Involvement of Two Different Photoreceptors in the Light Regulation of Glutamine Synthetase Activity in a Chlorophyll-Free *Chlorella* Mutant

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Glutamine synthetase (EC 6.3.1.2) activity of a non-photosynthetic mutant of *Chlorella kessleri* is markedly enhanced under blue and slightly increased under red light. In both cases, the effect is largest after 6 h of irradiation. In blue light, saturation is reached at about 10 $\mu E\ m^{-2}\ s^{-1}$; in red light, it is not even indicated at 62 $\mu E\ m^{-2}\ s^{-1}$.

Semilogarithmic plots of both intensity dependencies reveal different slopes, indicating envolvement of two separate photoreceptors. This feature is supported by different effects of pulse irradiation: The response to 15 min of red light irradiation (λ_{max} 650 nm) increases in subsequent darkness. It reaches the same value as in continuous light after 6 h. The response to 15 min of blue light irradiation (λ_{max} 441 nm) increases also in subsequent darkness. However, after 6 h it reaches only 30% of the value obtained by continuous irradiation.

It is concluded that, glutamine synthetase of *Chlorella* is controlled by two different photoreceptors both independent of photosynthesis. There is evidence of two forms of glutamine synthetase, the intracellular distribution and specific light regulations of these are discussed.

Introduction

Recently it has been reported that *Chlorella kessleri* contains two forms of glutamine synthetase, each of which appeared separately regulated by light (Meya, 1993). GS 2, supposed to reside in the chloroplast, proved to be influenced by blue light, while GS 1, probably located in the cytosol, seemed to be affected by red light. However, in recent experiments GS 1 reacts similarly to enhanced levels of endogenous carbohydrates, so that the question arises whether the effect of red light might be a consequence of photosynthetic activity and not be the result of a true regulatory effect. We tried to answer this question by examining the respective behaviour of a *Chlorella* mutant unable to perform photosynthesis due to the complete lack of both chlorophylls. Although this mutant possesses several carotenoids (Dresbach and Kowallik, 1974) – whose shading effect is certainly disadvantageous for studies on blue light-absorbing photoreceptors – it has successfully been used in this connection in the past (for review: Kowallik, 1987; Ruyters, 1987).

In this paper we present data supporting the assumption that two different photoreceptors are involved in the regulation of glutamine synthetase activity in *Chlorella.*

Materials and Methods

Organism and growth conditions

All the experiments were performed with a chlorophyll-free mutant of *Chlorella kessleri* Fott et Nováková (formerly *Chlorella vulgaris*) 211-11 h/20 from the Culture Collection of the Institute for Plant Physiology at Göttingen University, Germany. The algae were grown in static cultures at 30 ± 1 °C in an inorganic medium (Ruppel, 1962) with iron supplied as an EDTA complex and a supplementation of 1% glucose. Culture tubes (46 cm length, ∅ 4.5 cm), the aeration with air and the light thermostat used have been described by Kuhl and Lorenzen (1964).

Abbreviations: DEAE-, diethylaminoethyl-; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GS, glutamine synthetase; pc, packed cells; TCA, trichloroacetic acid.

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Light regimes

Blue light (λ 400–530 nm, max. at 441 nm) was produced by passing the radiation of two high-pressure mercury lamps (Philips HPL-N 250 W 9 A) through a $CuSO_4$ solution ($E_{623\,nm}$ 0.250) and a 3 mm blue plexiglass filter (Nr. 627, Röhm GmbH, Darmstadt). Red light (λ 570–736 nm, max. at 650 nm) resulted from shining the radiation of four tungsten lamps (Osram Concentra 120 W Flood) through a $CuSO_4$ solution ($E_{623\,nm}$ 0.087) and a 3 mm red plexiglass filter (Nr. 501, Röhm GmbH, Darmstadt). Spectral distributions of the light fields and photon fluence rates used were measured with a quantum spectrometer QSM 11-2500 from Techtum Instruments, Sweden. When small wavelength regions were needed, a set-up described by Dresbach and Kowallik (1974) with Xenon arcs (150 W, 450 W) and interference filters (AL, Schott & Gen., Mainz) was used. Light intensities were determined by means of a thermopile (CA 1) and a galvanometer (Microva AL 4; all instruments from Kipp & Zonen, Holland). The intensities could be adjusted by altering the lamp distance or by applying neutral filters (NG 5, Schott & Gen., Mainz).

