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# On The Regulation of Spinach Nitrate Reductase[1]

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## ABSTRACT

**A coupled assay has been worked out to study spinach (*Spinacea oleracea* L.) nitrate reductase under low, more physiological concentrations of NADH. In this assay the reduction of nitrate is coupled to the oxidation of malate catalyzed by spinach NAD-malate dehydrogenase. The use of this coupled system allows the assay of nitrate reductase activity at steady-state concentrations of NADH below micromolar. We have used this coupled assay to study the kinetic parameters of spinach nitrate reductase and to reinvestigate the putative regulatory role of adenine nucleotides, inorganic phosphate, amino acids, and calcium and calmodulin.**

Beside $CO_2$ assimilation, nitrate assimilation is a major function of a leaf cell. NR[3] can be regarded as a key enzyme in this process. In spite of a contradicting report (14), most evidence supports the view that nitrate reductase is located in the cytosolic compartment (2, 21, 32). As sources of redox equivalents for the generation of NADH required for nitrate reduction, the oxidation of glyceraldehyde-3-phosphate via the cytosolic NAD-GAPDH (15) and/or the oxidation of malate catalyzed by the cytosolic MDH (17) have been discussed. In both cases, the redox equivalents could be ultimately provided by the photosynthetic light reaction in the chloroplast, either by a triose phosphate-phosphoglycerate shuttle catalyzed by the phosphate translocator (7) or by a malate-oxaloacetate shuttle mediated by specific malate and oxaloacetate transport (10) across the chloroplast envelope. Alternatively, the cytosolic NAD can also be reduced by NADH generated in the mitochondria by tricarboxylic acid cycle via a malate-oxaloacetate shuttle between the mitochondrial matrix and the cytosol (6, 35). This way could make it possible that the redox equivalents required for nitrate reduction might also be provided during darkness.

The product of NR reaction, nitrite, is imported into the chloroplast, where it is further reduced to ammonia via the ferredoxin-dependent nitrite reductase. This reaction is directly coupled to the photosynthetic electron transport and is, therefore, essentially light dependent, although some nitrite reduction in the absence of light, although at a lower rate, has also been suggested (1, 13, 33).

Despite the high rate of nitrate assimilation in leaves, even during a light-dark transient, nitrite does not markedly accumulate in leaf tissues (B Riens, HW Heldt, unpublished results). The question arises in which way the activity of NR in the cytosol is regulated such that it can respond instantaneously to a decrease of nitrite reduction in the chloroplast. Previous reports on the inhibition of NR by ADP (5, 20) made it seem possible that the cytosolic ATP/ADP ratio may be a regulatory factor. Also phosphate has been reported to stimulate NR activity (20, 23). NADH, as well as being substrate, has been postulated to be involved in a regulatory mechanism by which the enzyme is activated by reduction with NADH and cyanide (19, 27), whereas the activity is restored by oxidation with blue light and flavines as the most efficient treatment (28). Feedback regulation by amino acids (22) or by the metabolic regulator calmodulin (26) have also been investigated. Finally, NR activity in spinach leaves has been reported to undergo only small daily fluctuations (29), which indicates that regulatory mechanisms based on the biosynthesis and degradation of enzyme protein are too slow to play any significant role in the rapid arrest of nitrate reduction in the dark.

Common to all the studies briefly summarized above is the fact that they have been carried out by using a standard assay in which the purified preparation of NR is incubated in the presence of the substrates, NADH and nitrate, and the activity is determined either by colorimetric measurement of the nitrite formed or by monitoring the oxidation of NADH (9). The concentration of reactants usually employed in the standard assay is in the range of millimolar for nitrate and around 0.1 mM for NADH. The cytosolic level of nitrate in the leaf cell has proved difficult to determine because most of these anions concentrate in the vacuole (18, 24), but the "metabolic" pool seems to be smaller than the concentration of this substrate normally present in the standard assay. Similarly, the cytosolic concentration of NADH has proved very difficult to measure accurately because of the low level of this reductant, which is essentially oxidized in all the cell compartments (11, 12), but we can safely assume that the concentration of NADH normally employed in the standard assay is one or two orders of magnitude higher than its physiological concentration in the cytosol of the leaf cell. The concentration of reactants in the standard assay is, therefore, far from physiological.

We have worked out a coupled assay system which allows the study of NR under low and steady-state concentrations of

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[3] Abbreviations: NR, nitrate reductase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GOT, glutamate oxaloacetate transaminase; MDH, malate dehydrogenase.

