Different Porphobilinogenases in Cytoplasm and Isolated Chloroplasts from Light-Grown *Euglena gracilis* Z.

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Lysed cells as well as different fractions of isolated, disrupted chloroplasts from light grown *Euglena gracilis* were tested for their capability of porphyrin biosynthesis. It is shown that the formation of the soluble porphobilinogenase (PBG-ase) fraction is inhibited by cycloheximide indicating its biosyntheses on 80S ribosomes, whereas the formation of the membrane bound chloroplast PBG-ase is chloramphenicol sensitive. Different pH-optima are demonstrated for the two enzymes. From the presented results it is deduced that PBG-ase from chloroplasts is different from those isolated from dark grown cells.

Introduction

5-Aminolevulinic acid (ALA) is the first specific precursor in the tetrapyrrole pathway leading to hemes, chlorophylls, cytochromes, corrins and bile pigments. In bacteria, fungi and animal tissue ALA is formed *via* the Shemin pathway by the condensation of glycine and succinyl-CoA, catalyzed by the enzyme ALA-synthetase (ALA-S, succinyl-CoA: glycine C-succinyl transferase, EC 2.3.1.37). In plants and algae ALA is mainly synthesized *via* the C_5-pathway from the intact carbon skeleton of glutamate and/or 2-oxoglutarate (for review see [1]).

Both pathways were found to exist in the photosynthetically active unicellular flagellate *Euglena gracilis* [2–5], being functionally compartmented [6]. The C_5-pathway provides ALA for the chloroplast tetrapyrrole synthesis while non-plastidic heme formation is related to the Shemin pathway.

For the subsequent steps in porphyrin biosynthesis Rossetti *et al.* [7] have recently reported the occurrence of porphobilinogenase (PBG-ase), an enzyme complex which catalyzes uroporphyrinogen III synthesis from porphobilinogen (PBG), in both, soluble and particulate fractions of dark grown *Euglena gracilis*. It is subject of this publication to investigate whether both enzyme activities are also developed in light grown *Euglena gracilis* cells. Attempts to measure porphyrin biosynthesis in isolated and afterwards fractionated chloroplasts will be described to prove whether the particulate PBG-ase from light or dark-grown *Euglena* cells can be attributed to thylakoid and prothylakoid structures, respectively.

Abbreviations: ALA, 5-aminolevulinic acid; ALA-S, ALA-synthetase; PBG, porphobilinogen; Chl, chlorophyll.

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Materials and Methods

Euglena Growth and harvesting

Euglena gracilis Z. strain was grown photoheterotrophically at 26 °C for 72–96 h in permanent white fluorescent light of 5 W m^{-2}. Cells were before precultured three times in light in a medium [8] containing low vitamin B_{12} concentrations (50 ng/l) [9].

For harvesting cells were first filtered through 3–4 layers of autoclaved Miracloth (Chicopee Mills, Inc.; Milltown, U.S.A.) to remove mucus and then sedimented by centrifugation for 10 min at $600 \times g$ yielding about 6 to 7 g wet weight of cells per liter culture medium. The pellet was washed twice with sodium phosphate buffer, 50 mM, pH 7.4.

Isolation of Euglena chloroplasts

Euglena gracilis chloroplasts were isolated following the method of Gomez-Silva *et al.* [3] at 0–4 °C in dim green safelight changing only the formation of the trypsin lysate: Cells were gently resuspended in hypotonic buffer and then lysed by vigorous stirring for 10 min as described by Ortiz *et al.* [9].

Linear Percoll (Sigma, Munich, F.R.G.) gradients (5–70% v/v) were loaded with the chloroplasts suspension and centrifuged for 20 min at $8000 \times g$ in a Kontron TGA 65 Ultracentrifuge using a TST 28.38 swinging bucket rotor. The lower green bands containing the intact chloroplasts were isolated from the gradients, washed and resuspended according to Gomez-Silva *et al.* [3].

Isolated chloroplasts were immediately used to prepare membranes by sonication.

During chloroplast isolation the intactness of the organelles was controlled by light microscopy applying the phasecontrast technique. Additional washings were performed if necessary to separate broken cells or cell debris.

Chloroplast membrane subfractionation

The intact, washed chloroplasts were resuspended in 50 mM phosphate buffer, pH 6.5, and subjected to ultrasonic treatment for 1 min in an ice bath using a MSE sonifier at an amplitude of 7 μ. The disrupted organelles were centrifuged for 30 min. The $15{,}000 \times g$ pellet was washed twice with the above described phosphate butter and employed for the experiments.

