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Action Spectra for Nitrate and Nitrite Assimilation in Blue-Green Algae[1]

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ABSTRACT

Action spectra for the assimilation of nitrate and nitrite have been obtained for several blue-green algae (cyanobacteria) with different accessory pigment composition. The action spectra for both nitrate and nitrite utilization by nitrate-grown *Anacystis nidulans* L-1402-1 cells exhibited a clear peak at about 620 nanometers, corresponding to photosystem II (PSII) C-phycocyanin absorption, the contribution of chlorophyll *a* (Chl *a*) being barely detectable. The action spectrum for nitrate reduction by a nitrite reductase mutant of *A. nidulans* R2 was very similar. All these action spectra resemble the fluorescence excitation spectrum of cell suspensions of the microalgae monitored at 685 nanometers—the fluorescence band of Chl *a* in PSII. In contrast, the action spectrum for nitrite utilization by nitrogen-starved *A. nidulans* cells, which are depleted of C-phycocyanin, showed a maximum near 680 nanometers, attributable to Chl *a* absorption. The action spectrum for nitrite utilization by *Calothrix* sp. PCC 7601 cells, which contain both C-phycoerythrin and C-phycocyanin as PSII accessory pigments, presented a plateau in the region from 550 to 630 nanometers. In this case, there was also a clear parallelism between the action spectrum and the fluorescence excitation spectrum, which showed two overlapped peaks with maxima at 562 and 633 nanometers. The correlation observed between the action spectra for both nitrate and nitrite assimilation and the light-harvesting pigment content of the blue-green algae studied strongly suggests that phycobiliproteins perform a direct and active role in these photosynthetic processes.

At present it is well established that the assimilation of inorganic oxidized nitrogenous compounds by microalgae and green tissues of higher plants is a genuine photosynthetic process (14, 21). The phenomenon is particularly clear in the case of blue-green algae, also called cyanobacteria, a group of photosynthetic prokaryotes which are able to perform oxygenic photosynthesis (11). Accumulated experimental evidence, both enzymological (5, 14, 22) and with reconstituted systems and *in vivo* (4, 10, 23, 30), speaks in favor of the photosynthetic nature of the process in these microorganisms. Nevertheless, no detailed studies have been performed so far on the light-quality dependence of nitrate and nitrite assimilation by blue-green algae, the information available being very incomplete and limited to a brief report (32).

Photosynthesis is efficiently sensitized by phycobilin-absorbed light in organisms having these chromophores as major accessory pigments, absorption of light by Chl *a* itself resulting in only a feeble O_2 production (19). Cyanobacteria are phycobiliprotein-containing microalgae in which the bulk of accessory pigments—tightly associated with PSII—is represented by these chromoproteins (13). In cyanobacteria containing the C-phycocyanin-Chl *a* pigment system the action spectra for a number of light-dependent processes, namely O_2 evolution (20, 26, 32), CO_2 fixation (27) and akinete (spore) germination (2), show in the wavelength range from 550 to 660 nm a broad peak with a maximum near 625 nm, which is coincident with the characteristic C-phycocyanin absorption band exhibited by this chromoprotein either in pure state or as a cellular component (7, 19). In contrast, the only action spectrum reported for nitrate reduction by a cyanobacterium has been obtained with C-phycocyanin-depleted cells of a nitrite reductase mutant of *Agmenellum quadruplicatum* and exhibited a Chl *a*-like shape, with two peaks at 425 and 680 nm (32).

In this work the action spectra for both nitrate and nitrite utilization and nitrate photoreduction in cyanobacterial cells having different light-harvesting pigment composition are reported. The results obtained demonstrate that the light-quality dependence of the assimilation of these inorganic nitrogenous compounds is tightly related to the phycobiliprotein content of the cells, the pigments acting as the main physiological antenna to channel the excitation energy required for these photosynthetic processes.

