Synthesis of Protoheme *via* Both, the C_5- and the Shemin-Pathway, in the Pigment Mutant C-2 A′ of *Scenedesmus obliquus*

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Scenedesmus obliquus, ALA-Biosynthesis, C_5-Pathway, Shemin-Pathway, Protoheme

During greening of pigment mutant C-2 A′ of *Scenedesmus obliquus* the iron tetrapyrrole protoheme is synthesized *via* 5-aminolevulinic acid (ALA). In this organism ALA can be formed either *via* the C_5- or the Shemin-pathway. [^{14}C]labeling with precursors of both pathways led to radioactive protoheme. [1-^{14}C]glutamate, the substrate of the C_5-pathway, labeled protoheme 7 times more efficiently than [2-^{14}C]glycine, a substrate most effective for the Shemin-pathway. Labeling experiments in the presence of different inhibitors were carried out to prove the specificity of the observed labeling. Gabaculine, a highly effective inhibitor of glutamate-1-semialdehyde aminotransferase, one of the enzymes of the C_5-pathway, led to a decrease in [1-^{14}C]glutamate incorporation, and to an increase in [2-^{14}C]glycine label, showing that the pathways work independently from each other. With DCMU only the incorporation of [1-^{14}C]glutamate was reduced, indicating that the C_5-pathway activity is depending on plastidic energy supply.

The presented data confirm the existence of both pathways to 5-aminolevulinic acid in the pigment mutant C-2A′ of *Scenedesmus* and their involvement in protoheme formation. It is concluded that the C_5-pathway provides the bulk of protoheme for the plastidic electron transport chain and the Shemin-pathway for the minor part of the hemes needed for the electron transport in the mitochondria.

Introduction

The common precursor for all tetrapyrrols is 5-aminolevulinic acid (ALA). It can be synthesized *via* two different pathways: the Shemin-pathway, condensing succinyl-CoA and glycine [1] and the C_5-pathway, incorporating all five carbons of glutamate into ALA [2]. In higher plants and most algae only the C_5-pathway seems to facilitate the biosynthesis of all tetrapyrroles [3]. However for *Euglena gracilis* [4] and for *Scenedesmus* [5–8] the existence of both pathways to ALA was shown. It has been proven by ^{13}C NMR analysis that in *Scenedesmus* [9], maize [10] and in some photosynthetic bacteria [11, 12] the porphyrin ring of chlorophylls is entirely formed *via* the C_5-pathway. This paper is concerned with the identification of the pathway used for the synthesis of the heme tetrapyrrols which are essential for the electron transport chains in mitochondria and chloroplasts. The phytoflagellate *Euglena gracilis* is reported to synthesize its protoheme for cytochromes *via* both pathways.

Heme *a*, which is only necessary for the mitochondrial electron transport chain, is synthesized entirely *via* the Shemin-pathway in this organism [4]. In *Cyanidium caldarium* [13] and maize [14] all hemes are formed exclusively *via* the C_5-pathway. In the unicellular green alga *Scenedesmus obliquus* the existence of both pathways up to ALA was demonstrated by specific labeling and ALA accumulation after the inhibition of ALA-dehydratase [5–8]. In a preceeding paper a method was presented describing as well the labeling of hemes in *Scenedesmus* with [^{14}C]precursors of both pathways to ALA as how to extract and purify them [15]. In the present paper further evidence for the participation of both pathways in heme biosynthesis is given, focussing on the distribution of the pathways in plastidic and mitochondrial heme biosynthesis in the light-dependent greening mutant C-2 A′ of the unicellular green alga *Scenedesmus obliquus*.

Abbreviations: ALA, 5-aminolevulinic acid; PCV, packed cell volume; DCMU, 3-(3,4-dichlorphenyl)-1,1-dimethylurea; TCA-cycle, tricarboxylic acid cycle.