Soluble and particulate Euglena fractions

The supernatant of the crude chloroplast preparation was centrifuged for 30 min at $35{,}000 \times g$ yielding the soluble protein fraction ($35{,}000 \times g$ supernatant). The $35{,}000 \times g$ pellet was also washed twice with an equal volume of hypotonic buffer, as above.

Euglena growth in the presence of antibiotics

For studies on light-induced chloroplast development in non-dividing cells, dark grown *Euglena* cells [10] were transferred to a resting medium [11] and the antibiotics treatment performed as described by Hovenkamp-Obbema *et al.* [12], always running controls without inhibitor.

Analytical methods

The incubation system for porphyrin formation and protein determinations as well as all other methods, not specified here, were identical with those described earlier [10].

The activity of PBG-ase is expressed as nmol porphyrins formed per 4 h and mg chlorophyll or mg protein of the corresponding protein fraction.

For chlorophyll determination cells were extracted with hot methanol in the presence of small amounts of magnesium hydroxyde carbonate (Merck, F.R.G., # 5827).

The total chlorophyll content of the extracts was determined spectrophotometrically using the absorption coefficient of Mackinney [13].

Results and Discussion

Porphyrin biosynthesis in light grown Euglena

Vitamin B_{12} was found to be essential for *Euglena gracilis* growth [14]. Drastic vitamin B_{12} depletion results in cell gigantism, prolongation of generation time, and an increase in the number of chloroplasts per cell [15, 16]. This type of cells also forms easily spheroblasts when treated with proteases [17]. Therefore cultivation of *Euglena* in a Vitamin B_{12} limited medium was found suitable for the isolation of chloroplasts from this organism [3, 8, 9]. Chloroplasts isolated by this method were disrupted by sonication and the obtained different membrane and supernatant fractions incubated with PBG. Table I shows porphyrin biosynthesis from PBG in the different chloroplast and cytoplasmic fractions from those vitamin B_{12} deficient light grown *Euglena* cells. The distribution of the porphyrin synthesizing activities was calculated by setting the value of the cell free lysate in nmol porphyrins per 4 h and 1 l of culture as 100%. By this calculation it becomes obvious that the soluble fraction ($35{,}000 \times g$ supernatant) contains about 85% of the total activity, whereas the light membrane fraction ($15{,}000 \times g$ supernatant) shows only 10% of the porphyrin synthesizing capacity. Neglectible activity was found when the $15{,}000 \times g$ pellet was incubated.

From a previous paper [18] it is known that the extent of chloroplast fragmentation depends strongly on the composition of the sonication medium and time of sonication. Katoh and San Pietro [19] reported a different chlorophyll distribution in sonicated chloroplast particles when spinach leave chloroplasts were disrupted by sonication for 10 min in variing phosphate buffer concentrations followed by differential centrifugation. Accordingly, 30% of the total chlorophyll was localized in the $10{,}000 \times g$ pellet when 50 mM phosphate buffer was employed during sonication.

In our experiments the $15{,}000 \times g$ pellet was found to contain 26% of the total chlorophyll after only 1 min of ultrasonic treatment (Table I). This fraction, however, shows maximum uroporphyrinogen

Table I. Porphyrin biosynthesis from PBG in different fractions of light grown *Euglena gracilis* Z.

Fraction	Specific activity		Activity*	Chlorophyll content**
	nmol porphyrin / mg protein · 4 h	nmol porphyrin / mg Chl · 4 h	[%]	[%]
1) Cell free lysate	0.214	29.55	100	100
2) Soluble fraction (35,000 × *g* supernatant)	0.394	–	85	–
3) Sonically disrupted chloroplasts				
a) 15,000 × *g* supernatant	2.424	9.60	10	11
b) 15,000 × *g* pellet	0.119	0.417	1	26

Experimental details are given in the text.

* Activity is expressed as nmol porphyrin/4 h·l culture suspension.
** Chlorophyll content [mg] was defined on the basis of 1 liter of culture suspension.

III formation. The pattern of activity becomes different with longer sonication time, leading to a dramatic decrease in porphyrin biosynthesis capacity accompanied by an unwanted increase in uroporphyrinogen I (data not shown).

In *Euglena* the distribution of the porphyrin synthesizing activity in isolates from chloroplasts disrupted by sonication varies from 90% for the 15,000 × *g* supernatant to 9% for the corresponding pellet. It is thus obvious that the PBG-ase system is mainly found in the soluble plastid fraction. As expected, a membrane associated activity was also detected (Table I). It is most likely that this particulate enzyme fraction has to be considered as an integral membrane protein [20] which most probably is identical with the membrane-bound PBG-ase from dark grown *Euglena* [7].

