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Role of nitrate in photosynthetic electron transport of *Chlorella vulgaris*

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Abstract

Addition of nitrate to a suspension of NO_3^--depleted *Chlorella vulgaris* cells raised the O_2-evolving capacity of the organism by 60 %. The rate of O_2-evolution under flash irradiation of the depleted cells was drastically reduced, which could be restored by addition of NO_3^-. The 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB)-insensitive O_2-evolution, *i.e.*, photosystem (PS) 2 activity of NO_3^--depleted cells, showed a 75 % stimulation by addition of NO_3^-. PS1-mediated electron transport was also stimulated (50 %) by addition of NO_3^-. Fluorescence yields of the NO_3^--depleted cells were significantly reduced. A normal fluorescence response was restored by the addition of NO_3^-. The fluorescence yield of the NO_3^--depleted and DCMU-treated-cells increased significantly after addition of NO_3^- ions, indicating a further reduction of the primary acceptor of PS2 (Q). In addition, the low temperature fluorescence emission spectra showed that energy transfer to PS2 and PS1 was much higher when nitrate was present. Hence nitrate accelerates the light-induced charge transfer from the intact O_2-evolving system to the primary electron acceptor of PS2 and stimulates the PS1-mediated electron transport.

Additional key words: chlorophyll fluorescence; DCMU; 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; oxygen evolution rate; photosystem 1 and 2 activities.

Introduction

The enhanced O_2-evolution of irradiated *Chlorella* cells by addition of NO_3^- was first observed by Warburg and Negelein (1920). The increased O_2-evolution in response to NO_3^--addition was attributed to an accelerated NO_3^--reduction (Losada and Guerrero 1979). Using thylakoid preparations, which under the chosen

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Abbreviations: Chl - chlorophyll; DBMIB - 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU - 3-(3,4)-dichlorophenyl)-1,1-dimethylurea; DCPIP - 2,6-dichlorphenol indophenol; DCQ - dichloro-*p*-benzoquinone, MV - methylviologen; NH_2OH - hydroxylamine; PS - photosystem.
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experimental condition could not reduce nitrate any more, the involvement of NO_3^- ions at the donor side of PS2 was demonstrated (Osman *et al.* 1982): they reported that nitrate did not activate the PS1-activity. This conclusion disagrees with the interpretation of Warburg *et al.* (1965), who considered nitrate as possible electron acceptor for Hill reaction. In *Chlorella,* nitrogen starvation decreases yield of PS2 and photosynthetic efficiency (Sayed 1998). Marek and Beranová (1989) demonstrated that NO_3^- (up to 20 mM) increased the photosynthetic rate, ribulose-1,5-bisphosphate carboxylase/oxygenase activity, and transpiration rate of spring barley. NO_3^- assimilation uses photosynthetically derived reductant ($NADPH_2$) and stimulates the rate of non-cyclic electron flow by acting as a second electron accepting process in addition to CO_2-fixation (De la Torre *et al.* 1991).

The present work was designed using partially NO_3^--depleted *C. vulgaris* as intact system to reevaluate the NO_3^- effect on the photosynthetic electron transport.

Materials and methods

Chlorella vulgaris, strain (211-11b) (Sammlung von Algenkulturen, Pflanzenphysiologishes Institut, Universität Göttingen, Germany) mass culture was grown as described by Lorenzen (1964). The culture grew under 12 fluorescent tubes (irradiance of 120 W m^{-2}) at 25±1 °C and continuous aeration with a mixture of 97 % air and 3 % CO_2. Chlorophyll (Chl) content was determined according to Metzner *et al.* (1965). Algal cells were harvested during the exponential phase (5 d). In order to obtain partially NO_3^-- and Cl^--depleted cells, they were centrifuged at 3 000×g, washed several times with bidistilled water and then in 3 mM NH_4SO_4 solution (to minimize the nitrate reductase activity), resuspended in phosphate buffer (pH 7.2), and finally irradiated by 300 W m^{-2} for one hour. The concentration of NO_3^- in the cells after this treatment, determined according to Allen *et al.* (1974), ranged between 0.2-0.3 mM. About 95 % of the oxygen evolution capacity of the NO_3^--depleted cells could be restored by adding 10 mM KNO_3.