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Materials and Methods

Growth conditions and incubation with radioactive substrates

For the current experiments the X-ray induced mutant C-2 A′ of *Scenedesmus obliquus* [16], which forms chlorophyll and a functioning photosynthetic apparatus only in the light [17, 18], was applied. Axenic cultures of the mutant were grown in 500 ml Erlenmeyer-flasks in the dark at 32 °C. The inorganic culture medium [19] was supplemented with 0.5% (w/v) glucose and 0.25% (w/v) yeast extract [20] for heterotrophic growth of the cells. The cultures were harvested aseptically at the end of the logarithmic growth phase 60 h after inoculation by centrifugation. At that time they had reached a density of 10–11 µl PCV ml^{-1} culture medium.

For greening and incubation with radioactive substrates the cells were resuspended in sterile inorganic growth medium to a final density of 10 µl PCV ml^{-1} and transferred to culture tubes (3.7 cm diameter; 42 cm length). The cultures were then illuminated with white light (20 W m^{-2}) in a light thermostat and continuously aerated with 3% CO_2 in air. Incubation with the radioactive substrates, either [1-^{14}C]glutamate or [2-^{14}C]glycine and [1-^{14}C]glycine, respectively, was done in three parallel experiments with algal cells from the same culture. The uptake of the radioactive precursors during the incubation was determined by measuring the amount of radioactivity left in the culture medium after separation from the cells after different periods of incubation. Physiological studies with inhibitors were done in parallel experiments without radioactive substrates. In each experiment the cultures were supplemented with both, unlabeled glutamate [1 mM] and glycine [1 mM], to ensure the availability of both substrates.

Isolation and determination of protoheme

Protoheme was extracted with acidified acetone and purified by anion exchange chromatography followed by HPLC, as described previously [15]. The amount of isolated protoheme was determined either by measurement of the reduced minus oxidized difference spectrum of the pyridine-hemochromogen [21] or by measurement of the absorption of air-oxidized protoheme in the fractions eluted from HPLC [4].

The amount of protoheme, which was synthesized from exogenous substrate during the incubation period was calculated under the assumption that the intracellular pool of the substrate is negligible compared to the amount of exogenous substrate. Furthermore, it was taken into account that eight molecules of substrate form one molecule of protoheme. The formula for the calculation is given in the legend to Fig. 4b. When expressed as the percentage of the total amount of protoheme isolated in one experiment this value does not depend on the specific activity of the substrate, the amount of culture material and the size of the intracellular protoheme pool. This allows the comparison of results from different labeling experiments.

Measurement of the radioactive label in protoheme

The volume of the protoheme containing HPLC-fractions was reduced to approximately 0.5 ml *in vacuo*. The samples were then dissolved in 8 ml Aqualuma Scintillation cocktail (Baker, Groß-Gerau, Germany). The amount of radioactivity incorporated into protoheme was determined by liquid scintillation counting with a Betamatic I liquid scintillation counter (Kontron, Neufahrn, Germany). Probes were kept in total darkness for several hours prior to counting to avoid chemoluminescence. The counting efficiency was ± 3%. The incorporation of label into protoheme is expressed as the specific radioactivity of the isolated protoheme.

Results

Influence of inhibitors on the greening process of mutant C-2 A′

When grown heterotrophically in the dark, cells of the developmental mutant C-2 A′ of *Scenedesmus obliquus* form only traces of chlorophyll, accumulate starch and appear yellow in color due to their carotinoids. This mutant was chosen for the experiments since the existence of the two pathways to 5-aminolevulinic acid had been proven before [5, 6] and, furthermore, since the formation of ALA and the subsequent steps of tetrapyrrole biosynthesis are performed at high rates during greening of the mutant in the light. The cultures were harvested aseptically when they had reached a density of 10–11 µl PCV ml^{-1}, were resuspended

in inorganic medium and then transferred into light for greening. Besides the start of chlorophyll-biosynthesis, the enhancement of respiration and the development of photosynthetic activity can be observed [17, 18]. The influence of the applied inhibitors, *i.e.* gabaculine and DCMU, on the greening process under the described conditions is shown in Fig. 1 and 2. Gabaculine inhibits the transamination reaction from glutamate-1-semialdehyde to 5-aminolevulinic acid in the C_5-pathway [22, 23] and thus blocks chlorophyll biosynthesis totally (Fig. 1). Accordingly, these cells could not develop photosynthetic activity in the light. However, the enhancement of respiration which involves catabolism of the accumulated starch in these cells was not influenced.

