**In Vivo Blue-Light Activation of Chlamydomonas reinhardii Nitrate Reductase**

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**ABSTRACT**

*Chlamydomonas reinhardii* cells, growing photoautotrophically under air, excreted to the culture medium much higher amounts of NO₃⁻ and NH₄⁺ under blue than under red light. Under similar conditions, but with NO₂⁻ as the only nitrogen source, the cells consumed NO₂⁻ and excreted NH₄⁺ at similar rates under blue and red light. In the presence of NO₃⁻ and air with 2% CO₂ (v/v), no excretion of NO₂⁻ and NH₄⁺ occurred and, moreover, if the bubbling air of the cells that were currently excreting NO₂⁻ and NH₄⁺ was enriched with 2% CO₂ (v/v), the previously excreted reduced nitrogen ions were rapidly reabsorbed. The levels of total nitrate reductase and active nitrate reductase increased several times in the blue-light-irradiated cells growing on NO₃⁻ under air. When tumescent replaced molybdate in the medium (conditions that do not allow the formation of functional nitrate reductase), blue light activated most of the preformed inactive enzyme of the cells. Furthermore, nitrate reductase extracted from the cells in its inactive form was readily activated *in vitro* by blue light. It appears that under high irradiance (90 m W⁻¹) and low CO₂ tensions, cells growing on NO₂⁻ or NO₃⁻ may not have sufficient carbon skeletons to incorporate all the photogenerated NH₄⁺. Because these cells should have high levels of reducing power, they might use NO₂⁻ or, in its absence, NO₃⁻ as terminal electron acceptors. The excretion of the products of NO₂⁻ and NH₄⁺ to the medium may provide a mechanism to control reductant level in the cells. Blue light is suggested as an important regulatory factor in the photosynthetic consumption of NO₃⁻ and possibly of the whole nitrogen metabolism in green algae.

Nitrate assimilation in green algae and higher plants is a basic metabolic process because it uses more than 20% of the reducing power generated by their photosynthetic apparatus (11). Among the different steps involved in this metabolic pathway, reduction of NO₃⁻ to NO₂⁻ catalyzed by nitrate reductase has become particularly relevant due to its regulatory features on nitrogen metabolism (5, 12, 36).

NADH-nitrate reductase from green algae is a multimeric enzyme of high mol wt with several electron transport components such as flavin adenine dinucleotide, protoheme b₅₉₃, and molybdenum (8). Recently, it has been reported that molybdenum is held in a special cofactor that contains an unidentified pterin (13).

Depending on the growth conditions, nitrate reductase can be extracted from algae cells in two interconvertible forms: active and inactive. In cells optimally grown on NO₃⁻ under air enriched in CO₂, nitrate reductase is usually found in its active form. However, after mid-term (1–2 h) NH₄⁺ addition to such cells, most of nitrate reductase appears in its inactive form (11, 15, 16).

*In vitro* nitrate reductase can be inactivated by incubation with NAD(P)H or S₂O₅²⁻ and more readily if CN⁻ or C₂H₂ are also present (14, 18). Both the *in vivo* inactive form and the *in vitro* CN⁻-inactivated spinach enzyme can be rapidly reactivated by oxidation with ferricyanide. *In vitro* blue-light irradiation of CN⁻ or C₂H₂-inactivated nitrate reductase, as well as the *in vivo* inactive enzyme from Chlorella fusca, promotes their full activation (1, 18a). Likewise, CN⁻-inactivated nitrate reductase from *Neurospora* is also fully reactivated by blue light (23). The photoactivation of spinach nitrate reductase is greatly accelerated by exogenous flavins, especially under anaerobic conditions. Therefore, excited flavins, but not some highly reactive oxygen species, are directly involved in the photoactivation. Accordingly, it has been proposed that excited flavins that are strong oxidants (38) reactivate nitrate reductase by oxidizing some of its electron transport component(s), probably molybdenum. Alternatively, the excited flavins could act by removing protein-bound CN⁻ in a photoaddition reaction that generates cyanoflavins as proposed for the CN⁻-inactivated enzyme (18a).