Oxygen evolution rate of a cell suspension (Chl content adjusted to 15 g m^{-3}) under continuous irradiation ("white", 150 W tungsten lamps) was measured polarographically with a Clark-type electrode at 20±1 °C. A dark adaptation time of 30 min after addition of NO_3^- was needed for 95 % restoration of the O_2-evolution capacity of the depleted cells. For measuring the PS1 activity (DCMU-insensitive O_2 uptake), sonnicated cells were used. For sonnication, algal culture was centrifuged at 3 000×*g* for 3 min. The pellet was suspended in cooled nitrate-free medium, sonnicated for 3 min at 15 amplitude micron with a *Sonniprep 150 MSE* equipped with 1.3 cm horn, and centrifuged again at 5 000×*g* for 5 min. The pellet was used for measuring PS1 activity (DCMU-insensitive O_2 uptake) using sonnicated algal cells equivalent to 22 g(Chl) m^{-3}. Flash induced oxygen yield was measured using a Joliot-type electrode (Joliot and Joliot 1968). According to this method, 10 cm^3 algal suspension was centrifuged at 3 000×*g*, then resuspended in 50 cm^3 phosphate buffer (pH 6.5). Sample aliquots of 150 mm^3 each [50 g(Chl) m^{-3}] were placed on the electrode surface and irradiated by a xenon flash lamp with a sequence of 300 ms

flashes (4 Hz frequency) following 5 min dark adaptation with or without NO_3^-. The produced signals were evaluated on a storage oscilloscope (*Hameg* model *MH 42*) and printed by a *Servogor* recorder.

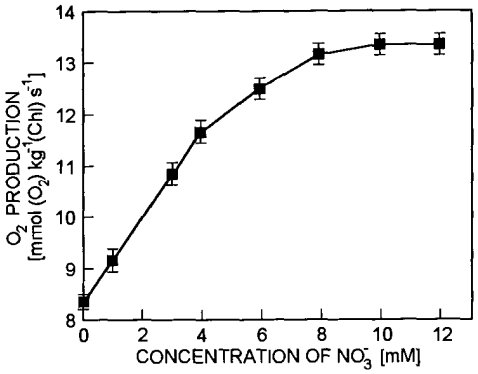

Fig. 1. Effect of different concentrations of NO_3^- (KNO_3) on the O_2 evolution of NO_3^--depleted *Chlorella vulgaris* cells. Measurements in phosphate buffer (pH 6.8). The vertical bars represent the standard error of the mean values of at least five determinations.

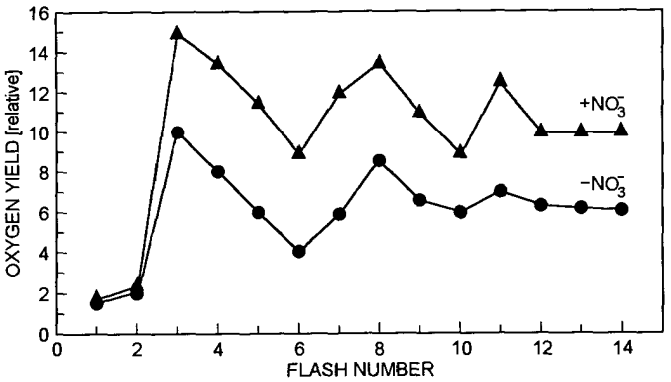

Fig. 2. Effect of NO_3^- (10 mM KNO_3) on the flash oxygen yields of NO_3^--depleted *Chlorella vulgaris* cells. The cells were suspended in 50 mM phosphate buffer, pH 6.5 and incubated for 3 min in the dark on the electrode surface prior to measurements.

Table 1. Effect of NO_3^- (10 mM KNO_3) on PS2 (DBMIB-insensitive O_2-evolution in the presence of 1 μM DBMIB and 1 mM DCQ) and PS1 activities (O_2-uptake from 40 μM DCPIP to 2 mM MV in presence of 10 μM DCMU and 2 mM sodium ascorbate) [mmol(O_2) kg^{-1}(Chl) s^{-1}] of nitrate-depleted *Chlorella vulgaris*. Means ± standard errors (SE).