During the initial phase of greening when energy is provided by enhanced respiration, DCMU showed no influence. However, in the later phase of greening when the photosynthetic apparatus should take over the energy supply, a complete inhibition of photosynthetic oxygen evolution was observed, but there was no influence of DCMU on respiration (Fig. 2, *cf.* [18]).

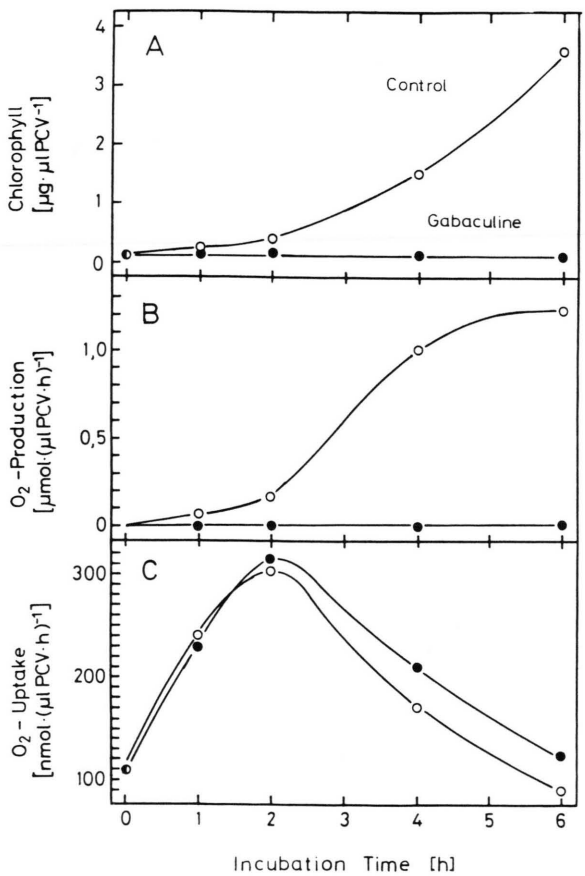

Fig. 1. Influence of gabaculine on the greening process. Chlorophyll biosynthesis (**A**), photosynthetic capacity as O_2-evolution in the light (**B**) and respiratory activity as O_2-uptake in the dark (**C**) of the *Scenedesmus* mutant C-2A′ during greening in white light (20 W m^{-2}) under standard greening conditions (○) and with 1 mM gabaculine (●).