In *C. fusca*, the assimilation of NO₃⁻ under nonstabilizing irradiation was higher under blue than under red light (7). However, no direct evidence was reported assigning to blue light an *in vivo* regulatory role of nitrate reductase activity and therefore of nitrogen metabolism. The results presented in this article show that inactive form of nitrate reductase extracted from *Chlamydomonas reinhardii* can be reactivated by blue light and that cells, growing on NO₃⁻ under air without supplementary CO₂, release NO₂⁻ and NH₄⁺ to the culture medium at much higher rates under blue than under red light. Furthermore, under these growth conditions, irradiation of the cells with blue light increases the enzyme level and maintains most of the intracellular nitrate reductase in active form.

**MATERIALS AND METHODS**

*Chlamydomonas reinhardii* 11-32b from the Alga Collection of Göttingen University was grown autotrophically in a mineral medium with NO₃⁻ as the only nitrogen source (31). The medium was adjusted to pH 7.1 with K-phosphate and gassed with air (containing approximately 0.03% CO₂). Cultures (800 ml) were illuminated with both 160-w model SBR-F, PAR 38, (Eye, Japan) and 100-w tungsten lamps under alternating light-dark periods of 7 and 5 h, respectively. Daily, in the middle of the light period, 300 ml of the growing culture were removed and the remaining culture was refilled up to the initial volume with fresh nutrient solution. The cells were collected by centrifugation and washed three times with the same nutrient solution except that the pH was 7.8, and in some cases NO₃⁻ was omitted. The resuspended cells were diluted with the pertinent nutrient solution to obtain a final Chl content of 35 μg ml⁻¹. Chl concentration did not change...
appreciably during the subsequent experimental period. From the resulting cell suspensions, 12 to 15 ml were held with magnetic stirring in glass cylindrical tubes (22 mm inner diameter, 68 mm high) placed in small transparent metacrylate baths at 26°C. Where indicated, air with 2% CO₂ (v/v) was bubbled through the cell suspensions with hypodermic needles, making stirring unnecessary. Two slide projectors fitted with DT 450- and 680-nm wide-band Balzers filters were used to illuminate the cell suspensions. Light intensity at the sample location was measured with a Yellow Springs Radiometer and adjusted for both color lights to 90 W m⁻¹ using neutral density filters.

To estimate the levels of nitrate reductase activity, 4 ml of cell suspension were supplemented with 20 μM flavin adenine dinucleotide, 2 mM cystein, and 5 mM EDTA and sonicated in translucent polyethylene tubes at 4°C either under background white light at the beginning of the experiment or under background blue or red light according to the particular experiment. The illuminating conditions were obtained with a 100-w tungsten lamp fitted with commercial metacrylate dip blue plate or Kodak safelight red filter No. 1A. Two 45-s periods of sonication with 1-min interval were carried out with a MSE sonifier at 10 kc and 6 μamps to disrupt the cells. Subsequently, parts of the broken cell suspensions were incubated for 2 to 5 min with 0.4 mM ferricyanide. Aliquots (0.5 ml) from both ferricyanide-treated and untreated broken cell suspensions were used both to estimate NADH-nitrate reductase activity and to determine indigenous NO₂⁻ content. To stop the enzymatic reaction and eliminate NADH interference with the diazotization reaction, zinc acetate and phenazine methosulfate were added to the reaction mixture after 20 min incubation at room temperature (25).

For the experiments on in vitro photoactivation of nitrate reductase, the cells were grown under the above conditions, although with continuous illumination. Cells (1.5 g) were harvested by centrifugation, washed three times with 50 mM K-phosphate (pH 7.5), and broken by sonication as described above. Cell fragments were removed by centrifugation (28,000g, 15 min), and the supernatant was used for the photoactivation studies. The cell-free extract (2.0 ml) was supplemented with 10 μM flavin adenine dinucleotide and illuminated at 4°C in glass tubes under the same conditions described above, but with 180 W m⁻² for both blue and red lights. Aliquots (0.2 ml) were taken out to estimate NADH-nitrate reductase activity.

Chl was determined according to Arnon (3). Nitrite was measured by the diazotization method of Snell and Snell (27) and NH₄⁺ by the phenolphthalein method of Solorzano (28). NADH-nitrate reductase activity was assayed as described elsewhere (18). One unit of nitrate reductase corresponds to 1.0 μmol of NO₂⁻ formed/min. Protein was estimated according to Lowry et al. (17) using BSA as standard.

