyll formation and CO_2 fixation in *E. gracilis* [14, 17]. In *Neurospora crassa* a repressor–like mitochondrial gene product seems to control the mitochondrial proteins synthesized on cytoplasmic ribosomes [18].

Taking into consideration that *Euglena* RuDP carboxylase is synthesized on chloroplast ribosomes while chlorophyll formation is controlled both by plastid and nuclear DNA [15, 16], we conclude from our results that chloroplast Leu-RS is synthesized merely on cytoplasmic ribosomes. The inhibitory influence of CAP may be referred as to the block, on 70 S plastid ribosomes, of a light-stimulated formation of protein(s) which by its role as a possible derepressor for specific nuclear gene information may regulate the enzyme synthesis. A partial inhibition of plastid Leu-RS synthesis by CAP (table 1) may be explained with the presence of a basic amount of the hypothetical derepressor in dark-grown cells and with the fact that the cell number is approximately doubled during the experimental period.

The results of adequate studies with 9 other plastid aa-RS species can be seen in table 2. According to the response of the individual aa-RS species towards the inhibitors one may discriminate two categories of the control of plastid aa-RS synthesis. Those enzymes showing high light-stimulated increases of activity (Asp-, Lys-, Phe-, Tyr-, Val-RS) seem to be synthesized and controlled in the way suggested for Leu-RS. The synthesis of other species (Arg-, Ile-, Ser-, Thr-RS) is influenced by CHI, CAP or the two drugs together in a similar manner as demonstrated for the synthesis of cytoplasmic Leu-RS (table 1). The insensitivity of their synthese against CAP suggests a control without the participation of the plastid ribosome protein synthesizing system.

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