Cell suspensions were irradiated in culture tubes kept in a temperature-controlled water bath (30 ± 0.1 °C). For all illumination experiments, the algae were separated from their growth medium by centrifugation (4300×*g*), washed twice with phosphate buffer (0.1 M; pH 7.5) and finally resuspended to a cell density of 7 µl packed cell volume per ml.

Preparation of crude cell extracts

For experimental use, the algae were separated from their growth medium and washed twice with distilled water by centrifugation (4300×*g*). The resulting cell sediments were resuspended in Tricine-KOH buffer (100 mM N-Tris-(hydroxymethyl)-methylglycine; pH 7.8) and in the case of ion exchange chromatography in Tris-HCl buffer consisting of 50 mM Tris-(hydroxymethyl)-aminomethane, 1 mM EDTA, 6 mM DTT, pH 8.0, to yield a final cell density of 100 µl packed cell volume per ml. Packed cell volume was determined with microhematokrit tubes which were centrifuged in a Varifuge S (Heraeus-Christ, Osterode, Germany) for 15 min at 4000×*g*. The cell suspensions were mixed with 1.6 volumes of glass beads (∅ 0.5 mm) and shaken for 10 min at 0–4 °C in a Vibrogen cell mill (Bühler, Tübingen, Germany). The resulting homogenates of broken cells (>90%) were centrifuged at 27000×*g* for 30 min at 4 °C in a Sorvall refrigerated centrifuge RC-5 (DuPont, Newton, CT, U.S.A.) and the supernatants (= crude extracts) were used for analyzing GS.

Ion exchange chromatography

For the purpose of protein separation, a DEAE-Sephacel (Pharmacia Ltd., Uppsala, Sweden) column (2.5×6.5 cm) was used. After equilibration with 50 mM Tris buffer containing 1 mM EDTA and 6 mM DTT (pH 8.0), it was loaded with 4 ml crude cell extract. Thus prepared, the column was washed with two bed volumes of the above buffer before the adsorbed proteins were eluted with a linear KCl gradient (0–400 mM). At a flow rate of 45 ml h^{-1}, the fraction volume was 1.8 ml. The KCl concentration of each sample was calculated from the index of refraction (Abbe universal refractometer/Schmid and Haensch, Berlin).

Enzyme assay

GS activity was determined by the so-called synthetase reaction (Miflin and Lea, 1977; Stewart *et al.*, 1980; Lea *et al.*, 1990). This enzyme assay is based on the formation of γ-glutamylhydroxamate (γ-GH) from the artificial substrate hydroxylamine. The assay mixture (1 ml) consisted of 20 mM $MgSO_4$, 100 mM Na-glutamate, 8 mM ATP, 12 mM hydroxylamine hydrochloride in 100 mM Tricine-KOH buffer (pH 7.8) and an adequate amount of enzyme (25 µl crude extract or 0.2 ml elution volume). The reaction mixture was incubated at 30 °C for 30 min. The reaction was started by addition of hydroxylamine hydrochloride (pH previously adjusted to 7.8) and stopped with 1 ml of a ferric chloride reagent (0.37 M $FeCl_3$, 0.67 N HCl, and 0.2 M TCA). This reagent formed a brown-coloured complex with the reaction product γ-GH. After removal of precipitated proteins (centrifugation at 4300×*g* for 5 min), the absorbance was determined at 546 nm and the GS activity computed by using a γ-GH standard ($[\Delta E\ ml^{-1}] \cdot 2.613 = \mu mol\ (\gamma\text{-GH})\ ml^{-1}$).

Soluble protein

Soluble protein was determined according to Lowry *et al.* (1951) with bovine serum albumin as a standard. Due to interfering substances in crude extracts (Peterson, 1979), the protein had to be precipitated with 0.27 M TCA. It was redissolved in sodium dodecyl sulphate (2% (w/v)).

Statistics

Statistics were calculated by the Pätau method (1943).

