Nitrate uptake and extracellular alkalinization by the green alga *Hydrodictyon reticulatum* in blue and red light

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Abstract

Nitrate uptake and the medium alkalinization related to it were studied with nets of the coenocytic, giant cell, green alga *Hydrodictyon reticulatum*. A comparison of red, blue and white light irradiation showed no special control of nitrate uptake and of the corresponding alkalinization of the external medium by light quality, but rather a response as expected for the photosynthetic apparatus. In the dark, nitrate uptake rates amounted to one-fifth of those in saturating white light. This is in contrast to the chlorococcal microalga *Monoraphidium braunii*, where blue light specifically switched on nitrate uptake-dependent alkalization and where uptake and reduction of nitrate strongly depended on blue light; the rates in pure red light and in the dark being very low. The stoichiometric ratio between nitrate taken up and extracellular alkalinization was close to 1 (0.86) in air with CO₂ but close to 2 (1.84) in N₂ for nitrate pre-loaded cells. In the absence of any carbon source, a high proportion of the absorbed and reduced nitrogen is released, most of it as ammonium which causes the excess alkalinization and some as nitrite, which lowers the ratio. Nitrite and ammonium release rates under anaerobic, CO₂-free conditions were also independent of red or blue light and continued for several hours when the medium was buffered at pH 6. The data indicate that nitrate uptake, but less its reduction, is regulated differently in vacuolate, coenocytic algae from microalgae. In *Hydrodictyon*, nitrate uptake and reduction seem to be controlled by energy supply; in various microalgae, in addition, it is controlled specifically by blue light.

Key words: Alkalinization, blue light, *Hydrodictyon*, nitrate uptake, stoichiometry nitrate/protons.

Introduction

In several species of microalgae, blue light signals are involved in nitrate uptake and nitrate reduction, but so far it has been unknown whether this is a general characteristic of all algae or specifically of microalgae. Both nitrate uptake and nitrate reduction depend on metabolic energy (Ullrich, 1983, 1992). Nitrate uptake depends on ATP via the plasma membrane H⁺-ATPase in a secondary active H⁺ co-transport process. Nitrate reduction requires reducing equivalents. The subsequent ammonium assimilation via GS-GOGAT requires ATP, reducing equivalents and the supply of carbon skeletons. Enzymes of nitrate assimilation, including the membrane carriers, have constitutive components to maintain a low level of activity in the dark or in the absence of substrate. Nitrate, as well as nitrite, usually induces high amounts and activities of the related proteins, but in many cases induction additionally requires light. In the microalga *M. braunii*, short-term N starvation for a few hours in the light promotes the greatest increase in nitrate assimilation (Ullrich et al., 1981). However, algal families or even individual species may behave differently in their response to light, particularly to light quality, whereas nitrate and nitrite always support the induction of their own metabolic machinery, particularly in the absence of external NH₄⁺. In these respects, pioneer studies were carried out with the unicellular microalgae *Chlorella* (Calero et al., 1980), *Monoraphidium* (Quiones and Aparicio, 1990; Aparicio et al., 1994) and *Chlamydomonas* (Azuara and...
Aparicio, 1983). In these algal species blue light has a very strong stimulatory effect; it totally controls nitrate assimilation independent of the photosynthetic energy supply. The blue light receptors contain flavins (probably in combination with pterins) as shown by action spectra of the activation of nitrate reductase (Quiñones and Aparicio, 1990) and of nitrate and chloride uptake (Stöhr et al., 1995; Witt and Aparicio, 1995; Quiñones et al., 1997). Nitrate reductase is immediately activated by blue light in Chlamydomonas (Azuara and Aparicio, 1983), Chlorella and Monoraphidium (Quiñones and Aparicio, 1990). In some cases, nitrate reductase of higher plants also showed reactivation by blue light (Maurín et al., 1983). Recent studies of the photoregulation of nitrate uptake in M. braunii showed that for nitrate transport low PFR of either UVC, UVA or blue light are absolutely required, thus corresponding to the action spectra of flavins and/or pterins (Witt and Aparicio, 1995).