NADH. By using this assay system we have reinvestigated the putative regulatory roles of adenine nucleotides and phosphate, as well as those of amino acids and calcium/calmodulin, under more physiological conditions.

## MATERIALS AND METHODS

### Enzyme Preparation

NR and MDH were prepared from 50 g of leaves harvested from 10 weeks old spinach (*Spinacea oleracea* L.) plants, which were ground in 100 mL of a buffer consisting of 50 mM potassium phosphate (pH 7.5), 1 mM DTT, 1 mM cysteine, and 10 μM flavin-adenine dinucleotide (buffer A). The grinding was performed in a Polytron homogenizer for 1 min. The homogenate was filtered through cheesecloth and Miracloth, and the grinding vessel was rinsed with 50 mL of the same buffer. The crude extract was centrifuged at 40,000*g* for 1 h and the pellet discarded. To the clear supernatant solid ammonium sulfate was added up to 50% saturation and, after stirring for 30 min, the suspension was centrifuged at 17,000*g* for 30 min. The pellet was resuspended in 10 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM cysteine, and 10 μM flavin-adenine dinucleotide (buffer B). The turbid suspension was clarified by centrifugation at 12,000*g* for 5 min. An aliquot of the clear solution, containing approximately 150 mg protein, was loaded onto a Blue Sepharose CL-6B affinity column of 10 mL of bed volume prepared in a plastic syringe with suitable tubing. The column was eluted with 50 mL of 0.1 M KCl in buffer B and then with 25 mL of 0.1 mM NADH in the same buffer. Fractions of 5 mL were collected, and active fractions were pooled and concentrated by ultrafiltration to approximately 2 mL; the same volume of glycerol was added, and the resulting extract in 50% glycerol was stored at −20°C. This procedure yielded a nitrate reductase preparation with a specific activity of 3 μmol $min^{-1}$ mg $protein^{-1}$. The purification was 150-fold.

### NR

NR activity was in many instances determined by measuring the formation of $NO_2^-$ (5). The enzyme extracts were incubated in 0.5 mL of a mixture containing 40 mM potassium phosphate (pH 7.5), 5 mM $KNO_3$, and 0.1 mM NADH. Incubations were carried out at 25°C for 15 min and were stopped by addition of 0.25 mL of 1% sulfanilamide in 1.5 N HCl followed by 0.25 mL of 0.025% *N*-1-napthylethylenediamine, then the red color was allowed to develop for 30 min and the absorbance of the solution was measured at 540 nm.

### MDH/NR Coupled Assay

In this assay (25), the reduction of $NO_3^-$ to $NO_2^-$ is coupled to the oxidation of malate to oxaloacetate catalyzed by the NAD-MDH. The reaction mixture contained 20 mM potassium phosphate (pH 7.2), 5 mM $KNO_3$, 0.5 mM NAD, 5 mM L-glutamate, 2 mM L-malate, 0.2 unit/mL of pig heart glutamate-oxaloacetate transaminase (from Boehringer-Mannheim), and variable amounts of spinach NR and MDH. The reaction was carried out in a 3 mL quartz cuvette at 20°C, in Sigma ZFP 22 spectrophotometer to monitor the concentration of NADH, and samples of suitable sizes were withdrawn at regular intervals to determine $NO_2^-$ formation as described above.

The concentration of NADH in the reaction mixture can be increased in the course of the incubation by addition of MDH. In such a way it is possible to measure rates of $NO_2^-$ formation at five or six different steady-state concentrations of the reductant.

## RESULTS AND DISCUSSION

### MDH/NR Coupled Assay

In this assay the reduction of nitrate to nitrite is coupled to the oxidation of malate to oxaloacetate according to the stoichiometry shown in Figure 1. Since the equilibrium of the MDH reaction strongly favors the formation of malate, the accumulation of oxaloacetate must be avoided. Addition of the auxiliary enzyme GOT, as well as glutamate, catalyzes the conversion of oxaloacetate to aspartate, thus allowing the reaction to proceed. This assay has been discussed in detail previously (25).

A mixture containing the enzymes NR and GOT and the substrates nitrate, malate, NAD, and glutamate in buffer is preincubated in a cuvette contained in a spectrophotometer until the signal corresponding to $A_{334}$ stabilizes. The addition of a certain activity of MDH results in the formation of NADH. After the concentration of NADH has reached a plateau reflecting the steady-state concentration, samples are taken at regular intervals from the reaction mixture to determine nitrite. From the values thus obtained, NR activity is calculated. In the same assay, the steady-state concentration of NADH can be successively increased by addition of MDH. After each addition the trace is allowed to stabilize at the new plateau, and then new aliquots are withdrawn from the cuvette to determine nitrate reductase activity via the measurement of nitrite formation.