Results

In crude extracts of the chlorophyll-free mutant 20 of *Chlorella kessleri* grown in darkness with an ample supply of exogenous glucose and all inorganic nutrients, a glutamine synthetase activity of 168.4 ± 0.6 nmol·min^{-1}·mg protein^{-1} (n = 3) can be measured. When grown under blue or red light instead, the respective activities are 228.0 ± 9.3 and 179.8 ± 11.0 nmol·min^{-1}·mg protein^{-1}. Although the small increase of 7% in red light is not significant, it corresponds in principle to the en-

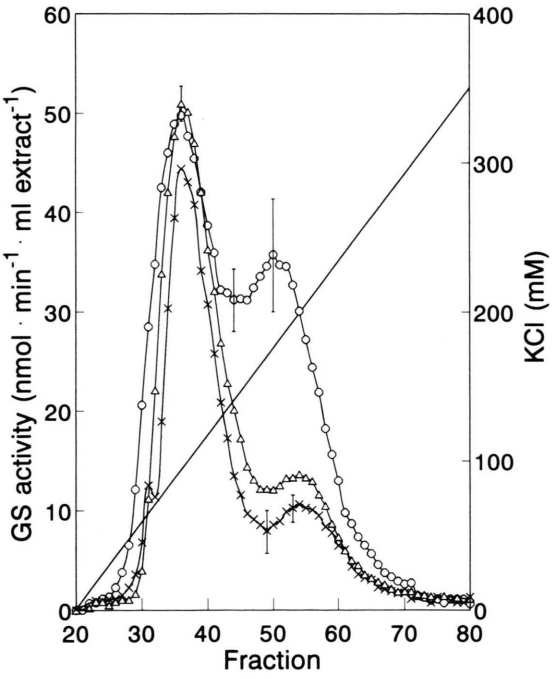

Fig. 1. Distribution after protein separation by ion exchange chromatography on DEAE-Sephacel of the GS activity of crude extracts from a chlorophyll-free mutant of *Chlorella kessleri*, exposed to darkness (×), blue (○) or red (△) light for 2 days; the total carbohydrate contents were 157 µg µl pc^{-1}, 60 µg µl pc^{-1} or 165 µg µl pc^{-1}, respectively. Photon fluence rates were 33 µE m^{-2} s^{-1}. Soluble protein per ml applied: dark 2.844 ± 0.965 (n = 3), blue 4.271 ± 0.591 (n = 3), red 3.907 ± 0.088 (n = 3). 50 mM Tris-HCl buffer, pH 8.0, containing 6 mM DTT and 1 mM EDTA was used for preparing cell extracts and with increasing concentration of KCl for protein elution from the column.

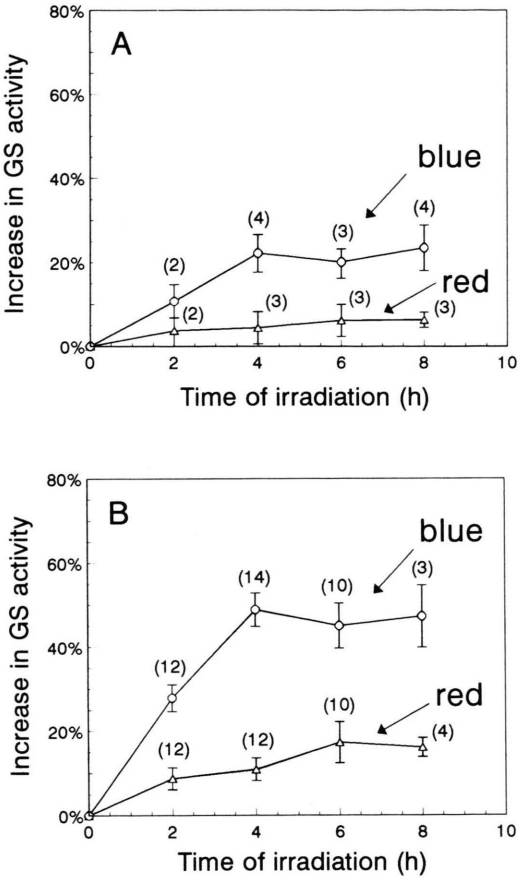

Fig. 2. Dependence of the increase in the activity of glutamine synthetase of a chlorophyll-free *Chlorella* mutant on the time of irradiation. Cells from logarithmic (A) and from stationary (B) phase of a static culture in darkness were used; the total carbohydrate contents were 157 µg µl pc^{-1} and 73 µg µl pc^{-1}, respectively. Incubation buffer: 0.1 M KH_2PO_4/Na_2HPO_4, pH 7.5. Fluence rate of blue light (λ 400–530 nm, max. at 441 nm) was 12.79 µE m^{-2} s^{-1}, that of red light (λ 570–736 nm, max. at 650 nm) 23.30 µE m^{-2} s^{-1}. Increase in GS activity is given as $\frac{L - D}{D} \times 100$. In brackets number of determinations.

