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Coordination of Chloroplastic Metabolism in N-Limited *Chlamydomonas reinhardtii* by Redox Modulation[1]

II. Redox Modulation Activates the Oxidative Pentose Phosphate Pathway during Photosynthetic Nitrate Assimilation

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The onset of photosynthetic NO_3^- assimilation in N-limited *Chlamydomonas reinhardtii* increased the initial extractable activity of the glucose-6-phosphate dehydrogenase (G6PDH), the key regulatory step of the oxidative pentose phosphate pathway. The total activated enzyme activity did not change upon NO_3^- resupply. The higher activity, therefore, represents activation of existing enzyme. No activation occurred during NH_4^+ assimilation. Incubation of extracts with DTT reversed the NO_3^- stimulation of G6PDH activity, indicating that the activation involved redox modulation of G6PDH. Phosphoribulosekinase, an enzyme activated by thioredoxin reduction, was inhibited at the onset of NO_3^- assimilation. A 2-fold stimulation of O_2 evolution and a 70% decrease in the rate of photosynthetic CO_2 assimilation accompanied the enzyme activity changes. There was an immediate drop in the NADPH and an increase in NADP upon addition of NO_3^-, whereas NH_4^+ caused only minor fluctuations in these pools. The response of *C. reinhardtii* to NO_3^- indicates that the oxidative pentose phosphate pathway was activated to oxidize carbon upon the onset of NO_3^- assimilation, whereas reduction of carbon via the reductive pentose phosphate pathway was inhibited. This demonstrates a possible role for the Fd-thioredoxin system in coordinating enzyme activity in response to the metabolic demands for reducing power and carbon during NO_3^- assimilation.

The assimilation of both N and carbon require the plant cell to supply electrons, ATP, and carbon skeletons. In the light, photosynthetic reactions provide the electrons and ATP, whereas organic carbon is principally supplied from the reductive pentose phosphate pathway (Syrett, 1981; Larsson et al., 1985; Robinson and Baysdorfer, 1985; Le Van Quy et al., 1991). When algal cells are grown with sufficient N, the demands of N metabolism are small in comparison to that of carbon metabolism (Turpin et al., 1991) and the interactions between these pathways is difficult to demonstrate. N limitation of *Selenastrum minutum*, a unicellular green alga, has been shown to increase its capacity for N assimilation relative to photosynthesis and has permitted detailed characterization of the metabolic response of this organism when N becomes available (Turpin et al., 1991). In *S. minutum*, the demand for carbon skeletons to synthesize amino acids at the onset of the assimilation of either NH_4^+ or NO_3^- stimulates respiration in the light and the dark (Weger and Turpin, 1989). The additional redox potential required to assimilate NO_3^- appears to be supplied in part by carbon respiration via the OPP pathway (Vanlerberghe et al., 1991; Huppe et al., 1992).

The change in metabolic priorities demonstrated for this system provides a means to examine the role that redox regulation may play in coordinating energy flow in the light. Unfortunately, the strength of the *S. minutum* cell wall does not permit the rapid extraction necessary to examine redox activation of enzymes. In the preceding paper, we developed a method using *Chlamydomonas reinhardtii* CW-15 cells that demonstrated that the redox activation states of two key enzymes in the coordination of chloroplastic carbon metabolism, G6PDH (EC 1.1.1.49) and PRK (EC 2.7.1.19), change relative to the photosynthetic electron supply (Farr et al., 1994). In this paper we, first, confirm that N-limiting *C. reinhardtii* causes an increase in the relative demands of carbon and N metabolism on cellular metabolism similar to that characterized in *S. minutum* and, second, examine the effect of altered metabolic demands for photosynthetic electrons on the poising of these redox-controlled enzymes. We show that redox regulation can be involved in more than sensing the presence or absence of light.

MATERIALS AND METHODS

Cells and Supplies

Chlamydomonas reinhardtii CW-15 cc-1883 cells were cultured as detailed by Farr et al. (1994). Biochemical reagents for determination of pyridine nucleotide levels were from Boehringer Mannheim and all other chemicals were as described by Farr et al. (1994).