In this manner the $K_m$ of NADH can be accurately measured under steady-state conditions. Figure 2 shows the double reciprocal plot of such an experiment, in which the $K_m$ for NADH is determined as 1.37 μM. In seven similar experiments the average $K_m$ value for NADH was 1.40 μM (± 0.3 μM standard deviation). To simulate physiological conditions, these measurements were done in the presence of a 0.5 mM NAD. Therefore, our results show that spinach NR is half saturated at a NADH/NAD ratio of $2.8 \times 10^{-3}$. This value is very close to the NADH/NAD ratio which has been evaluated

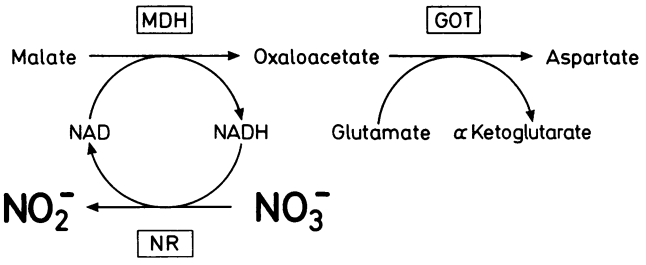


**Figure 1.** Reaction scheme of the NR activity assay.

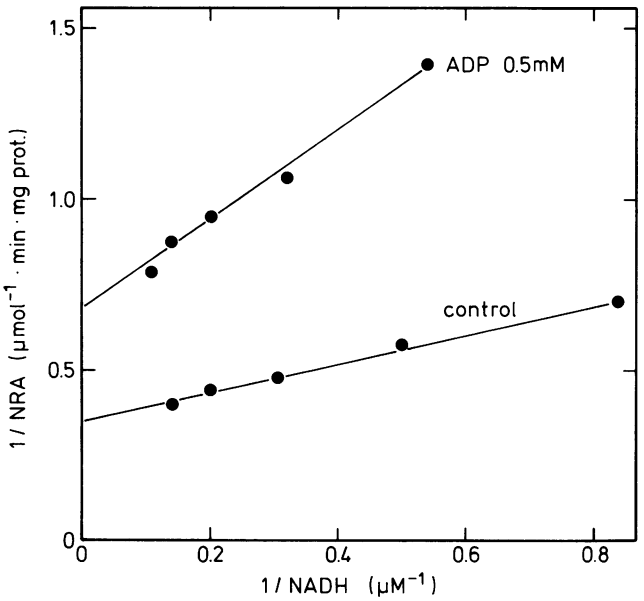


**Figure 2.** Effect of ADP on the dependence of NR activity (NRA) on the steady-state concentration of NADH. Double reciprocal plot of the data. Control: $K_m$ = 1.37 μM, $V_{max}$ = 2.98 μmol min$^{-1}$ mg protein$^{-1}$. In the presence of 0.5 mM ADP: $K_m$ = 2.09 μM, $V_{max}$ = 1.53 μmol min$^{-1}$mg protein$^{-1}$.

for the cytosolic fraction of spinach leaves by nonaqueous fractionation (B Riens, HW Heldt, in preparation), and it is also similar to the NADH/NAD ratio in the cytosol of animal cells (4). In many experiments, instead of using commercially available MDH from yeast, a 40-fold purified preparation of MDH from spinach leaves was employed. The results obtained were identical with those shown above.

In another approach, the reduction of nitrate was coupled to the oxidation of D-glyceraldehyde-3-phosphate as catalyzed by glyceraldehyde-3-phosphate dehydrogenase. This assay also contained triosephosphate isomerase in order to use dehydroxyacetone phosphate as substrate, and phosphoglycerate kinase and ADP in order to convert 1,3-biphosphoglycerate into 3-phosphoglycerate. This coupled assay of nitrate reductase activity yielded essentially the same results as the MDH/NR coupled assay. Since the ADP required for the coupled GAPDH/NR assay results in a partial inhibition of nitrate reductase activity, as shown in the following, the MDH/NR coupled assay appeared to be more suitable for our studies.