zyme's behaviour in the wild type (Meya, 1993). As in the wild type, in the mutant two types of GS can also be distinguished by ion exchange chromatography. Only one of them, GS 2, increases in blue light. The small enhancement in red light cannot be assigned to either enzyme form (Fig. 1). The maximum increase in total GS activity develops within 4–6 h at both light qualities (Fig. 2 A). This also holds true for cells from older static cultures in which carbon and nitrogen sources are largely exhausted. In such cells, the enzyme responds even more pronouncedly to both light qualities (Fig. 2 B), thus yielding a significant effect of about 20% in red light as well. Therefore, there are influences on glutamine synthetase activity of the chlorophyll-free *Chlorella* mutant of either end of the visible spectrum. Since the cells are suspended in a glucose-free phosphate buffer the increase of GS activity cannot result from an enhancement of intracellular carbohydrates in both light qualities. It must be independent of photosynthesis in blue and red light.

A measurement of the respective intensity dependencies has been used in order to discover whether the effects of blue and of red light are mediated by a photoreceptor with short and long wavelength absorption, or by two different photoreceptors. Fig. 3 shows that the semilogarithmic plot of GS activity enhancement *versus* photon fluence rates exhibits a much steeper slope at application of blue light of 448 nm and of 399 nm than at irradiation with near red light of 649 nm and with far red light of 720 nm. Such a difference is usually taken as an indication for the existence of two different photoreceptors (Shropshire, 1972; Hartmann, 1982; Pratt *et al.*, 1990). While the deviation of the 448 nm plot, at fluence rates

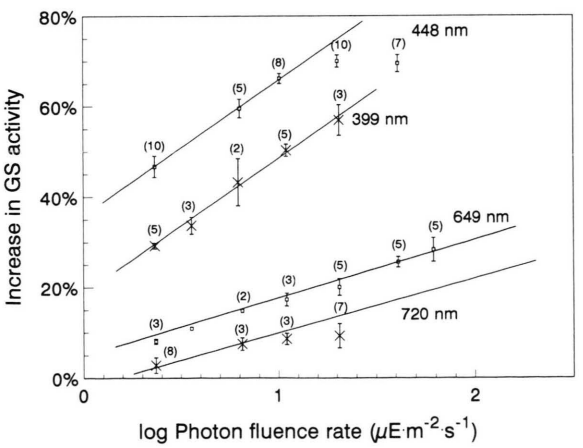

Fig. 3. Dependence of the activity of glutamine synthetase of *Chlorella* on photon fluence rates (semilogarithmic) at different wavelengths of light. Cells from stationary phase of the static culture in darkness – suspended in 0.1 M KH_2PO_4/Na_2HPO_4 buffer, pH 7.5 – were used. Time of irradiation was 6 h. Increase in GS activity is given as $\frac{L - D}{D} \times 100$. In brackets number of determinations.