	$-NO_3^-$	$+NO_3^-$	Stimulation [%]
PS2	4.4 ± 0.6	7.8 ± 0.6	75
PS1	2.2 ± 0.3	3.3 ± 0.6	50

Fluorescence transient measurements on algal suspension (Chl content 10 g m^{-3}) were done at room temperature (22±1 °C) according to Wiessner *et al.* (1981). The cells were centrifuged at 3 000×*g*, resuspended in phosphate buffer (40 mM, pH 7.8), and then incubated with 10 mM KNO_3 for 30 min before measurements. The blue actinic radiation was filtered by a 420 nm broad-band *BG 38 Schott* filter, switched on by a magnetic shutter (0.6 ms opening time), and focused on the sample cuvette. Fluorescence emission was filtered through two red cut-off filters (*Rubilith-Amberlith ulano* + *Kodak Wratten* no. *70*) before reaching a *RTCXP 1002* photomultiplier. Variations of the signal as a function of time were recorded on the memory screen of *Tektronix DII 5103 N* oscilloscope. A dark adaptation time of 10 min between measurements was sufficient for complete relaxation of the photosynthetic flow of algal cells between the light periods. Fluorescence emission spectra at liquid nitrogen temperatures were measured according to Harnischfeger (1977) using the cheese-cloth method (Cho *et al.* 1966). 10 cm^3 of the NO_3^- and Cl^--depleted algal suspension was centrifuged at 3 000×*g*, then resuspended in 40 mM phosphate buffer (pH 7.2) to a standard Chl concentration (150 g m^{-3}). The samples were spotted carefully on two layers cheese cloth, fixed between two hard gummi rings, then chilled rapidly in liquid nitrogen (-196 °C). The excitation radiation was a blue broad band at 470 nm. The fluorescent radiation was passed through photomultiplier, then printed by the *Servogor* recorder.

Results and discussion

The addition of NO_3^- to NO_3^-- and Cl^--depleted *C. vulgaris* cells stimulated the O_2-evolution capacity of the organism; this stimulation was concentration-dependent (Fig. 1). The maximum stimulation (60 %) was observed after addition of 10 mM NO_3^-. O_2 emission from dark-adapted algae under flashes was also drastically raised by adding NO_3^- (Fig. 2). However, although NO_3^--depleted cells evolved less O_2 per flash than the nitrate-treated cells, both showed a similar pattern of O_2 emission. Thus NO_3^- may interfere with an event associated with PS2, since the reduction in O_2-evolution under flashing irradiation indicates that electron transport to the pool of PQ is reduced in the absence of NO_3^-. This conclusion was supported by our results on the DBMIB-insensitive O_2-evolution (Table 1), which showed marked stimulation of O_2-evolution (75 %) in response to NO_3^- addition. PS1-mediated electron transport was also stimulated (by 50 %) after adding NO_3^- to NO_3^--depleted cells (Table 1).

In order to localize the action sites of NO_3^- in photosynthetic electron transport, we studied the fluorescence induction kinetics at room temperature as well as the fluorescence emission spectra at liquid nitrogen temperature. The addition of NO_3^- to dark-adapted nitrate-depleted cells raised the fluorescence yield which resulted in a significant increase of the F_{max} value of variable fluorescence (Fig. 3*A*). Addition of DCMU to NO_3^--depleted cells raised the fluorescence yield significantly, which was further increased by addition of NO_3^- (Fig. 3*B*). Because the yield of variable fluorescence is taken as an indicator of the redox state of Q (primary acceptor of

PS2) (Duysens and Sweers 1963, Butler 1966), the increased F_{max} value means that NO_3^- leads to a stronger reduction of Q.

There are four possibilities for the accumulation of Q^-:

(*1*) Blocking of the electron transport between the two photosystems by a poison such as DCMU.

(*2*) Enhanced photoreduction of Q by increasing irradiance.

(*3*) Chemical reduction by the addition of a reducing agent, *e.g.*, sodium dithionate.

(*4*) Stimulation of the electron transport at the donor side of PS2 to a level which brings the intermediate pool (*A*) between the two photosystems into a nearly completly reduced state.