### Effect of Pi

NR activity has been reported to be stimulated by Pi, although this stimulatory effect was only detected at very high concentrations of NADH, 0.1 to 0.2 mM (20, 23). The cytoplasmic Pi concentration in leaves has been estimated as 20 to 30 mM (31), and measurements of phosphorylated intermediate provides indirect evidence that this could decline by 10 mM during photosynthesis (8). It was investigated how physiological concentrations of Pi in the reaction mixture affected nitrate reductase in the presence of 3 μM NADH. In this experiment, contrary to the others, Hepes buffer was used instead of phosphate. The results (Fig. 3a) show a clear inhibition of nitrate reductase by potassium phosphate, which contrasts with previous reports and our own experiment carried out with the standard assay (Fig. 3b), although the magnitude of the effect still seems to be insufficient to account for the regulation of nitrate reductase in the light/dark transition.

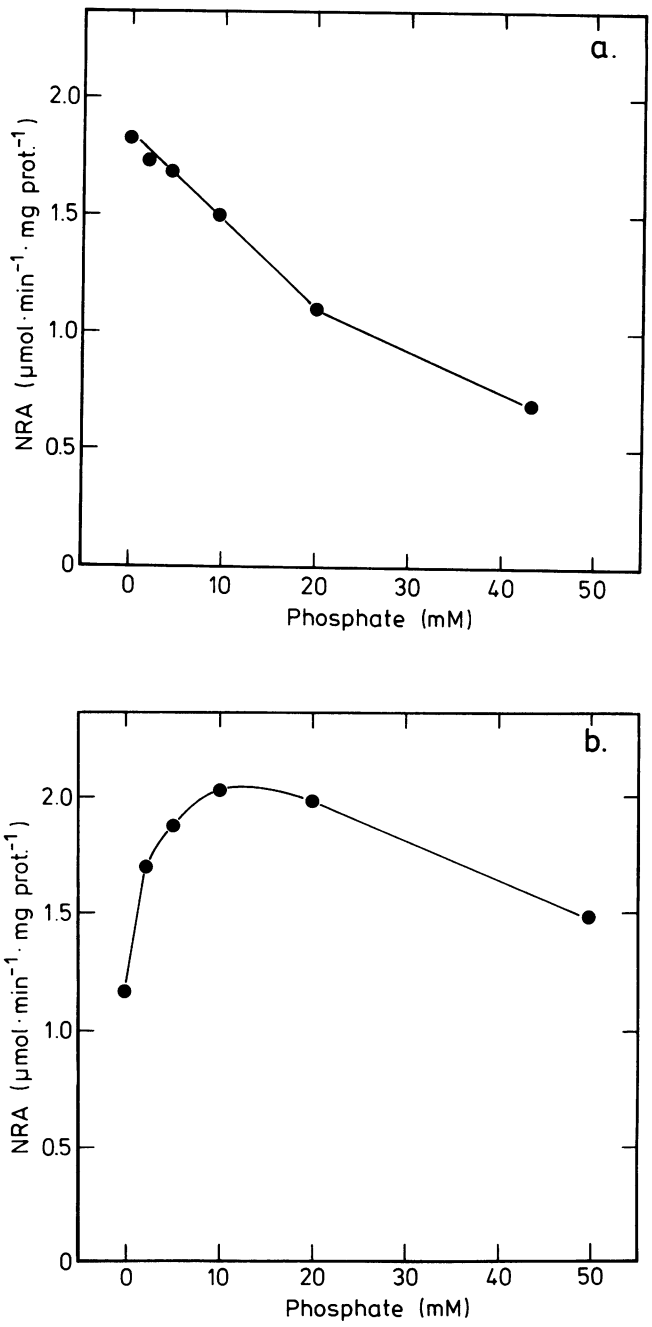


**Figure 3.** Effect of phosphate on NR activity (NRA). (a) Experiment carried out by using the MDH/NR coupled assay at a steady-state concentration of NADH of approximately 3 μM, and 50 mM Hepes (pH 7.4). (b) Experiment performed using the standard assay as described in "Materials and Methods," but with 50 mM Hepes (pH 7.4), 2 mM $KNO_3$, and 0.2 mM NADH.

Since we have been interested in testing the regulation of nitrate reductase under physiological conditions, in all the experiments described below phosphate buffer was used, in spite of its inhibitory effect on the enzyme.