RESULTS

Figure 1 shows that Chlamydomonas cells placed in a medium containing both NO₃⁻ and NO₂⁻ and gassed with air supplemented with 2% CO₂ assimilated NO₂⁻ at similar rates under blue or red light of the same intensity. However, when the cells were deprived of supplementary CO₂, NO₂⁻ uptake eventually stopped and subsequently a net release of NO₂⁻ began. The rate of NO₂⁻ excretion increased during light exposure and finally reached much higher values under blue than under red light. Figure 2 shows that, under air, cells growing on NO₂⁻ not only released NO₂⁻ but also NH₄⁺, blue light being again much more effective than red light. However, the release of NH₄⁺ was slightly delayed when compared with that of NO₂⁻. These results indicate that, under air, blue light may promote NO₂⁻ entrance and/or reduction at such high rates that a major portion of NO₂⁻ and NH₄⁺ formed in the cells is released to the culture medium. Strøtmann

Fig. 1. Effect of CO₂ and light quality on NO₂⁻ utilization and NO₃⁻-dependent NO₂⁻ excretion. Cells were suspended in a medium containing 10 mM NO₃⁻ and 0.25 mM NO₂⁻. Initially, the cell suspensions were gassed with air containing 2% CO₂ (v/v). Where indicated by the arrow, the cell suspensions were gassed with air containing approximately 0.03% CO₂. Blue (©) and red (Δ) light illumination.

Fig. 2. Blue-light-promoted NO₂⁻ and NH₄⁺ excretion by cells suspended in a nutrient solution with 10 mM NO₃⁻ under air. NO₂⁻ excretion (——), NH₄⁺ excretion (———), blue (©), and red (Δ) light.
Fig. 3. NO₃⁻ consumption and NH₄⁺ release under blue and red light in cells suspended in a nutrient solution with 0.3 mm NO₃⁻ under air. NO₃⁻ consumption (--), NH₄⁺ release (---), blue (○), and red (△) light.

FIG. 4. a. Effect of light quality on NO₃⁻ consumption and NO₃⁻-dependent NO₂⁻ excretion by cells growing under air. At zero time, cells were suspended in a medium containing 0.2 mm NO₃⁻. When indicated by the arrow, 10 mm NO₃⁻ was added to the cultures. Blue (○) and red (△) light. b. Activity levels of NADH-nitrate reductase in the corresponding broken cell suspensions. Activity of the active form (○, △); total activity, after ferricyanide incubation (●, △); blue light (○, ●); red light (△, △).

and Ried (29) have observed NO₂⁻ excretion from Chlorella when NO₃⁻ was the only nitrogen source in the medium.

To investigate if light quality also modulates NO₃⁻ consumption, cell suspensions with NO₂⁻ as the only nitrogen source were illuminated with blue or red light under air. NO₂⁻ uptake was mostly independent of light quality and proceeded until completion accompanied by a parallel NH₄⁺ excretion (Fig. 3).

Under low CO₂ tensions, excretion of both NO₂⁻ (Figs. 1 and 2) and NH₄⁺ (Figs. 2 and 3) is probably determined by the low levels of available carbon skeletons for further assimilation, since, if air was subsequently supplemented with 2% CO₂, eventually all excreted combined nitrogen was rapidly taken up (data not shown).

To study the internal levels of active and inactive nitrate reductase in relation to both light quality and NO₃⁻ excretion, the cells were disrupted at different stages and conditions of growth. When 10 mm NO₃⁻ was added to cells suspended in a NO₃⁻ medium and bubbled with air, the cells became exporters instead of importers of NO₃⁻ (Fig. 4). The rates of NO₃⁻ excretion increased during light exposure reaching again much higher values under blue than under red light. Figure 4b shows the NADH-nitrate reductase activity of the corresponding cells at different times. At the beginning, nitrate reductase was found predominantly in its inactive form, in part probably due to the obligate dark intervals necessary to prepare the cells for the experiment. At the end of the NO₃⁻ consumption period, either under blue or red light, the levels of both the ferricyanide-reactivated enzyme and the standing active enzyme appreciably decreased. However, 2 h after NO₃⁻ addition, cells illuminated with blue light showed a higher level of nitrate reductase and a fully active enzyme. In the extracts of the cells illuminated with red light, the levels of both the active form and the ferricyanide-reactivated enzyme were much lower. It is worth noting that, on a Chl basis, the different rates of NO₃⁻ excretion and the levels of the active form of the extracted nitrate reductase show a reasonable relationship (157 nmol NO₃⁻ excreted min⁻¹·mg⁻¹ Chl and 228 enzymic units·mg⁻¹ Chl for the cells illuminated with blue light; and 46 and 78, respectively, for the red-light cells), taking into account that part of the NO₃⁻ formed undergoes further reduction to NH₄⁺.