In contrast to these microalgae, giant algal cells such as those of Characeae or Hydrodictyon, have a large storage capacity for ions due to their vacuolar system. Thus, even if nitrate reduction is prevented by the lack of induction of the related enzymes or by external circumstances, transport through the plasma membrane may continue. Similar to Monoraphidium (Ullrich, 1974; Ullrich and Eisele, 1977), H. africam can use nitrate as the photosynthetic electron acceptor to produce the corresponding amount of O2 (Raven, 1977) even in the absence of a carbon source. Inhibition by external electron acceptors of the consumption of nitrate and other anions from the medium was studied in H. reticulatum in relation to the plasma-membrane redox system (Nešpurková et al., 1993).

The aim of the present study was to analyse by physiological experiments, how, in the vacuolate, giant-celled and coenocytic alga, H. reticulatum, nitrate uptake and nitrate reduction respond to monochromatic light and whether a blue light regulation system is found under the longer delay periods for nitrate reduction respond to monochromatic light and of the algal cells required dihydrogen at various light intensities. In the experiments, blue light was shown by action spectra of nitrate uptake in H. reticulatum (Ullrich, 1971). Prior to the experiments, these algae were kept in N-free medium for 2 h in the dark and aerated with CO2-free air. With H. reticulatum nitrate uptake experiments were prepared by washing the cell nets, transferring them to 1 mM CaSO4 and started by adding Ca(NO3)2 of the concentrations indicated in the legends to the figures. Two slide projectors with tungsten-halogen lamps (24 V, 150 W) were used for irradiation with white light or together with interference filters (Schott, Mainz, Germany, or Balzers, Liechtenstein) for red light and blue light (for details see legends to figures). Photon flux rates were determined with a Quantum Radiometer (Li–185 B from LICOR Lincoln, NE, USA).

Nitrate uptake rates were calculated from the depletion of the medium. Nitrate was determined at intervals by UV-spectrophotometry at 202 against 250 nm (Cawse, 1967) or colorimetrically after reduction to nitrite with hydroxylamine sulphate (“Braun-Systematic, sheet N60”), nitrite colorimetrically (Snell and Snell, 1957) and ammonium colorimetrically by the phenol blue method (Solórzano, 1969). For these measurements samples were withdrawn from the stirred medium, without cells in experiments with H. reticulatum, with cells in those with M. braunii where the cells had to be separated by rapid filtration through membrane filters (0.45 μm pore size). The different size of the algal cells required different experimental protocols and longer delay periods for H. reticulatum.

Continuous measurements of NO3− concentrations and pH (Fig. 4) were performed with the electrodes inserted in the same cuvette with the illuminated algae. The pH was measured with a glass electrode (G 2040 C), NO3− concentration with a nitrate electrode (F2412 NO3), both against a double junction reference electrode (K 701, all electrodes from Radiometer, Köbenhavn, Denmark) of which the lower compartment was filled with 1 M Na2SO4 as electrolyte. In stoichiometry experiments, the pH was kept constant at 5.6 by automatic titration with 2.5 mM H2SO4. The pH, NO3− concentration and K+ concentration, the latter to detect possible K+ fluxes, were monitored by pH and ion meters (pH M84, TTT 80 Titratormeter, ABU 80 Autoburette, ION 83 Ion Meter, all from Radiometer, Köbenhavn, Denmark) and recorded by a HP 86 B personal computer (Hewlett-Packard, Palo Alto, CA, USA). Nitrate electrode values were calibrated by photometric control measurements, K+ values by flame photometry (Eppendorf, Hamburg, Germany).

The numbers of experiments (n) represent data from different cultures on different days. Where meaningful, mean values are...
given with SE bars, otherwise representative experiments out of n separate experiments are shown.

**Results**

**Nitrate uptake and light quality**

With cell nets of *H. reticulatum* pretreated for 17 h in the dark, nitrate uptake rates were determined in strong white light, weaker red light (654 + 684 nm) and weaker blue light (425 + 454 nm). After a small lag, the rates were constant for the experimental period (Fig. 1). They were only a little higher in saturating white light than at the much lower PFR of red light, thus characterizing *H. reticulatum* as a ‘shade’ alga. The blue light showed no specific stimulation; nitrate uptake rates were distinctly lower than in red light, while in the dark the rate was still between 20% and 25% of that in saturating white light. This is in a sharp contrast to *M. braunii* (Witt and Aparicio, 1995), where red light alone has no effect. Also in combination with monochromatic red light, blue light did not produce the stimulatory effect known from microalgae (Table 1; Fig. 2). Pretreatment effects could be excluded by changes from red to blue light or vice versa during the experiments (Fig. 2) showing immediate changes in the specific rates of the respective light quality.