### Inhibition by Adenine Nucleotides

The *in vitro* inhibition of NR from tomato leaves was first reported by Nelson and Ilan (20). Their assays, however, were carried out at very high concentrations of both NADH (1 mM) and ADP (2 mM). Later, this inhibitory effect was thoroughly investigated by Eaglesham and Hewitt (5) using spinach nitrate reductase. Their results showed a complex inhibition pattern depending on the concentration of NADH and the presence of thiol reagents in the assay mixture. They concluded that inhibition of NR by ADP in the dark would never exceed 20% of the activity measured in the light, which is really modest.

We investigated the effect of ADP on the activity of nitrate reductase under steady-state conditions. As shown from Figure 2, the addition of 0.5 mM ADP, a concentration within the physiological range (30), the $K_m$ for NADH is found to be increased from 1.37 to 2.09 $\mu$M, whereas the $V_{max}$ decreased from 2.98 to 1.53 $\mu$mol $min^{-1}$mg $protein^{-1}$. AMP at the physiological concentration of 0.25 mM did not show any marked effect on the enzyme.

The question arose whether the $Mg^{2+}$ concentration had an effect on the inhibitory action of adenine nucleotides on nitrate reductase activity. The effect of $Mg^{2+}$ was investigated in the presence of ATP/ADP at a ratio of 8:1 (2.22 mM ATP and 0.28 mM ADP) and keeping a steady-state concentration of NADH of approximately 1 $\mu$M. Results (Fig. 4) clearly show that addition of $Mg^{2+}$ counteracts the inhibitory effect of adenine nucleotides. We also studied the effect of $Mg^{2+}$ and adenine nucleotides on the kinetic parameters of NR (Table I). As discussed above, the presence of adenine nucleotides clearly affects kinetic parameters of NR but addition of $Mg^{2+}$ clearly counteracts the inhibitory effect of adenine nucleotides.

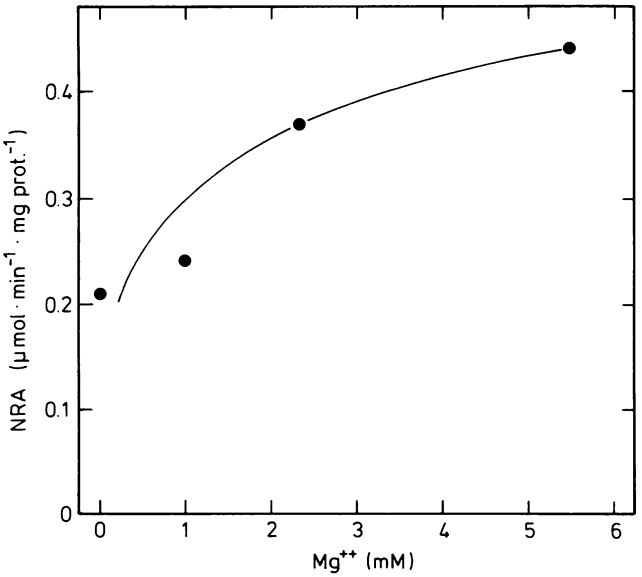


**Figure 4.** Effect of $Mg^{2+}$ concentration on the activity of NR in the presence of 1 $\mu$M NADH, 2.22 mM ATP, and 0.28 mM ADP.

**Table I.** *Effect of Adenine Nucleotides and $Mg^{2+}$ on Kinetic Parameters of NR*

The total concentration of adenine nucleotides, ATP + ADP, was 2.5 mM.

| Treatment | $K_m$ NADH | $V_m$ |
|---|---|---|
| | $\mu$M | $\mu$mol/min·mg |
| Control | 1.44 | 2.46 |
| ATP/ADP=2:1 | 4.58 | 0.97 |
| + 5 mM $Mg^{2+}$ | 2.80 | 1.58 |
| ATP/ADP=8:1 | 4.94 | 0.89 |
| + 5 mM $Mg^{2+}$ | 2.67 | 1.01 |

**Table II.** *Effect of Phosphate, Adenine Nucleotides, and $Mg^{2+}$ on Kinetic Parameters of NR*

The experiment was performed in 50 mM Hepes buffer (pH 7.4).

| Pi | ATP/ADP | 5 mM $Mg^{2+}$ | $K_m$ NADH | $V_m$ |
|---|---|---|---|---|
| mM | | | $\mu$M | $\mu$mol/min·mg |
| 5 | 8:1 | − | 5.12 | 6.15 |
| 5 | 8:1 | + | 1.92 | 4.97 |
| 20 | 1:1 | − | 14.25 | 7.36 |
| 20 | 1:1 | + | 4.25 | 6.08 |

Table II shows the results of an experiment in which the effect of extreme fluctuations of Pi, ATP/ADP ratio and $Mg^{2+}$ on NR activity was tested. The results show that a change from a very high phosphorylation state (Pi 5 mM, ATP/ADP = 8:1) to a very low phosphorylation state (Pi 20 mM, ATP/ADP = 1:1), which may be more extreme than normally observed (30), decreased the catalytic efficiency of nitrate reductase by a factor of about two.