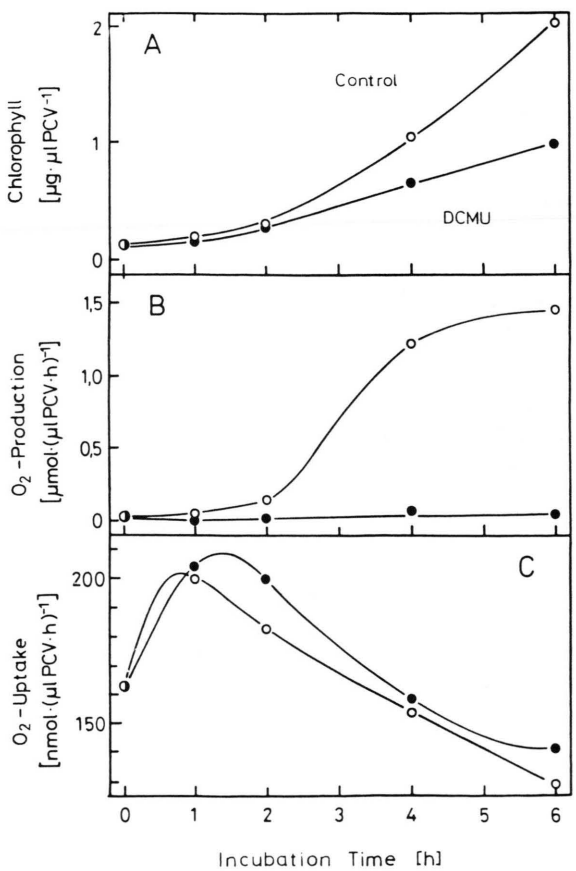

Fig. 2. Influence of DCMU on the greening process. Chlorophyll biosynthesis (**A**), photosynthetic capacity as O_2-evolution in the light (**B**) and respiratory activity as O_2-uptake in the dark (**C**) of the *Scenedesmus* mutant C-2A′ during greening in white light (20 W m^{-2}) under standard greening conditions (○) and with 10 µM DCMU (●).

Influence of inhibitors on the uptake of substrates by the algae

The influence of the inhibitors on the uptake of the radioactive substrates [1-^{14}C]glutamate, [2-^{14}C]glycine and [1-^{14}C]glycine from the medium was monitored by measuring the radioactivity remaining in the culture medium after separation of the cells at different intervals during the incubation period.

Under standard greening conditions glycine was taken up faster and more efficiently than glutamate (Fig. 3). After 2 h of incubation more than 90% of the available glycine was imported into the cells, whereas only 60% of the supplied glutamate was taken up. Finally, at the end of the 6 h incubation period 80% of the added glutamate had been taken up by the cells. When the above mentioned inhibitors were added to the culture medium, the time courses of the uptake of the different substrates changed differently. The uptake of glycine remained faster and at higher rates than the uptake of glutamate in the presence of both inhibitors (Fig. 3). With gabaculine, however, the uptake of the substrates was generally decreased.

Labeling of protoheme during greening

According to the uptake kinetics (Fig. 3) 6 h of incubation with [1-^{14}C]glutamate are needed for a maximum uptake of the substrate by greening cultures of the pigment mutant. The specific activities of protoheme and the amount of protoheme synthesized from the exogenous substrates after 6 h of greening in the presence of radioactive precursors are shown in Fig. 4a and 4b. The most effective precursor for labeling protoheme was [1-^{14}C]glutamate, which is incorporated into this molecule *via* ALA formed by the C_5-pathway. In all experiments a significant amount of radioactivity from [2-^{14}C]glycine, one of the ALA-precursors in the Shemin-pathway, was also incorporated into protoheme. The specific activity of protoheme obtained after incubation with [1-^{14}C]glutamate was 7 times higher than the specific activity of protoheme isolated from cells after incubation with [2-^{14}C]glycine. The amount of label incorporated into protoheme from [1-^{14}C]glycine was negligible.

Influence of gabaculine

The mode of action of gabaculine on the labeling of protoheme by its blocking of the C_5-pathway provides a possibility to examine the specificity of the labeling of protoheme with [2-^{14}C]glycine. It can thereby be distinguished whether the labeling of protoheme with [2-^{14}C]glycine is achieved by the conversion of the added glycine into glutamate and its subsequent incorporation into the tetrapyrrole *via* the C_5-pathway, or whether glycine is directly incorporated *via* ALA from the Shemin-pathway.

Gabaculine obviously showed an inhibitory effect on the labeling of protoheme by [1-^{14}C]glutamate, but not on that by [2-^{14}C]glycine (Fig. 4a