MATERIALS AND METHODS

Organisms and Culture Conditions. The cyanobacterial strains used in this study were: *Anacystis nidulans* (reclassified *Synechococcus leopoliensis*) strain L-1402-1 (from Göttingen University's Culture Collection), *Calothrix* sp. (formerly *Fremyella diplosiphon*) PCC 7601 (from the Pasteur Institute Culture Collection), and a nitrite reductase mutant of *A. nidulans* strain R2 which was obtained (16, 24) by transposon mutagenesis from the wild type (strain PCC 7942 from the Pasteur Institute Culture Collection). All strains were cultured photoautotrophycally under continuous fluorescent illumination (25 W m^{-2}) at 40°C, or 30°C for *Calothrix* sp., on 2% (v/v) CO_2 in air using a modified BG-11 synthetic medium (15) with either 20 mM KNO_3, for *A. nidulans* L-1402-1 and *Calothrix* sp., or 5 mM $(NH_4)_2SO_4$, in the case of the nitrite reductase mutant of *A. nidulans* R2, as the nitrogen source. Nitrogen-starved *A. nidulans* L-1402-1 cells, depleted of C-phycocyanin, were obtained from normal-pigmented cells as follows: the cells were harvested by centrifugation, washed twice and resuspended in the same volume of a nitrogen-free medium and cultured as above for 24 h.

Action Spectra. Cyanobacterial cells were harvested at the exponential phase of growth (5–10 μg Chl *a* ml^{-1}) by centrifugation of cultures (2000*g*, 5 min), washed twice with 50 mM

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Tricine-NaOH/KOH buffer (pH 8.3), and resuspended in the same buffer to a cell density equivalent to 10 μg Chl *a* ml^{-1}. Before illumination with monochromatic light, cell suspensions were preincubated for 20 min at 40°C, or 30°C in the case of *Calothrix* sp., with continuous shaking under saturating white light (100 W m^{-2}) in the presence of 1 mM MSX.[2] Where indicated, the cells were previously incubated in the dark in the presence of 25 mM DLG under otherwise the same conditions.

Uptake experiments to estimate inorganic nitrogen utilization were started by simultaneously adding 0.2 μmol of either KNO_3 or KNO_2 to 1-ml aliquots of the cell suspensions and switching on the light. Incubations under monochromatic light were carried out with continuous stirring, at 30°C for 30 to 60 min, in air opened 1-cm diameter cylindrical cuvettes. Nitrate and nitrite uptake activities were estimated by following the disappearance of these compounds from the medium. Photoreduction of nitrate by the nitrite reductase mutant of *A. nidulans* R2 was monitored by determining the nitrite released by the cells in the assay medium. Starting at zero time, 0.15 ml aliquots were withdrawn at regular time intervals and, after rapid removal of cells using an Eppendorf microcentrifuge, nitrate or nitrite was determined in the supernatants. The choice of an initial external concentration of 200 μM was based on previous studies (10) which showed it to be saturating for nitrate and nitrite uptake rates in *A. nidulans*. The kinetics observed for nitrate or nitrite uptake and nitrite photoproduction were linear with time along the intervals followed. Under the experimental conditions chosen, maximal rates exhibited by normal-pigmented *A. nidulans* cells (15–25 μmol mg^{-1} [Chl] h^{-1}) were in the range expected from the reported light-dependence profile of nitrate uptake by this cyanobacterium (17).

The exciting monochromatic light (half-band width, 12 nm) was provided by an Oriel grating monochromator, using a 200 W mercury-xenon arc lamp (Hanovia 901-B1) modulated at a current of 10 amp with a 8500 Oriel universal power supply. Overheating of assay medium during the experiments was prevented by a water infrared filter. Incident photon flux on the surface of the cuvette was measured at each wavelength with a Li-Cor LI-188 integrating quantum/radiometer/photometer provided with a LI-190 SB quantum sensor cell. The light intensity (30–40 μE m^{-2} s^{-1}) at the wavelengths selected for illumination was adjusted so that it lay within the region of linear photosynthetic response (17, 33).