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Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; NaTT, sodium tetrathionate; OPP, oxidative pentose phosphate; 6PG, 6-phosphogluconate; PRK, phosphoribulosekinase.

Measurements

Cells were incubated in darkness or saturating light (400 $\mu E\ m^{-2}\ s^{-1}$) for all experiments in sampling cuvettes as detailed by Vanlerberghe et al. (1991). Enzyme activation analysis was as described by Farr et al. (1994). Ammonia disappearance was measured according to Strickland and Parsons (1972). NO_3^- assimilation was typically determined as the disappearance of NO_3^- because when the appearance of NO_2^- was determined in an initial experiment, only very small amounts (less than 5 μM after 60 min) were produced during NO_3^- assimilation. NO_3^- concentrations were determined from the absorption difference at 202 nm and 250 nm of cell samples treated with 5% perchloric acid and 0.4% sulfamic acid (Larsson et al., 1982). NO_3^- was determined according to Weger et al. (1989). Photosynthetic CO_2 fixation was determined by measurement of incorporation of $^{14}CO_2$ into acid-stable products before and after addition of either NO_3^- or NH_4^+ to 5× concentrated cells (Elrifi and Turpin, 1986). All data were calculated from averaging rates determined in three separate time-course experiments, and the SE on any time point was less than 5% in all studies. The determination of pyridine nucleotide changes was as described by Huppe et al. (1992), except that cells were concentrated to approximately 10 μg Chl mL^{-1} and the sample size for cycling was optimized for each type of nucleotide determined. O_2 evolution was determined with an O_2 electrode (Hansatech, King's Lynn, UK).

RESULTS

Effect of N Assimilation on Physiological Parameters

N-limited *C. reinhardtii* cells assimilate either NO_3^- or NH_4^+ immediately upon resupply (data not shown). Assimilation continued linearly until the N source was depleted and the rate for NO_3^- assimilation was approximately 50% that of NH_4^+ (Table I). The photosynthetic carbon assimilation rate declined by approximately 70% immediately upon resupply of NO_3^- to N-limited *C. reinhardtii* cells (Table I) and NH_4^+ caused a decline of approximately 20%. Net O_2 evolution did not change when cells were supplied with NH_4^+, whereas NO_3^- addition stimulated O_2 release almost 2-fold (Table I).

Table I. *The effect of the assimilation of different N sources on photosynthesis*

Measurements of CO_2 assimilation and N assimilation were done with 5-fold-concentrated cells and O_2 evolution was measured with 2-fold-concentrated cells. Experiments to determine the effect of NH_4^+ and NO_3^- on the assimilation of CO_2 were performed at different times. All rates are in μmol mg^{-1} Chl h^{-1}.

Physiological Property	Light Control	NH_4^+ Addition	NO_3^- Addition
N assimilation rate		105	55
CO_2 assimilation, experiment 1	115		35
CO_2 assimilation, experiment 2	83	70	
O_2 evolution	276	284	480

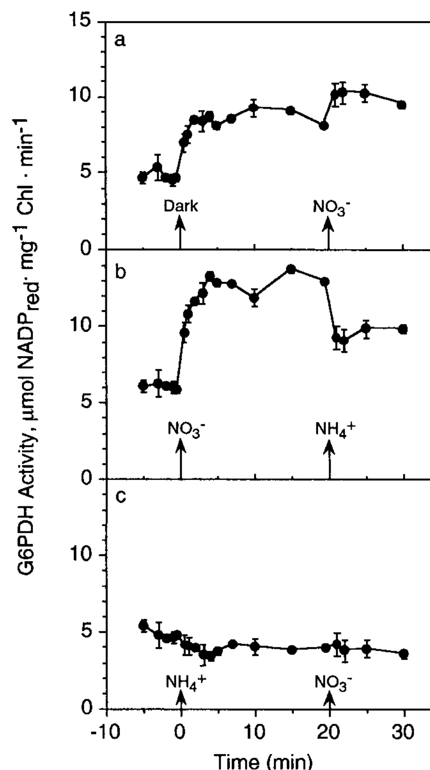

Figure 1. Response of G6PDH activity to the addition of darkness (a), NO_3^- (b), or NH_4^+ (c) as indicated. The sixth point is taken 30 s after the first treatment commenced. A second treatment as indicated was begun after 20 min. Activities were assayed in samples that had been frozen in liquid N_2 throughout the time course and were later thawed rapidly for immediate assay. The data are the average of three replicates.