To cleave integral proteins from the membrane, drastic treatments have to be employed. Thus a chaotropic agent [7, 21], was used to solubilize the membrane bound enzyme complex. As previously reported [7] the 15,000 × *g* pellet was resuspended in 0.5 M NaSCN and then exposed to sonication. Although 20% of the total membrane proteins had dissociated, the Sephadex G-25 eluate, as described earlier for dark grown cells [7], was in this case devoid of PBG-ase. This indicates that the plastid enzyme in contrast to the membrane bound enzyme in dark grown cells is much more labile and cannot be isolated by the described procedure.

pH optima of soluble and integral PBG-ase

PBG-ase activity in the 35,000 × *g* supernatant could be shown to be optimum at pH 7.4 (Fig. 1), whereas a pronounced shift in the pH optimum to pH 6.5 was observed for the enzyme in the 15,000 × *g* supernatant arising from sonicated chloroplasts disrupted in the same buffer. Thus it became clear that PBG-ase from chloroplasts shows different properties, such as shifted pH optimum and inactivation by NaSCN, compared to the particulate and soluble PBG-ase from dark grown *Euglena* with pH-optima of 7.2 to 8.1 [7, 22].

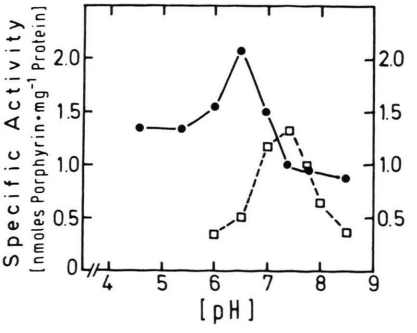

Fig. 1. pH-Dependence of porphyrin formation by the 35,000 × *g* soluble protein fraction (□——□) and the 15,000 × *g* supernatant of disrupted chloroplasts (●——●). The experimental procedure is described in the text.

Antibiotic effect

Isolated *Euglena* chloroplasts incubated in the light can incorporate [^{35}S]methionine or [^{3}H]leucine into proteins and [^{14}C]ALA into chlorophyll *a*. Incorporation was found to be strictly light-dependent and insensitive to cycloheximide, but to be inhibited by chloramphenicol [3, 9]. In contrast Gomez-Silva and Schiff [23] reported that *Euglena* chloroplasts, incubated under specific conditions, could perform protein synthesis and most of the chlorophyll formation steps in darkness. When dark grown *Euglena* was cultivated for 6 growth periods in a medium containing chloramphenicol they measured no change in the aminolevulinate dehydratase (ALA-D) EC 4.2.1.24 activity. The ALA-D activity distribution in various protein fraction was also reported by these authors. As inhibition by chloramphenicol verifies that protein synthesis takes place at the 70S ribosomes of the chloroplasts, the authors concluded that the enzyme should be synthesized in the cytoplasm and afterwards be transferred into the proplastids [24].

When dark grown *Euglena* cells were exposed for 36–48 h to light in the presence of 1 mg/ml chloramphenicol, greening of the cells, measured as chlorophyll content (μg per 48 h and g cells), was inhibited by 75%. When 10 μg/ml cycloheximide were added under the same conditions a total inhibition of chlorophyll biosynthesis was observed.

It is known that the inhibition of pigment synthesis is a secondary effect, as the inhibition of the synthesis of enzymes necessary for the *de novo* synthesis of chlorophyll is primarily affected. Moreover, at least one specific protein synthesized at the 70S chloroplast ribosomes must be present to allow normal chlorophyll biosynthesis. On the contrary Perl [25] suggested that *Euglena* greening is controlled by a cytoplasmic repressor synthesized on the 80S ribosomes which may be inactivated by a 70S ribosomal product.

When porphyrin biosynthesis activity was analyzed, we found on the one hand that the soluble PBG-ase (35,000 × *g* supernatant) seems to be synthesized by the 80S ribosomes, since in greening *Euglena* cells PBG-ase synthesis is inhibited up to 70% by cycloheximide.

On the other hand a depletion of 80% of the PBG-ase activity in the 15,000 × *g* supernatant was observed for cells grown in the presence of chloramphenicol.

As conclusion it is assumed that the chloroplast PBG-ase is involved in chlorophyll biosynthesis and that the cytoplastic enzyme in heme formation.

It is a matter of present research to clarify whether the two PBG-ases are isoenzymes.

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