To study light effects in comparison with transport of other ions, pH changes were followed during nitrate uptake with various light qualities, with *H. reticulatum* (Fig. 3A) as well as with *M. braunii* in the same light conditions (Fig. 3B). While with *H. reticulatum* blue light attained the lowest rate of alkalization, according to the low PFR, for *M. braunii* blue light was an absolute requirement in addition to red light. With *H. reticulatum*, red light provided similar rates as saturating white light and there was almost no stimulation when blue light was added to red light.

**Stoichiometries of NO₃⁻ and H⁺, efflux of NO₃⁻ and NH₄⁺**

Stoichiometries between nitrate taken up and alkalization had to be studied when net K⁺ influx or efflux did not occur. Under such conditions, titration of alkalization and nitrate uptake gave stoichiometric ratios close to 1:1 (0.86). This ratio remained almost unchanged when the light was switched from blue to red + blue, red or white (Fig. 4). However, when the aeration with air containing CO₂ (Fig. 4A) was exchanged for pure nitrogen and the algae were preloaded with nitrate (Fig. 4B) a stoichiometric ratio close to 2:1 (1.84) was attained, as with *Monoraphidium* (Eisele and Ullrich, 1975) and explainable by stoichiometric release of ammonium and nitrite (see below). When the pH was kept constant by automatic titration with H₂SO₄, constant rates and stoichiometries could be followed over several hours.

As long as the cell nets of *H. reticulatum* were aerated with CO₂-containing air no release of ammonium or nitrite could be observed (data not shown). When gassed with argon or pure nitrogen, ammonium was released as the main product of reduction, at least at pH 5.6 or 6.0 (Figs 4, 5). In an unbuffered medium, ammonium release stopped after a short time (data not shown, but cf. Fig. 3A),

Table 1. Nitrate uptake by *H. reticulatum* in red and red + blue light in sequence

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Nitrate uptake (μmol g⁻¹ FW)</th>
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<tbody>
<tr>
<td>0–120</td>
<td>r + r</td>
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<tr>
<td></td>
<td>5.98 ± 0.09</td>
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<tr>
<td>120–240</td>
<td>r + b</td>
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<td>6.21 ± 0.40</td>
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Ullrich et al.

Nitrate uptake-dependent pH changes under different light conditions. Wavelengths and PFR as in Fig. 1, single red 17 µmol m⁻² s⁻¹; single blue 4 µmol m⁻² s⁻¹, 1 mM KNO₃, air; (A) *H. reticulatum*, irradiation kept constant, FW 37 mg ml⁻¹ (n=5, representative experiment); (B) *M. braunii*, irradiation changing as indicated by arrows. PFR same as for (A) (n=7, representative experiment shown).

probably due to alkalinization beyond pH 8. In a buffered medium the rates were constant over several hours as in the pH-stat experiments (Fig. 5). Nitrite was also released in these experiments, but at much lower rates. In all those cases differences between red and blue or red + blue light did not show any preferences of a special light quality.

Discussion

Algae, among them Chlorophyceae and even separate genera of Chlorococcales, can differ widely in their cell organization, even more in physiological and ecological properties. *H. reticulatum* has coenocytic cells that can reach several millimetres in size. They multiply by intracellular division and formation of new cell nets within the mother cells. This type of development necessarily causes strong physiological changes during the life cycle. For the experiments in this study, actively growing cell nets were used. In contrast to the more thoroughly investigated microalgae, *Chlorella*, *Monoraphidium*, *Chlamydomonas* or *Tetrahedron*, the *Hydrodictyon* species have large storage vacuoles which allow for a certain independence of the regulation between ion transport through the plasma membrane and nitrate reduction and assimilation. High amounts of ions can be stored in the vacuoles and accumulation can continue in the dark. Such cells should be helpful to distinguish processes with respect to energy supply and carbon skeletons. In former studies (Rybova et al., 1972, 1980; Raven, 1977; Raven and Smith, 1977) as well as in those dealing with extracellular electron acceptors and the plasma membrane redox activities (Metlicka et al., 1991; Nešpurková et al., 1993), anion uptake and its relationship with photosynthesis has been investigated. However, the regulation of ion transport by monochromatic light and the implication of putative light receptors have remained unknown for this coenocytic alga.