G6PDH and PRK Activities Are Affected by the Addition of NO_3^- or Darkness

G6PDH activity increased immediately upon addition of either NO_3^- or transition to darkness, but no activation occurred when NH_4^+ was added (Fig. 1). *C. reinhardtii* assimilates NH_4^+ in preference to NO_3^- (Florencio and Vega, 1983). Interestingly, inhibition of NO_3^- assimilation by NH_4^+ addition decreased G6PDH activity but the enzyme activity was still higher than before NO_3^- addition. Addition of NO_3^- to the NH_4^+-assimilating cells did not influence the activity of G6PDH. When NO_3^- was added to darkened cells, there was a further activation of G6PDH. Neither G6PDH nor PRK activity was affected by the addition of either NO_3^- or NH_4^+ salts directly to extracts or assay mixtures (data not shown).

In contrast to G6PDH, PRK activity was inhibited upon addition of NO_3^- or dark to the *C. reinhardtii* (Fig. 2). The addition of NH_4^+ caused a small, transient increase in PRK activity. Addition of NO_3^- to the NH_4^+-assimilating cells did not change the activity of PRK. As with G6PDH, inhibiting NO_3^- assimilation by the addition of NH_4^+ partially reversed the effect of NO_3^- assimilation on PRK. The dark inactivation of the PRK was not influenced by the addition of either NO_3^- or NH_4^+ (data not shown).

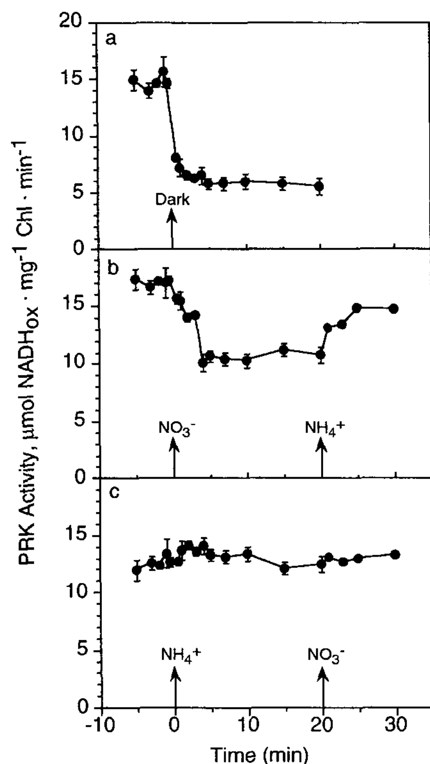

Figure 2. Response of PRK activity to the addition of darkness (a), NO_3^- (b), or NH_4^+ (c) as indicated. The sixth point is taken 30 s after the first treatment commenced. A second treatment as indicated was begun after 20 min. Activities were assayed in samples that had been frozen in liquid N_2 throughout the time course and were later thawed rapidly for immediate assay. The data are the average of three replicates.

Pyridine Nucleotide Pool Changes during N Assimilation

The onset of photosynthetic NO_3^- assimilation resulted in a marked drop in the NADPH/NADP ratio, which reflected an increase of NADP and a decrease of NADPH, within 5 s of N addition (Fig. 3, a and b). The NADP concentration remained greater than before the onset of assimilation, whereas the NADPH pool recovered to its pre-addition level within 10 min. Increased levels of NADP, however, caused the NADPH/NADP ratio to remain below its pre-addition value. The ratio of NADPH/NADP also dropped within 5 s of NH_4^+ addition but then oscillated (Fig. 3, c and d). There was little significant change in the actual concentrations of either NADPH or NADP during NH_4^+ assimilation. The small size of the NADH pool made it difficult to determine small changes in its level. There was no significant change initially in either NADH, NAD, or the NADH/NAD ratio with either NO_3^- or NH_4^+ addition; however, a slow, long-term increase in NADH levels occurred with both N sources that was not accompanied by any decrease in NAD (data not shown).