Absorbance and Fluorescence Spectra. Absorbance and fluorescence excitation spectra of cell suspensions were measured in 1 × 1 cm four-side cuvettes at room temperature using, respectively, a Pye Unicam SP8-150 recording spectrophotometer equipped with a turbid sample holder and a Perkin-Elmer LS-5 luminiscence spectrometer. The small apparent absorption at 740 nm was attributed to scattering and subtracted from the absorbance spectrum as an approximate correction. Fluorescence emission spectra were monitored at 685 nm, the half-band width of both excitation and analyzing monochromators being 2.5 nm.

Analytical Methods. Chl *a* was estimated after extraction with methanol using the extinction coefficient given by McKinney (25). Nitrate was determined by optical absorption at 210 nm in acid solution (6). Nitrite was estimated by the method of Snell and Snell (31).

Chemicals. DLG, MSX, and Tricine were from Sigma Chemical Co. Other chemicals were analytical grade products of Merck (Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

Since radioactive isotopes of nitrogen are not easily available, *in vivo* research on the photosynthetic assimilation of oxidized inorganic nitrogenous compounds by microalgae are normally restricted to their uptake and sequential reduction to ammonia, a study that can be greatly facilitated if appropriate mutants are available (9–11, 24). Uptake and reduction are indeed the initial stages in inorganic nitrogen metabolism before ammonium-N becomes integrated as amido- and amino-N into the various nitrogenous compounds of the cell (11). Incorporation of ammonia into carbon skeletons—through the reaction catalyzed by the enzyme glutamine synthetase—leads to specific nitrogen products that have been claimed as negative feedback effectors of nitrate assimilation in cyanobacteria (12). Thus, in order to assure maximal uptake rates under the conditions used (9, 11), all the experiments described below have been performed with cells treated with MSX, a specific and effective inhibitor of glutamine synthetase (29).

Figure 1 shows the nitrate assimilation rate per unit of photon flux as a function of wavelength of incident light by nitrate-grown *A. nidulans* L-1402-1 cells. It can be seen that the action spectrum of nitrate utilization efficiency clearly exhibits a single peak at about 620 nm. The action spectrum of nitrite utilization by these cells presented also a similar shape, with a peak near 620 nm (Fig. 2). A broad band with a maximum at 625 nm occurs likewise in the fluorescence excitation spectrum of cell suspensions monitored at 685 nm, the fluorescence band of Chl *a* in PSII (28), and it is showed for comparison in Figures 1 and 2. Taking into account that the maxima of fluorescence excitation and action spectra are coincident with the broad absorption band of C-phycocyanin (7)—the predominant phycobiliprotein in *A. nidulans* cells—and considering that Chl *a* fluorescence excitation spectra are a form of action spectra of photosystems operation (19), the observed parallelism strongly suggests that this chromoprotein, tightly associated *in vivo* with PSII (13), behaves as the main light-harvesting pigment for the assimilation of both nitrate and nitrite.

Figure 3 shows the action spectrum for nitrite production resulting from nitrate reduction by a mutant of *A. nidulans* R2 defective in nitrite reductase activity. It should be noted that this mutant strain exhibits moreover a high level of nitrate reductase even in the presence of ammonium (24). An outstanding peak at about 620 nm in the action spectrum and a close correlation with excitation of Chl *a* fluorescence monitored at 685 nm are again evident. For comparison, the absorbance spectrum of the cell suspension is superimposed in Figure 3 to both fluorescence excitation and nitrite production action spectra.