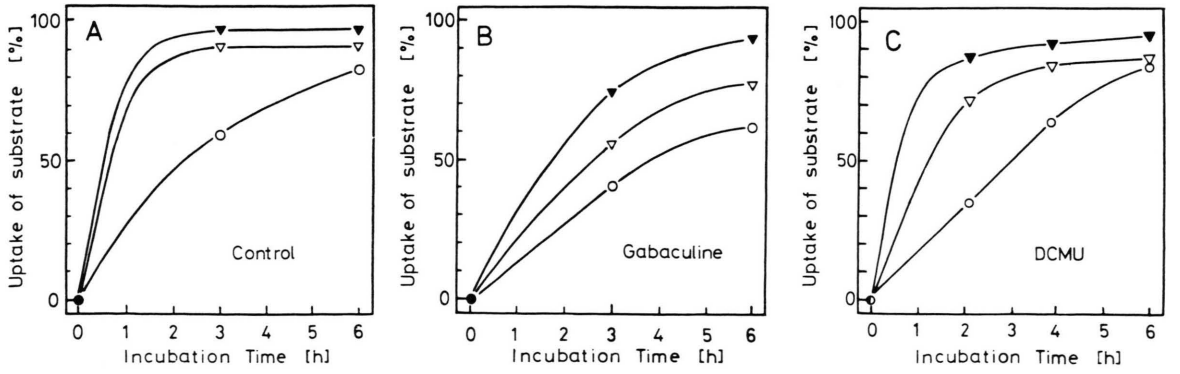

Fig. 3. Uptake of the labeled substrates during incubation. [1-^{14}C]glutamate (○), [2-^{14}C]glycine (▽) and [1-^{14}C]glycine (▼) uptake by cells of the pigment mutant C-2A′ of *Scenedesmus obliquus* during the early phase of greening in white light (20 W m^{-2}) under standard greening conditions and under the influence of inhibitors. **A:** standard greening conditions; **B:** 1 mM gabaculine; **C:** 10 M DCMU. For comparison of the uptake of the substrates by cells of the wild type and cells of the mutant C-2A′ see [15].

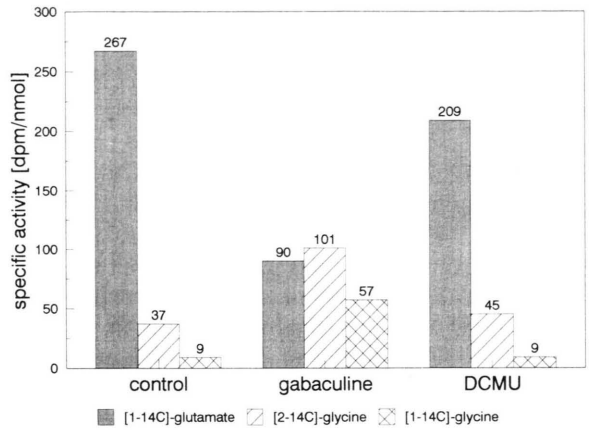

Fig. 4a. Specific activity of the label in protoheme. Protoheme was labeled by [^{14}C]precursors in the pigment mutant C-2A′ during 6 h of greening in white fluorescent light (20 W m^{-2}) with or without the addition of the two inhibitors, using the light-dependent greening mutant C-2A′ of *Scenedesmus*.

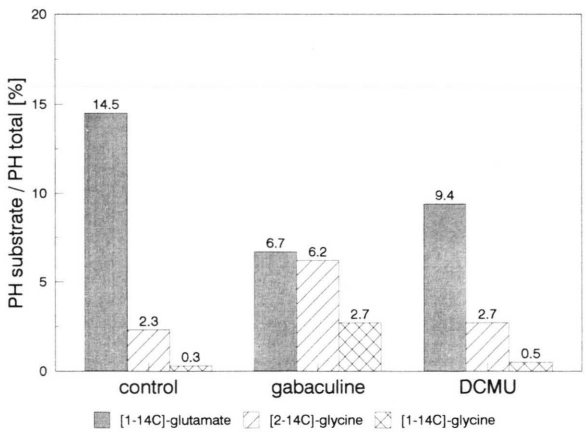

Fig. 4b. Amount of protoheme synthesized from exogenous substrate expressed as percentage of the total amount of protoheme isolated in the experiments. See Fig. 4a. The amount of protoheme synthesized from exogenous substrate is calculated according to the following formula [4]:

$$\frac{\text{Radioactivity}_{\text{product recovered}}}{\text{Spec. activity}_{\text{substrate}}\,[\text{dpm nmol}^{-1}] \times 8} = \text{Product}_{\text{exogenous substrate}}\,[\text{nmol}].$$

and 4b). With [1-^{14}C]glutamate the specific activity of protoheme was only one third of the control value for the incorporation without the addition of the inhibitor (Fig. 4a). The specific activity in protoheme increased by a factor of three compared to the control when [2-^{14}C]glycine was the substrate and was nine times higher when [1-^{14}C]glycine was used. The total amounts of protoheme synthesized from exogenous substrates changed, accordingly (Fig. 4b).

Influence of DCMU

The influence of photosynthetically formed precursors of protoheme was investigated by adding DCMU which blocks both, CO_2-fixation and glycine formation by the C_2-oxidative photosynthetic carbon cycle [24]. By blocking the CO_2-fixation $^{14}CO_2$ deriving from metabolized [1-^{14}C]glutamate or [^{14}C]glycine could not reenter the metabolic pool for ALA-formation.

When [1-^{14}C]glutamate was the substrate, the amount of radioactivity incorporated into protoheme decreased. The specific activity of protoheme was 20% lower (Fig. 4a) and the amount of protoheme synthesized from exogenous substrate dropped to two thirds of the control value (Fig. 4b). Even though DCMU blocks glycine formation by the C_2-oxidative photosynthetic carbon cycle, protoheme labeling with [1-^{14}C]- and [2-^{14}C]-glycine was identical to the control experiments without DCMU.

Discussion

In *Scenedesmus obliquus* both, the Shemin-pathway and the C_5-pathway are involved in the synthesis of ALA [25]. Our current investigation was addressed to the question how much both pathways contribute to the formation of protoheme, incorporated into the cytochromes of the photosynthetic as well as of the respiratory electron transport chain. The incorporation of ALA formed *via* the C_5-pathway into protoheme is straight forward, since [1-^{14}C]glutamate is specifically transferred into ALA and subsequently into protoheme with high efficiency. The application of the C_5-pathway inhibitor gabaculine efficiently prevents the formation of ALA and thus the incorporation of [1-^{14}C]glutamate label into ALA and protoheme.

[2-^{14}C]glycine labels ALA *via* the Shemin-pathway and subsequently protoheme with only 15% of the efficiency of the [1-^{14}C]glutamate label *via* C_5-pathway [Fig. 4]. Since glycine is metabolized in a more general way than glutamate, the weak la-

beling of protoheme with [2-^{14}C]glycine has to be discussed as part of the general metabolism. The first argument against such assumption is the fact that [1-^{14}C]glycine labels only in negligible amounts (Fig. 4). An elegant method to show incorporation of glycine *via* the Shemin-pathway into protoheme independently from the chloroplast located C_5-pathway [26] would be the application of isolated, intact organells. However, *Scenedesmus* does not allow such isolation of intact organelles, especially plastids. Because of this drawback we applied specific inhibitors. With gabaculine as inhibitor of the C_5-pathway the incorporation of [2-^{14}C]glycine into protoheme was not only unaffected, but rather enhanced.

DCMU, inhibiting photosynthesis and thus energy supply required in the C_5-pathway, reduced the rate of [1-^{14}C]glutamate incorporation into protoheme, however, did not influence the incorporation of [2-^{14}C]glycine *via* the Shemin-pathway.

The results of the experiments with both inhibitors, gabaculine and DCMU, prove that the two pathways operate independently in the biosynthesis of protoheme. Since respiration reaches only about 10% the rate of photosynthesis the demand for protoheme in the respiratory electron transport chain is much lower than that for the photosynthetic electron transport chain. Accordingly, the bulk of protoheme formed in the greening pigment mutant C-2A′ is synthesized *via* the C_5-pathway located in the chloroplast, whereas the Shemin-pathway synthesizes the much smaller fraction of the mitochondrial iron-porphyrins.

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