In Vitro Investigation of the Activation Changes of G6PDH and PRK

Incubation of extracts from NO_3^- or dark cells with DTT reversed the activation of G6PDH (Table II) so that the same level of activity remained regardless of whether cells had been incubated in light, in darkness, or with NO_3^-. When extracts were oxidized with NaTT there was no difference in the total amount of G6PDH activity between treatments. DTT was effective in activating PRK inhibited by either the NO_3^- or dark treatments, and oxidation of PRK with NaTT inhibited all extracts to a similar level of activity. The inhibition of G6PDH and the activation of PRK by DTT treatment followed time courses similar to those reported for extracts of darkened cells (Farr et al., 1994) (data not shown).

The metabolite 6PG is an allosteric inhibitor of spinach PRK and is reported to be key to the dark deactivation of this enzyme (Gardemann et al., 1983). To test if the activity changes found in our treatments resulted in part from the presence of 6PG, this metabolite was added during extraction and/or to the assay mixture at concentrations above the physiological concentration reported for darkened spinach cells. *C. reinhardtii* PRK was inhibited by approximately 50% by the addition of 4 mM 6PG to the assay mixture (Table III); however, a similar concentration present only in the extract itself resulted in essentially no inhibition (Table III).

DISCUSSION

Net O_2 evolution doubled during the photosynthetic assimilation of NO_3^- by *C. reinhardtii*, whereas NH_4^+ assimilation had little effect (Table I). NO_3^- assimilation initially requires the reduction of NO_3^- to NH_4^+ by the addition of eight electrons, six of which are added by the chloroplastic enzyme nitrite reductase. The rate of noncyclic electron transport is thought to be limited in saturating light by the rate of NADPH oxidation via carbon assimilation (Farquhar et al., 1980). The increased sink strength for photosynthetic electrons created by NO_3^- reduction likely increases the rate at which electron acceptors recycle, thereby allowing increased

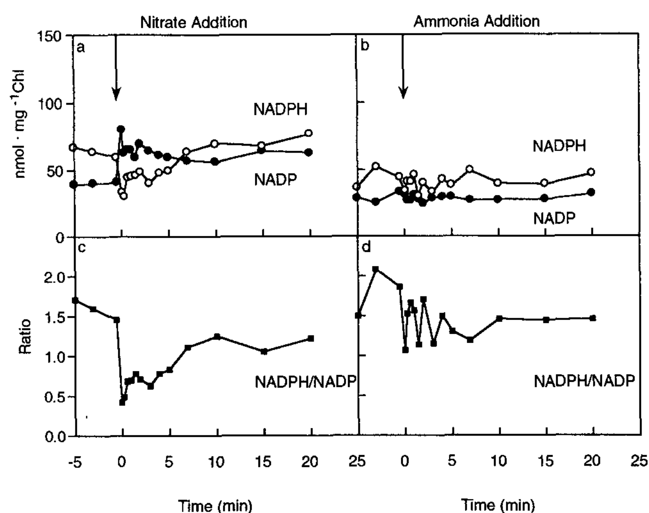

Figure 3. Response of NADP (●), NADPH (O), and the NADPH/NADP (■) ratios at the onset of NO_3^- (a and c) and NH_4^+ (b and d) assimilation (at the arrow) by *C. reinhardtii*. The fourth point is taken 5 s after N addition. The data are the average of three replicates and SE values for all points were less than 10%.

Table II. *Response of PRK and G6PDH activities in extracts from cells incubated in the light, in the dark, and in the light plus nitrate*

Cells were incubated for 25 min in the dark prior to sampling, moved into the light for 25 min, and finally NO_3^- was added and samples were taken after 10 min. Initial activity was recorded immediately after thawing. Aliquots for PRK activity were treated with 40 mM DTT for 1 h to reduce and 5 mM NaTT for 10 min to oxidize the sample. Aliquots for G6PDH activity were treated with 2.5 mM DTT for 10 min to reduce and 2 mM NaTT for 10 min to oxidize the sample. PRK activity is in μmol NADH oxidized mg^{-1} Chl min^{-1}; G6PDH activity is in μmol NADP reduced mg^{-1} Chl min^{-1}.