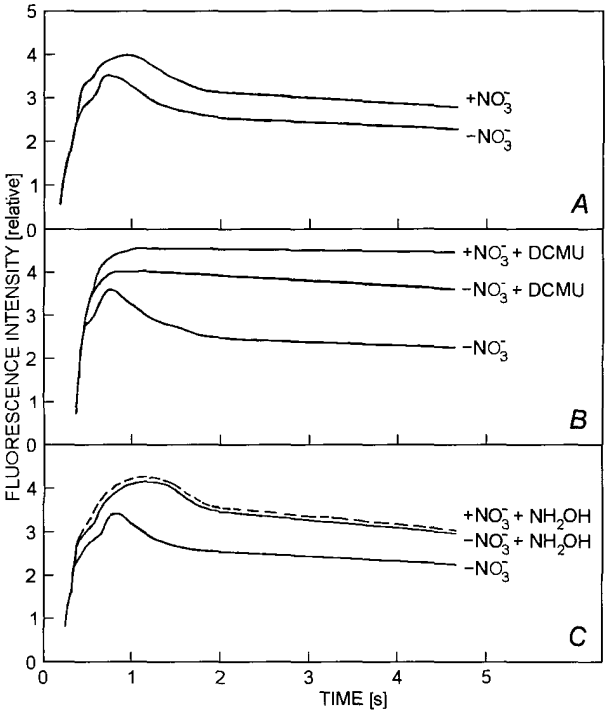

Fig. 3. Fluorescence induction kinetics of NO_3^--depleted *Chlorella vulgaris* cells: (*A*) Before and after addition of 10 mM KNO_3. (*B*) Nitrate-depleted cells, NO_3^--depleted + 10 μM DCMU, and NO_3^--depleted + 10 μM DCMU + 10 mM KNO_3. (*C*) NO_3^--depleted cells, NO_3^--depleted cells + 25 mM NH_2OH added before the dark adaptation period, and NO_3^--depleted cells + 25 mM NH_2OH + 10 mM KNO_3. Experimental conditions as described in Materials and methods.

Our results eliminated the first two possibilities. For the third possibility there was no evidence which could indicate a direct reduction of Q by nitrate. The relative position of the redox potentials of Q/Q^-, respectively $NO_3^-/NO_3^{\cdot}$, makes this assumption unlikely. Hence we have to accept the fourth possibility, which is supported by our results on the fluorescence yield of DCMU-treated cells. The increase in F_{max} of these cells after NO_3^- addition indicates that this anion stimulates

the charge transfer from the intact O_2-evolving system to the primary acceptor of PS2. In other words, nitrate stimulates the electron flow from the oxidizing side of PS2 to the primary electron acceptor Q, causing increase in the fluorescence yield.

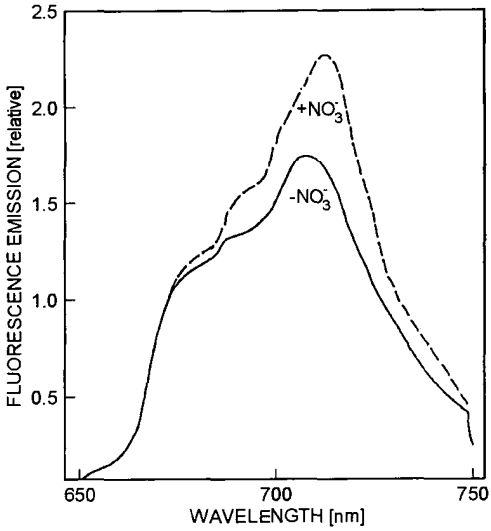

Fig. 4. Fluorescence emission spectra of NO_3^--depleted *Chlorella vulgaris* cells (——) and of these cells after addition of 10 mM KNO_3 (- - -) at liquid nitrogen temperature. Excited by broad blue band at 470 nm. Experimental conditions as described in Materials and methods.

To localize the site of NO_3^- action on the donor side of PS2 we used hydroxylamine as artificial electron donor for PS2, which donates its electron to the donor side of PS2 thus inducing strong reduction of Q. As shown in Fig. 3*C*, the induced fluorescence caused by addition of hydroxylamine was not affected by addition of NO_3^- and thus the role of NO_3^- is effective only with a natural electron donor of PS2 (X-H_2O), *i.e.*, the site of NO_3^- action is before the site of electron donation by hydroxylamine at the donor side of PS2.

The influence of NO_3^- on the efficiency of distribution of excitation energy between the two photosystems could be seen from the fluorescence emission spectra at liquid nitrogen temperature (Fig. 4). At this temperature, F_{718} is assigned to PS1 and F_{693} to PS2. The NO_3^- addition to NO_3^--depleted cells raised the fluorescence yield of both photosystems indicating that the energy transfer to PS2 and PS1 is much higher when nitrate is present and showing an involvement of this anion in the energy transfer mechanism of the two photosystems.

In conclusion, our results suggest that in addition to the unique role of NO_3^- in nitrogen metabolism of higher plants and algae it acts as a stimulating anion similar to Cl^- (Kelley and Izawa 1978) and HCO_3^- (Stemler 1982, Mende and Wiessner 1985) on the photosynthetic electron transport at the two photosystems. The action site of this anion in PS2 is somehow engaged in the electron release on the PS2-donor side before the action site of hydroxylamine as electron donor.

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