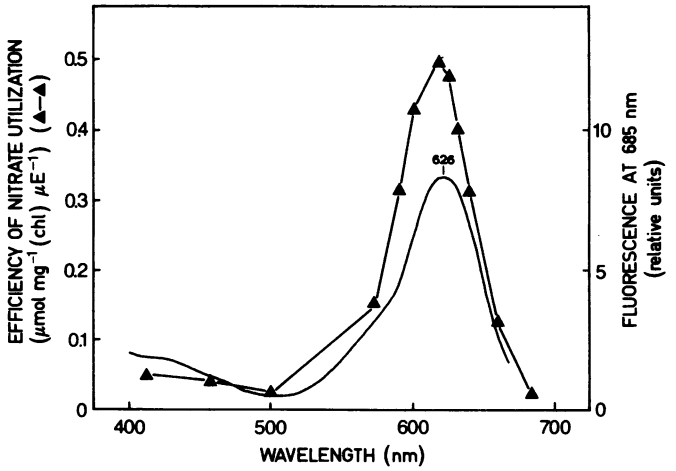

FIG. 1. Action spectrum for nitrate utilization by *A. nidulans* L-1402-1 cell suspensions. For comparison, the fluorescence excitation spectrum monitored at 685 nm (——) of the cell suspension is plotted against the same wavelength scale.

[2] Abbreviations: MSX, L-methionine-D,L-sulfoximine; DLG, D,L-glyceraldehyde.

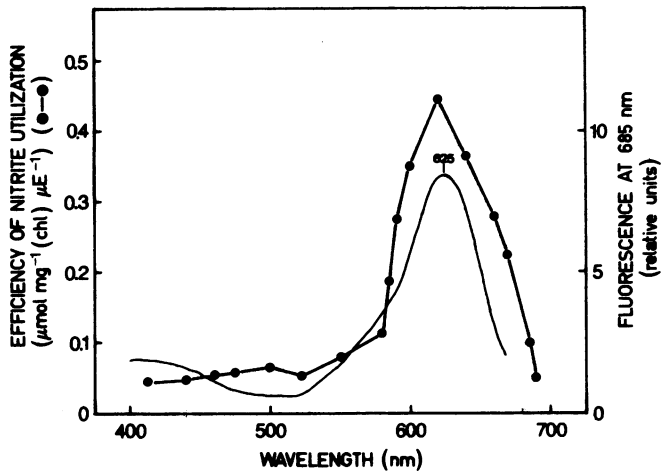

FIG. 2. Action spectrum for nitrite utilization by *A. nidulans* L-1402-1 cell suspensions. The fluorescence excitation spectrum monitored at 685 nm (——) of the cell suspension is plotted against the same wavelength scale.

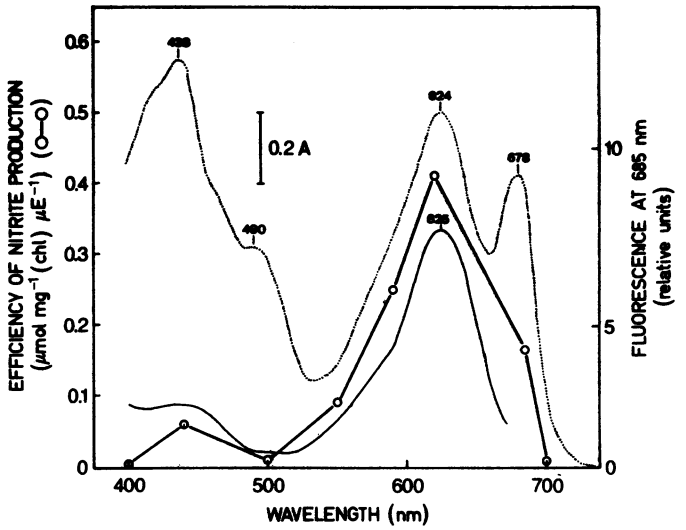

FIG. 3. Action spectrum for nitrite production resulting from nitrate reduction by cell suspensions of a nitrite reductase mutant of *A. nidulans* R2. For comparison, both the fluorescence excitation spectrum monitored at 685 nm (——) and the absorbance spectrum (. . . . .) of the cell suspension are plotted against the same wavelength scale.