Treatment	Initial Activity	Reduced Activity	Oxidized Activity
PRK			
Light	11.3	14.8	0.7
Dark	3.3	13.0	0.7
Nitrate	4.5	12.5	1.4
G6PDH			
Light	4.6	1.4	15.5
Dark	9.0	1.9	15.2
Nitrate	13.4	1.4	16.0

rates of noncyclic photosynthetic electron flow. The capacity to double the rate of electron transport shows that it is not a limitation of photosynthetic light harvesting that causes the decrease of CO_2 fixation that occurs during NO_3^- assimilation. *C. reinhardtii* CW-15 cc-1883 grown under N limitation appears to adjust the relative demands of carbon and N metabolism in a manner similar to N-limited *S. minutum* (Weger and Turpin, 1989). Therefore, we can use this cell wall-less alga to investigate the response of enzyme activity during the activation of N assimilation.

Recent work in our laboratory on the metabolite changes in *S. minutum* at the onset of N assimilation in the light and dark has provided strong evidence for an increased carbon flux through G6PDH (Vanlerberghe et al., 1991; Huppe et al., 1992), the key regulatory enzyme of the OPP pathway. The increased activity of this enzyme should increase the rate at which carbon is respired via the OPP pathway and implicates the OPP pathway in providing some reductive energy during both photosynthetic and heterotrophic NO_3^- assimilation. Using the wall-less alga *C. reinhardtii* CW-15, the activation of G6PDH was monitored during this activation to help determine the regulatory mechanisms involved in increasing OPP pathway activity.

G6PDH was activated immediately at the onset of photosynthetic NO_3^- assimilation in *C. reinhardtii*, whereas NH_4^+ addition had no effect on the activity of this enzyme (Fig. 1). Nitrate addition stimulated a greater increase in G6PDH activity than occurs during a transition from light to dark, and there was a further enhancement of dark-activated G6PDH activity when NO_3^- was supplied. The NO_3^- activation of G6PDH was dependent on NO_3^- reduction and not on the presence of NO_3^- per se. Three lines of evidence support this view: (a) NO_3^- salts added directly to extracts did not affect the amount of G6PDH that could be assayed; (b) suppression of NO_3^- reduction by NH_4^+ addition (Florencio and Vega, 1983) resulted in a decrease in G6PDH activity (Fig. 1); (c) NO_3^- addition to cells assimilating NH_4^+ did not change G6PDH activity (Fig. 1).

It has been reported previously that addition of NO_3^- to NH_4^+-grown cells leads to an increase in the amount of G6PDH protein in the cells, which can be blocked by protein synthesis inhibitors (Hipkin and Cannons, 1985). In our experiments, the total activity of G6PDH measured by oxidation with NaTT was the same regardless of treatment; therefore, the increase of activity represented an activation of existing enzyme protein. The G6PDH activation by NO_3^- assimilation in both light and dark supports the hypothesis that the OPP pathway provides reductant and carbon for both photosynthetic and heterotrophic NO_3^- assimilation.

G6PDH activation by NO_3^- assimilation was reversed by treatment with DTT (Table II), resembling the redox regulation found upon transition from light to dark (Farr et al., 1994). The response of G6PDH activity to the onset NO_3^- assimilation in the light appears to involve a reversal of the reductive inhibition of this enzyme by light. Further, the additional activation of G6PDH in the dark upon addition of NO_3^- (Fig. 1) suggests that redox regulation may have a role in metabolic regulation in the dark as well as during photosynthesis.

Unlike G6PDH, PRK is inhibited by oxidation and activated by reduction during light/dark transitions (Wolosiuk and Buchanan, 1978; Porter et al., 1986; Rault et al., 1991). The onset of NO_3^- assimilation inhibited PRK activity in our cells (Fig. 2), and treatment with DTT reactivated PRK, implying that the inhibition must result from redox modulation of the enzyme activation state (Table II). Interestingly, the inhibition of NO_3^- assimilation by NH_4^+ addition led to partial recovery of PRK activation (Fig. 2). The decrease in PRK activation during NO_3^- reduction resembled the inactivation by darkness, but was somewhat slower (Fig. 2). Although *C. reinhardtii* PRK, like the spinach enzyme, is inhibited by 6PG (Table III), which is produced by G6PDH, an increase of this metabolite does not explain the decrease of activity during NO_3^- assimilation. 6PG appears to allosterically inhibit the algal PRK; therefore, our standard conditions