From the preceding results, we concluded that, in the presence of NO₃⁻, blue light reactivates the inactive enzyme and also promotes the de novo synthesis and/or assembly of some preexisting enzyme precursors (12, 32, 33, 36). To further establish that, among other possible effects, blue light indeed activates in vivo nitrate reductase, the cells were placed in a medium where tungstate replaced molybdate. In green algae, where suitable concentrations of tungsten are present in the culture medium, this metal becomes incorporated in de novo synthesized nitrate reductase, presumably replacing the constitutive molybdenum, and generates an enzyme that is not able to reduce NO₃⁻ (22, 35). However, tungsten cannot displace molybdenum from the native nitrate reductase (20, 21). When tungstate was added to a molybdenum-free cell suspension, again high NO₃⁻ excretion took place under blue light (Fig. 5a). The values of the ferricyanide-reactivated enzyme remained almost unchanged in the blue-light cells and decreased only moderately in the red-light cells, while the level of active nitrate reductase increased more than four times in the blue-light cell extracts and only a slight increase was detected in the red-light cell extracts (Fig. 5b). These results seem to corroborate that blue light promotes a definite activation of the in situ inactive enzyme.

When an extract of Chlamydomonas cells containing in vivo inactivated nitrate reductase was exposed to blue light, the enzyme was reactivated more than three times in 25 min. In contrast, there was little effect on enzymic activity when the extract was kept in the dark or illuminated with red light (Fig. 6). The observed initial lag on photoreactivation might be due to the presence in the cell-free extract of some internal reductants that, like ascorbate or reduced pyridine nucleotides, may quench excited flavins before they become completely photooxidized (24). As described elsewhere for the Chlorella nitrate reductase (2), these data show that blue light is capable of activating the inactive form of the Chlamydomonas nitrate reductase.

**DISCUSSION**

Under the actual prevailing photorespiratory conditions of plant growth, i.e. low CO₂ air and high light intensity, C. reinhardii...
nitrogen metabolism appears to be photochromically regulated, inasmuch as cells under these conditions consume NO₃⁻ and release to the culture medium a major portion of the NO₃⁻ reduction products, namely NO₂⁻ and HN₄⁺, at much higher rates under blue than under red light. In this regard, preliminary results indicate that Chlorella pyrenoidosa as also behaves similarly. Blue light in vivo may act primarily as a trigger directly activating nitrate reductase and also promoting the biosynthesis and/or the assembly of additional enzyme. The observed in vitro blue light activation of different nitrate reductases, including that of Chlamydomonas, strongly supports the idea of the in vivo regulation of the enzyme activity by the blue component of the light.

More likely, under low CO₂ tensions, the CO₂-fixing capability of the cells is not able to supply sufficient carbon skeletons to incorporate the high amounts of NH₄⁺ that can be potentially generated from the excess of internal reducing power caused by high irradiance. Thus, cells that cannot allocate their light-generated reducing power in the assimilation of CO₂ may use the oxidized forms of nitrogen as electron sinks, thus releasing to the culture medium the possibly harmful excess of internal reductants.

Nitrate reductase from photosynthetic eukaryotes is currently considered as an assimilatory enzyme to differentiate it from nitrate reductases of the NO₃⁻-respiring prokaryotes (11). The experimental evidences described above indicate, however, that nitrate reductase in green algae might have an additional role, reducing NO₃⁻ in a photosynthetic way that is regulated, among other factors, by blue light. In the long term and with limited amounts of NO₃⁻ in the culture medium, it can be inferred that, once NO₃⁻ would become exhausted by cell growth, NO₃⁻ and NH₄⁺ might be subsequently reassimilated as further CO₂ fixation occurs. Hence, photosynthetic NO₃⁻ reduction does not imply the disappearance of the nitrogen source from the alga environment as it happens to be the case in the NO₃⁻-respiring prokaryotes that currently evolve dinitrogen (39).