Maximal efficiency of nitrate and nitrite utilizations is achieved in the same wavelength range, from 550 to 660 nm, clearly coinciding with the main absorption band of PSII-associated C-phycocyanin (7). The fact that both fluorescence excitation and action spectra exhibit their maxima in regions of shorter wavelength than those characteristic of Chl *a* absorption (see Figs. 1–3), suggests that, in *A. nidulans* cells with a normal phycobiliprotein content, Chl *a* is not able by itself to harvest light energy efficiently. On the other hand, since in the range from 550 to 660 nm the fluorescence and action spectra exhibit a shape similar to the absorbance spectrum (see Fig. 3), the activities of both photosystems shall be balanced, probably through energy transfer (the so-called (3) spillover) from PSII to PSI under illumination at these wavelengths. It is besides worthy to underline that in cyanobacteria both nitrate and nitrite reductases use PSI-reduced ferredoxin as physiological electron donor (14). Therefore, although both photosystems seem to be involved in the assimilatory reduction of oxidized nitrogenous compounds by *A. nidulans* cells, the action spectra indicate that the bulk of the excitation energy which drives both photosynthetic light reactions is provided by the PSII-associated C-phycocyanin.

No changes in the shape of the action spectrum for nitrite utilization by *A. nidulans* L-1403-1 cells were observed when CO_2 assimilation was selectively inhibited by the presence of DLG. Actually, higher rates of nitrite uptake (about twofold) were measured in the spectral range from 550 to 660 nm in the presence of the inhibitor than in its absence (data not shown). This phenomenon should be expected if at the light intensity range used for action spectra determinations, rather below that required for saturation (17), a competition exists between CO_2 and oxidized nitrogenous compounds for photosynthetic reducing power, as it has been claimed for *A. nidulans* cells (18). Our results are, therefore, consistent with the direct photosynthetic nature of the reduction of oxidized nitrogen compounds by cyanobacteria (11) and rule out the involvement in the process of photosynthetically generated carbon intermediates.

Additional evidence for a key role of phycobiliproteins in harvesting light energy for the assimilation of oxidized nitrogenous compounds in blue-green algae was obtained from the action spectrum for nitrite utilization employing cell suspensions of *Calothrix* sp. PCC 7601, a cyanobacterium that contains both C-phycoerythrin and C-phycocyanin as predominant accessory pigments (8). Figure 4 shows the corresponding action spectrum as well as the absorbance and fluorescence excitation spectra of *Calothrix* sp. cell suspensions. Both absorbance and fluorescence spectra exhibit in the yellowish-orange region two overlapped peaks at 560 to 570 and 620 to 630 nm, which correspond to C-phycoerythrin and C-phycocyanin, respectively. Accordingly, the action spectrum for nitrite utilization by *Calothrix* sp. cells shows a plateau in the wavelength region from about 560 to 620 nm (Fig. 4), thus suggesting that in this cyanobacterial species, in contrast with *A. nidulans*, both chromoproteins are actively involved in harvesting the light energy needed for the process.

When cyanobacteria are illuminated in the absence of a nitrogen source but in the presence of CO_2, the phycobiliproteins are destroyed, although viability and Chl *a* content of the cells remain high (nitrogen chlorosis) (1). Action spectrum for nitrite utilization was determined in nitrogen-starved cells of *A. nidulans* L-1402-1 that were depleted of phycobiliproteins, as indicated by the absorbance spectrum (Fig. 5). These cells still retained a substantial capacity to assimilate nitrite, but the action spectrum was different from that of cells having a normal pigment composition. Chl *a* is evidently the principal light-harvesting pigment