### Other Effectors

We further investigated whether major metabolites of cytosolic metabolism, *e.g.* amino acids and glycolytic intermediates, when added at physiological concentration had any effect on nitrate reductase activity assayed in the presence of a steady-state NADH concentration of 1 to 2 $\mu$M. A number of amino acids, Glu (40 mM), Asp (45 mM), Gln (35 mM), Asn (5 mM), Ala (10 mM), Ser (50 mM), Gly (10 mM), Cys (10 mM), as well as $NH_4Cl$ (3 mM), and $\alpha$-ketoglutarate (1 mM) were tested without finding any significant effect (results not shown), which agrees with a previous report (22). Glycolytic intermediates like phosphoglycerate (2 mM), glucose-6-P (2 mM), fructose-6-P (1 mM), fructose-1,6-$P_2$, (1 mM), and phosphoenolpyruvate (1 mM) were also without any significant effect (results not shown). We also did not observe any effect of $10^{-2}$ to $10^2$ $\mu$M $Ca^{2+}$, either in the absence or in the presence of 0.5 $\mu$M calmodulin, on NR activity assayed at a steady-state concentration of about 1 $\mu$M.

## CONCLUSIONS

It was the aim of our investigation to try to elucidate the regulatory mechanism by which NR is switched off during

the light to dark transition. Our starting assumption was that nitrate reduction is essentially light dependent, for the chloroplastic nitrite reductase is linked to the photosynthetic electron transport through ferredoxin. If this postulation is correct, and there is no other system to dispose the nitrite formed, then a NR activity of 6 $\mu$mol/h·mg Chl would result in an accumulation of nitrite equivalent to 0.1 $\mu$mol/min·mg Chl. Such an accumulation of nitrite has not been observed in spinach plants abruptly transferred to darkness in a growth chamber (B Riens, HW Heldt, unpublished results).

The inhibitory effect of adenine nucleotides on nitrate reductase has been so far investigated by using very high concentrations of NADH. We have put much emphasis in testing this effect under physiological conditions. The coupled assay described here allows the assay of nitrate reductase at very low, steady-state concentrations of NADH, conditions which are certainly more physiological. The results derived from these experiments are in good agreement with other previous reports (5, 20), and little regulatory function can be attributed to adenine nucleotides by themselves.

The effect of phosphate clearly illustrates the importance of assaying enzyme activities under physiological conditions. At high, non-physiological concentrations of NADH, Pi was found to stimulate nitrate reductase (20, 23) (see also Fig. 3b), at least until certain concentrations, and this effect was reported to depend on the concentration of NADH. At low NADH concentrations, phosphate was found to inhibit NR activity at the whole range of physiological concentrations. No significant effect has been observed by amino acids and calcium/calmodulin. The effects of adenine nucleotides, in the presence of $Mg^{2+}$, and phosphate seem to be insufficient to switch off NR activity even when assayed together at concentrations supposed to be close to those prevailing in the cytosol of the leaf cell in the light or in darkness. Thus, for these effects to have a regulatory function *in vivo*, the fluctuations of Pi, $Mg^{2+}$, and adenine nucleotides should be accompanied by variations in the level of substrates.

It seems, therefore, that there are other factors responsible for an immediate stop of nitrate reduction upon darkening. One possibility might be that upon darkening the availability of nitrate is restricted. The cytosolic level of nitrate is difficult to evaluate since by far most of this anion is located in the vacuole. In the case of cyanobacteria, it has been shown (16) that nitrate taken up into the cells may regulate nitrate assimilation. Since in illuminated spinach leaves a large portion of the assimilated nitrate is delivered from the leaf vacuole (29), an immediate stop of NR due to restricted availability of nitrate would imply an immediate stop not only of the nitrate uptake into the leaf cell, but also of the nitrate release from the leaf vacuole. On the other hand, the filling of the leaf vacuoles with nitrate observed in spinach leaves during the dark period (29) clearly shows that nitrate can be rapidly taken up into leaf cells also during darkness. For these reasons, an instantaneous stop of nitrate reductase due to a restriction of nitrate availability during a light-dark transient is difficult to visualize from our present knowledge. An alternative explanation for the apparent rapid decrease of NR could be lack of NADH. Although the export of redox equivalents from the chloroplast stroma via malate-oxaloacetate shuttle will come to an immediate stop upon darkening, mitochondrial metabolism should be capable of providing the necessary redox equivalent via a malate-oxaloacetate shuttle (6). Because of the high redox gradient supposed to exist between the NADH/NAD couple in the mitochondrial matrix and the cytosol (25), the very active malate-oxaloacetate shuttle between the mitochondrial matrix and the cytosol would have to be strongly regulated in order to restrict the generation of NADH in the cytosol. Further investigations are required to elucidate this.