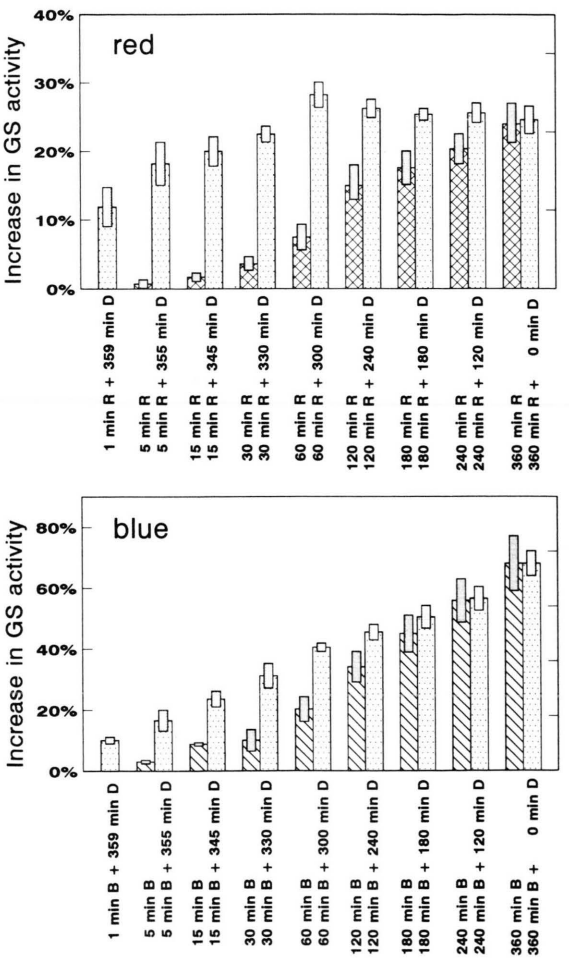

Fig. 4. Increase of the activity of glutamine synthetase of a chlorophyll-free *Chlorella* mutant after blue and red light pulses of different duration and subsequent darkness. Cells from the stationary phase of a static culture in darkness were used. For irradiations cells were suspended in 0.1 M KH_2PO_4/Na_2HPO_4 buffer, pH 7.5. Red light: λ 570–736 nm, max. 650 nm; 144.5 μE m^{-2} s^{-1}. Blue light: λ 400–530 nm, max. 441 nm; 23.3 μE m^{-2} s^{-1}. Increase in GS activity is given as $\frac{L - D}{D} \times 100$.

higher than 10 $\mu E\ m^{-2}\ s^{-1}$, reflects saturation of the effect (at about 20 $\mu E\ m^{-2}\ s^{-1}$), the deviation of the 720 nm plot is not significant. The small effectiveness of this wavelength makes further analysis difficult.

An involvement of two photoreceptors is also indicated by different kinetic responses of the enzyme to pulse irradiation. Exposure to red light of 144 $\mu E\ m^{-2}\ s^{-1}$ for 15 min leads to an enhancement in GS activity of less than 5%, which increases in following darkness to about 20% after a total of 6 h. This final increase is not significantly different from that obtained by continuous irradiation for 6 h (24%). The exposure to blue light of the saturating fluence rate of 23.3 $\mu E\ m^{-2}\ s^{-1}$ for 15 min leads to an approximately 10% higher GS activity, which also increases in subsequent darkness (Fig. 4). However, with less than 25% it reaches only 30% of the maximum enhancement present at continuous irradiation for 6 h.

Discussion

The chlorophyll-free *Chlorella* mutant possesses two forms of glutamine synthetase distinguished by different surface charges. This corresponds to the situation in wild type cells for which one of these forms, GS 1, has been assumed to be located in the cytosol, the other, GS 2, residing in the chloroplast. There is strong evidence that both of these forms are under separate light control. GS 2 is clearly influenced by blue light. The enhancing effect of red light cannot be comparably defined to GS 1; it is too small. Nevertheless, it is clear from the data presented here that both light effects exist in mutant and wild type cells, thus indicating that they are independent of photosynthesis.

The scarce data on the blue light-absorbing photoreceptor reported here correspond with those for the so-called blue/UV photoreceptor, obviously widely distributed among algae. They offer no new information on the nature of its light-absorbing component. Discussion on this subject is found in Kowallik (1967, 1982), Shropshire (1980); Senger and Briggs (1981), Senger and Schmid (1986) and Galland and Senger (1988). The data on the red light-absorbing photoreceptor correspond to the known switch-like behaviour of phytochrome. Phytochrome-like pigments influencing cell metabolism have been assumed to be present in algae before (Taylor and Bonner, 1967; Cordonnier *et al.*, 1986; Haupt, 1982; Dring, 1988; Ruyters, 1988; López-Figueroa *et al.*, 1989, 1990; Ruyters *et al.*, 1991; Figueroa, 1993).

In defining the significance of the two light effects on glutamine synthetase, blue light might be considered to be part of a regulatory system furnishing the chloroplast for its function during illumination. Beside CO_2, nitrite is also reduced to ammonium inside the chloroplast by light-dependent reducing power. To prevent inhibitory action of this cation on photophosphorylation, its concentration has to be kept at a low level. Formation of glutamine would be a suitable solution to this problem, providing storage of reduced nitrogen available on demand in addition. No detailed discussion is possible on the effect of red light with the data available. Considering the data for the wild type, it is tempting to speculate that red light influences cytosolic GS 1. However, more information is needed before this question can clearly be answered.

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