The fact that the cells in the presence of NO₃⁻ and under low CO₂ tensions excrete not only NH₄⁺ but also NO₂⁻ may imply some unknown regulatory mechanism related to the reduction of NO₂⁻ to NH₂⁺. Preliminary results indicate that in Chlamydomonas, as already reported for other photosynthetic eukaryotes (11, 33), nitrate reductase activity is 5 times higher than the values of nitrate reductase from the cells illuminated with blue light and that nitrate reductase exhibited similar activity levels after either short dark periods or under blue or under red light. On the other hand, the results described above show that, in the presence of 2% CO₂ and NO₃⁻, NO₂⁻ is rapidly assimilated, and according to the data from Chlamydomonas and other green algae (6, 14, 31), it might be even preferentially utilized to NO₃⁻.

Under high CO₂ tensions (2–5% v/v) or in the presence of bicarbonate, the regulation of NO₃⁻ assimilation in green algae may vary appreciably. Actually, the main difference concerns the strong inhibitory effect exerted by NH₄⁺ under high CO₂ tensions or bicarbonate concentrations on the NO₃⁻ uptake system and on nitrate reductase activity in several green algae including Chlorella (6, 11, 27, 30, 36) and Chlamydomonas (9, 31). On the contrary, in Chlamydomonas kept under air, NO₃⁻-dependent NH₄⁺ and NO₂⁻ excretion proceeded for long periods of time without any appreciable change in its rate, although NH₄⁺ concentration in the medium rose continuously, which indicates that both NO₃⁻ uptake system and nitrate reductase operated unrestrictively, provided that the cells were illuminated with blue light. Hence, in contrast to recent proposals (9), it seems unlikely that NH₄⁺ by itself would block the uptake of NO₃⁻, unless this effect also would be reversed by blue light under low CO₂ tensions. Nevertheless, Florencio and Vega (9) have recently shown that darkness inactivated nitrate reductase in Chlamydomonas cells kept under high bicarbonate concentrations, and that subsequent illumination

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**Fig. 5.** a, Photochrome effect on NO₃⁻ excretion in cells growing under air with tungstate replacing molybdate. The cells were suspended at zero time in a medium containing 10 mM NO₃⁻ and 200 μM tungstate. Blue (○) and red (△) light. b. Activity levels of NADH-nitrate reductase in the corresponding cells suspensions. Activity of the active form (○, △); total activity, after ferricyanide incubation (◇, ●); blue light (○, ●); red light (△, △).

**Fig. 6.** Activation of nitrate reductase by blue light irradiation of the cell-free extract. Blue light (○); red light (△); dark (●).
of the cells resulted in the full reactivation of the enzyme in short periods of time (30 min). However, no light quality experiments were conducted by the authors. The actual chemical species that contribute to the in vivo inactivation of nitrate reductase is still a matter of controversy (5, 11, 36). Notwithstanding, in all cases tested, in vitro blue light irradiation of the enzyme leads to its activation (2, 18a).

The in vitro requirement of NO$_3^-$ for the increase in internal levels and activation of nitrate reductase promoted by blue light is in good agreement with the results of Florencio and Vega (9) and others (11, 16). In vitro, however, although only spinach nitrate reductase has been studied, photoactivation of CN$^-$-inactivated enzyme was independent of NO$_3^-$, unless enzyme irradiation was carried out in the presence of NADH, the physiological electron donor of the enzyme. In this case, NO$_3^-$ contributed markedly to the initiation and acceleration of the photoreactivation (23). These experimental conditions might be more close to the physiological ones. On the other hand, NO$_3^-$ by itself does not reactivate in vitro nitrate reductase at the same rate that blue light irradiation does (24, 34).

The well-documented fact that plants grown under blue light have relatively a higher protein content, while those under red light are relatively rich in carbohydrates, has contributed greatly to stimulate research on photochromic regulation of carbon metabolism (37; note the papers in Ref. 26). Miyachi and Miyachi, studying *Chlorella* (19) and Gnanam et al., studying higher plants (10), have observed that the presence of NH$_4^+$ can mimic most of the metabolic effects caused by blue light. According to the present data, their observations could be explained by the blue light effect on the activation, biosynthesis and/or assembly of nitrate reductase, which in turn leads to a definite increase in the availability of NH$_4^+$ for a number of metabolic purposes.

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