An alternative explanation for the lack of nitrite accumulation observed in spinach leaves upon darkening could be a continuation of nitrite reduction in the chloroplast compartment at the expense of either NADPH provided by oxidative pentose phosphate pathway or by mitochondrial respiration via the malate/oxaloacetate shuttle. Since the activation of the key enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase requires the oxidation of the enzyme proteins by oxidized thioredoxin (3), this activation should take considerable time, during which a strong accumulation of nitrite were to be expected, which is not observed. The involvement of mitochondrial respiration in nitrite reduction in the dark has been reported for green algae (34), but its putative role in higher plants is still debatable.

On the other hand, regulatory mechanisms at the level of biosynthesis and degradation of enzyme protein, or daily variations of enzyme activity, seem to us too slow to account, by themselves, for the lack of accumulation of nitrite in spinach leaves within the first few minutes of the light to dark transient.

Although the present publication clarifies the effect of metabolites on the activity of nitrate reductase, it cannot answer the question whether and how nitrate reductase activity is adjusted to photosynthetic electron transport. Therefore, a better understanding of the modulation of nitrate reduction in the leaf cell would require the knowledge of the cytosolic level of the substrates, NADH and nitrate, both in the light and darkness.

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## LITERATURE CITED

1. **Aslam M, Huffaker RC, Rains DW, Rao KP** (1979) Influence of light and ambient carbon dioxide concentration on nitrate assimilation by intact barley seedlings. Plant Physiol **63:** 1205–1209
2. **Beevers L, Hageman RH** (1980) Nitrate and nitrite reduction. *In* BJ Miflin, ed, The Biochemistry of Plants, Vol. 5. Academic Press, New York, pp 115–168
3. **Buchanan BB** (1980) The role of light in the regulation of chloroplast enzymes. Annu Rev Plant Physiol **31:** 341–374
4. **Bücher T, Klingenberg M** (1958) Wege des Wasserstoffs in der lebendigen Organisation. Angew Chemie **70:** 552–570
5. **Eaglesham ARJ, Hewitt EJ** (1975) Inhibition of nitrate reductase from spinach (*Spinacea oleracea* L.) leaf by adeninenuclotides. Plant Cell Physiol **16:** 1137–1149
6. **Ebbighausen H, Chen J, Heldt HW** (1985) Oxaloacetate translocator in plant mitochondria. Biochim Biophys Acta **810:** 184–199