Table III. *Response of PRK activity to treatments with 6PG*

Cells were incubated for 25 min in the light prior to sampling. 6PG was added directly to the extraction buffer mixture or was added to the assay mixture to the final concentration indicated. Fifty microliters of extract was assayed in a total volume of 1 mL. PRK activity is in μmol NADH oxidized mg^{-1} Chl min^{-1}.

[6-PG] Added	To Extract		To Assay	
	Activity	Percent activity	Activity	Percent activity
mM				
0.0	12.0	100	13.8	100
0.1	14.0	117	14.1	102
0.5	13.6	113	12.6	91
1.0	13.3	111	11.2	81
2.0	12.9	108	9.6	70
4.0	12.2	102	7.2	52

sufficiently diluted the effector to overcome the effect of inhibitory concentrations added directly to the extract (Table III). It should be noted that the spinach PRK was shown to be inhibited by 300 μM 6PG, a concentration close to the estimated level of this metabolite in darkened chloroplasts (Gardemann et al., 1983). The algal PRK required 4 mM 6PG to reach 50% inhibition (Table III).

The probable redox regulator of both the G6PDH and PRK in *C. reinhardtii* is the Fd-thioredoxin system. Our observations are consistent with the onset of rapid NO_3^- reduction increasing the demands for electrons and oxidizing the Fd-thioredoxin system. The lack of electron flow to the Fd-thioredoxin system would permit activation of G6PDH and the OPP pathway in the light and would reverse the light activation of PRK. Decreasing PRK activity would decrease competition for reducing power between the reductive pentose phosphate pathway and NO_3^- reduction, whereas increasing G6PDH activation would increase the reductant produced via the OPP pathway and provide carbon skeletons for amino acid synthesis.

Two observations support this hypothesis. First, an immediate decrease in NADPH and a reciprocal increase in NADP occurs in *C. reinhardtii* cells upon NO_3^- addition, which results in a dramatic decrease in the NADPH/NADP ratio (Fig. 3, a and c). There is no significant effect on the NADP and NADPH levels when NH_4^+ is added (Fig. 3, b and d) and no significant change in the other reductant pool in the cell, NAD(H) (data not shown).

Second, a 70% decrease of CO_2 fixation occurs during NO_3^- assimilation by *C. reinhardtii*, whereas NH_4^+ assimilation resulted in only a minor change (Table I). Suppression of photosynthesis during N assimilation by *S. minutum* has been shown to involve a limitation of ribulose-1,5-bisphosphate carboxylase by ribulose bisphosphate (Elrifi and Turpin, 1985; Elrifi et al., 1988), which indicates inhibition of the regeneration phase of the reductive pentose phosphate pathway (Buchanan, 1991). The inhibition of *C. reinhardtii* PRK found upon NO_3^- resupply provides a biochemical explanation for this phenomenon.

Upon NO_3^- resupply to N-limited *C. reinhardtii*, photosynthetic carbon assimilation decreases and carbon oxidation is stimulated, a response that closely resembles the redirection of carbon in the chloroplast upon a light/dark transition. We have previously demonstrated that the supply of electrons from photosynthesis is important in poising the redox activation of two enzymes, PRK and G6PDH (Farr et al., 1994), which are key controllers of the carbon flux in the chloroplast (Buchanan, 1991). In this study we have demonstrated that the reductive regulation of PRK and G6PDH is also sensitive to the demand for electrons in biosynthesis, specifically NO_3^- reduction. Under such demands, the chloroplastic OPP pathway can be activated during photosynthesis. The additional activation of G6PDH in the dark by NO_3^- implies a role for redox modulation in the dark that merits further investigation. These results show that the redox regulation, probably involving the Fd-thioredoxin system, senses not only the supply of energy from light but the demands for energy by metabolism and that both factors are important in regulating the flux of carbon in the chloroplast.

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