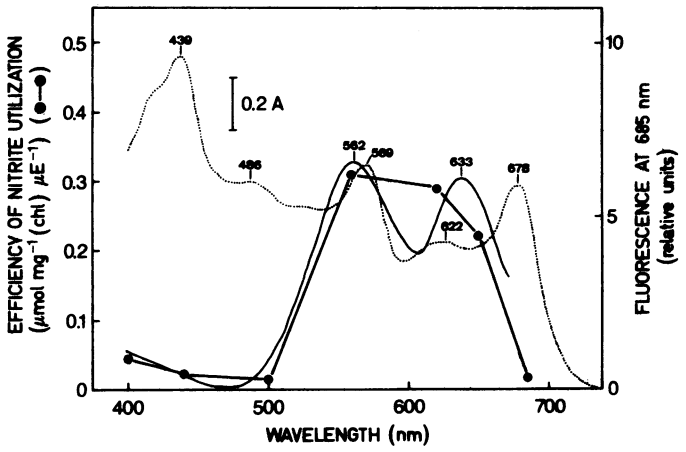

FIG. 4. Action spectrum for nitrite utilization by *Calothrix* sp. PCC 7601 cell suspensions. The fluorescence excitation spectrum monitored at 685 nm (——) and the absorbance spectrum (. . . . .) of the cell suspension are plotted against the same wavelength scale.

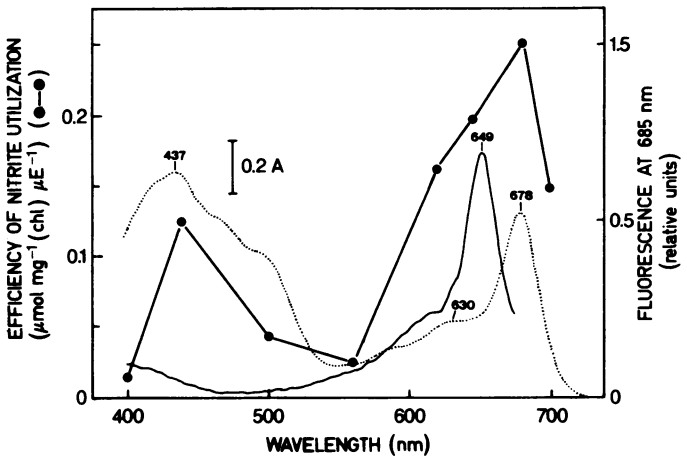

FIG. 5. Action spectrum for nitrite utilization by nitrogen-starved (phycocyanin-depleted) *A. nidulans* L-1402-1 cell suspensions. The fluorescence excitation spectrum monitored at 685 nm (——) and the absorbance spectrum (. . . . .) of the cell suspension are plotted against the same wavelength scale.

in the depleted cells, although the shoulder at 620 to 650 nm might be attributable to residual phycobiliproteins, probably allophycocyanin, as is also suggested by the peak at 650 nm showed by the fluorescence excitation spectrum of the cell suspension monitored at 685 nm (see Fig. 5). These results indicate that when the cellular phycobiliprotein level becomes too low, the light-harvesting role in inorganic nitrogen reduction is assumed by Chl *a*, as has been shown to be also the case for other photosynthetic processes like O_2 evolution (20). A similar action spectrum, exhibiting a Chl *a*-like shape, has been reported for nitrate reduction by nitrogen-starved cells of a nitrite reductase mutant of the cyanobacterium *Agmenelum quadruplicatum* (32).

To the best of our knowledge, this report is the first detailed study on the light-quality dependence of oxidized inorganic nitrogen assimilation by cyanobacteria. The results demonstrate that phycobiliproteins, closely associated *in vivo* to PSII, are actively involved in photoreduction of both nitrate and nitrite. These data are, moreover, in agreement with enzymological and physiological evidence on the genuine photosynthetic nature of these processes in cyanobacteria (11, 14). The results previously obtained using nitrogen-starved cells of *A. quadruplicatum* were interpreted as nitrate reduction being accomplished by a partial reaction of photosynthesis involving only PSI (32), but must be reconsidered in the light of our data in terms of PSII-associated phycobiliproteins depletion rather than nonparticipation of PSII in the process. In this regard it is interesting to note that a direct participation of PSII in nitrate and nitrite photoreduction has been recently demonstrated for cell suspensions of *A. nidulans*, which exhibited both a nitrate (nitrite)-dependent oxygen evolution and the stoichiometry values expected if the electrons required for these processes are derived from water photolysis (10).

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