Concluding from the results of this study, no special sensitivity to blue light of nitrate uptake or nitrate reduction exists in *H. reticulatum*. Photosynthetic energy seems to play an essential regulatory role instead. In this alga, non-cyclic electron flow drives nitrate reduction also under anaerobic conditions (Raven, 1977) as it does in *Monoraphidium* (Ullrich, 1971), *Chlorella* (Calero et al., 1980) and *Cyanidium caldarium* (Fuggi, 1990). This may allow the algal cells to avoid photo-damage and/or photo-inhibition of their photosynthetic apparatus (Azuara and Aparicio, 1983, 1985) and, at the same time, to produce ATP for phosphate storage in inorganic polyphosphates (Ullrich, 1971). By contrast, in spinach and other higher plants, nitrate reductase is usually inactivated by enzyme conditions. Wavelengths and PFR as in Fig. 1, single red 17 µmol m⁻² s⁻¹; single blue 4 µmol m⁻² s⁻¹, 1 mM KNO₃, air; (A) *H. reticulatum*, irradiation kept constant, FW 37 mg ml⁻¹ (n=5, representative experiment); (B) *M. braunii*, irradiation changing as indicated by arrows. PFR same as for (A) (n=7, representative experiment shown).

Fig. 3. Nitrate uptake-dependent pH changes under different light conditions. Wavelengths and PFR as in Fig. 1, single red 17 µmol m⁻² s⁻¹; single blue 4 µmol m⁻² s⁻¹, 1 mM KNO₃, air; (A) *H. reticulatum*, irradiation kept constant, FW 37 mg ml⁻¹ (n=5, representative experiment); (B) *M. braunii*, irradiation changing as indicated by arrows. PFR same as for (A) (n=7, representative experiment shown).
Nitrate uptake in blue light

Figure 4. Stoichiometry between nitrate uptake and extracellular alkalinization by *H. reticulatum*. pH kept constant at 5.6 by titration. Ordinates show nitrate concentration and titrated H⁺ consumption. 0.4 mM Ca(NO₃)₂ added at zero time. Data recorded with 6 s intervals. PFR for all light qualities 200 μmol m⁻² s⁻¹ (i.e. saturation). (A) Pretreatment without NO₃⁻, with aeration; during experiment flushing with air; irradiation 0–20 min: blue + blue light (b+b); 20–60 min: blue + red light (b+r); blue 50 μmol m⁻² s⁻¹; 60–80 min: red + red light (r+r); 80–120 min: white light (see arrows). FW 30 mg ml⁻¹ (n=5, representative experiment). (B) Pretreatment with NO₃⁻, without aeration, during experiment flushing with N₂, light sequence similar to (A), see arrows. FW 22 mg ml⁻¹ (n=2, representative experiment).

Figure 5. Nitrite and ammonium release by *H. reticulatum* during nitrate uptake under anaerobic conditions (flushing with Ar) at constant pH 6.0, buffered with 20 mM MES/KOH. Constant light: either red 130 μmol m⁻² s⁻¹ (680 nm) or red 80 μmol m⁻² s⁻¹ (670 nm) + blue 50 μmol m⁻² s⁻¹ (450 nm). FW 74 mg ml⁻¹ (n=3, representative experiment shown).

In the absence of any carbon source, nitrate is reduced to nitrite and further to ammonium, but both ions are released to the medium. When ammonium is released as an additional cation it will stoichiometrically increase alkalinization, at least when completely dissociated at medium pH as applied in these experiments. Release of nitrite will re-neutralize part of the cations left in the medium from nitrate uptake, hence less change in the pH of the medium can be expected (Aparicio et al., 1994). Therefore, stoichiometric release of ammonium may lead to an alkalinization ratio of 2:1 (NO₃⁻ taken up plus NH₄⁺ released). This ratio will be lower, the larger the proportion released as nitrite. In any case, such high ratios will only be attained when all nitrate taken up is reduced, i.e. when the storage compartments had been loaded with nitrate during the pretreatment.

As long as the vacuolated cells have sufficient energy, they will reduce nitrate to nitrite and ammonium. Uptake can continue in the dark. In the non-vacuolated, microalgal cells, the uptake of nitrate and nitrite directly depends on reduction in the light. Thus very little nitrate is taken up in the dark and the transport process is dependent on blue light. Under carbon limitation in the light, both types of algal cells take up nitrate and reduce it to ammonium indicating that the post-translational inactivation of nitrate reductase of higher plants does not occur.
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References