7. **Flügge UI, Heldt HW** (1984) The phosphate-triose phosphate-phosphoglycerate translocator of the chloroplast. Trends Biochem Sci **9:** 530–533
8. **Gerhardt R, Stitt M, Heldt HW** (1987) Subcellular metabolite levels in spinach leaves. Regulation of sucrose synthesis during diurnal alterations in photosynthetic partitioning. Plant Physiol **83:** 399–407
9. **Hageman RH, Reed AJ** (1980) Nitrate reductase from higher plants. Methods Enzymol **69:** 270–280
10. **Hatch MD, Dröscher L, Flügge UI, Heldt HW** (1984) A specific translocator for oxaloacetate transport in chloroplasts. FEBS Lett **178:** 15–19
11. **Heber UW, Santarius KA** (1965) Compartmentation and reduction of pyridine nucleotides in relation to photosynthesis. Biochim Biophys Acta **109:** 390–408
12. **Heineke D** (1988) Zur Bedeutung des Phosphates fur die Regulation der Photosynthese. PhD thesis, University of Goettingen
13. **Jones RW, Sheard RW** (1978) Accumulation and stability of nitrite in intact aerial leaves. Plant Cell Lett **11:** 285–291
14. **Kamachi K, Amemiya Y, Ogura N, Nakagana H** (1987) Immunogold localization of nitrate reductase in spinach (*Spinacea oleracea*) leaves. Plant Cell Physiol **28:** 333–338
15. **Klepper L, Flesher D, Hageman RH** (1971) Generation of nicotinamide adenine dinucleotide for nitrate reduction in green leaves. Plant Physiol **48:** 580–590
16. **Lara C, Romero JM, Coronil T, Guerrero MG** (1987) Interactions between photosynthetic nitrate assimilation and $CO_2$ fixation in cyanobacteria. *In* WR Ullrich PJ Aparicio, PJ Syrett, F Castillo, eds, Inorganic Nitrogen Metabolism. Springer-Verlag, Berlin, pp 45–52
17. **Mann AF, Hucklesby DP, Hewitt EJ** (1978) Sources of reducing power for nitrate reduction in spinach leaves. Planta **140:** 261–263
18. **Martinoia E, Heck U, Wiemken A** (1981) Vacuoles as storage compartments for nitrate barley leaves. Nature **289:** 292–294
19. **Maurino SG, Maldonado JM** (1987) In vivo and in vitro inactivation of spinach leaf nitrate reductase by cyanide photogenerated from carbonylcyanide *m*-chlorophenylhydrazone. Plant Sci **51:** 141–146
20. **Nelson N, Ilan I** (1969) Inhibition of nitrate reductase from tomato leaves by adenosine-5′-diphosphate. Plant Cell Physiol **10:** 143–148
21. **Oelmuller R, Schuster C, Mohr H** (1988) Physiological characterization of a plastidic signal for nitrate-induced appearance of nitrate and nitrite reductase. Planta **174:** 75–83
22. **Oji Y, Hamano T, Ryoma Y, Miki Y, Wakiuchi N, Okamoto S** (1985) Purification and characterization of NADH-nitrate reductase from leaves of 2-row barley, and its activity as affected by some metabolites. J Plant Physiol **119:** 247–256
23. **Oji Y, Ryoma Y, Wakiuchi N, Okamoto S** (1987) Effect of phosphate on *in vitro* activity of NADH:nitrate reductase isolated form the second row barley leaves. Plant Physiol **83:** 472–474
24. **Robin P, Conejero G, Passama L, Salsac L** (1983) Evaluation de la fraction metabolisable du nitrate par la mesure in situ de sa reduction. Physiol Veg **21:** 115–122
25. **Sánchez J, Heldt HW** (1989) Determination of the apparent Km of nitrate reductase from spinach leaves for NADH by a coupled assay. Bot Acta **102:** 186–188
26. **Sane PV, Kumar N, Baijal M, Singh KK, Kochar VK** (1987) Activation of nitrate reductase by calcium and calmodulin. Phytochemistry **26:** 1289–1291
27. **Solomonson LP** (1974) Regulation of nitrate reductase by NADH and cyanide. Biochim Biophys Acta **334:** 297–308
28. **Solomonson LP, Spehar AM** (1977) Model for the regulation of nitrate assimilation. Nature **265:** 373–375
29. **Steingrover E, Ratering E, Siesling J** (1986) Daily changes in uptake, reduction and storage of nitrate in spinach grown at low light intensity. Physiol Plant **66:** 550–556
30. **Stitt M, Lilley RCM, Heldt HW** (1982) Adenine nucleotide levels in the cytosol, chloroplasts, and mitochondria of wheat leaf protoplasts. Plant Physiol **70:** 971–977
31. **Stitt M, Wirtz W, Gerhardt R, Heldt HW, Spencer C, Walker DA, Foyer C** (1985) A comparative study of metabolite levels in plant leaf material in the dark. Planta **166:** 354–364
32. **Vaughn KC, Campbell WH** (1988) Immunogold localization of nitrate reductase in maize leaves. Plant Physiol **88:** 1354–1357
33. **Watt MP, Gray VM, Cresswell CF** (1987) Control of nitrate and nitrite assimilation by carbohydrate reserves, adenosine nucleotides and pyridine nucleotides in leaves of *Zea mays* L. under dark conditions. Planta **172:** 548–554
34. **Weger HG, Turpin DH** (1989) Mitochondrial respiration can support $NO_3^-$ and $NO_2^-$ reduction during photosynthesis. Plant Physiol **89:** 409–415
35. **Woo KC, Joniken M, Canvin DT** (1980) Reduction of nitrate via a dicarboxylate shuttle in a reconstituted system of supernatant and mitochondria from spinach leaves. Plant Physiol **65:** 433–436