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# Decrease of Nitrate Reductase Activity in Spinach Leaves during a Light-Dark Transition[1]

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## ABSTRACT

**In leaves of spinach plants (*Spinacia oleracea* L.) performing $CO_2$ and $NO_3^-$ assimilation, at the time of sudden darkening, which eliminates photosystem I-dependent nitrite reduction, only a minor temporary increase of the leaf nitrite content is observed. Because nitrate reduction does not depend on redox equivalents generated by photosystem I activity, a continuation of nitrate reduction after darkening would result in a large accumulation of nitrite in the leaves within a very short time, which is not observed. Measurements of the extractable nitrate reductase activity from spinach leaves assayed under standard conditions showed that in these leaves the nitrate reductase activity decreased during darkening to 15% of the control value with a half-time of only 2 minutes. Apparently, in these leaves nitrate reductase is very rapidly inactivated at sudden darkness avoiding an accumulation of the toxic nitrite in the cells.**

In a green plant cell, photosynthetic nitrate assimilation involves two reductive steps, located in different subcellular compartments. In the cytosol, nitrate is reduced to nitrite via NADH-dependent NR[2] (27), and after transport into the chloroplasts (3), nitrite is further reduced to ammonia by nitrite reductase located in the stroma (2). Because this reaction needs reduced ferredoxin, which is provided by PSI, it is essentially light dependent, although a minor amount of nitrite reduction may also occur during darkness at the expense of reducing equivalents generated by the oxidative pentose phosphate pathway (1, 24) and starch breakdown (13). The NADH required as substrate for the nitrate reductase in the cytosol could be provided in multiple ways, either by photosynthetic electron transport via malate-oxaloacetate or triosephosphate-3-phosphoglycerate shuttles (for references, see ref. 9) or from mitochondrial substrate oxidation via malate-oxaloacetate shuttle (6). In contrast to nitrite reduction, nitrate reduction, therefore, does not depend on chloroplast PSI activity. To avoid an accumulation of nitrite in the plant cells, the rate of nitrite formation by NR in the cytosol has to be matched to the rate of nitrite reduction in the chloroplasts. The question arises in which way the activity of NR in the cytosol is regulated to respond to a decrease of photosynthesis, *e.g.* by sudden shading of a leaf (21).

Daily fluctuations of NR activity occurring in leaves as the result of biosynthesis and degradation of the enzyme protein (27) are too slow to account for a rapid decrease of enzyme activity during darkening. Although a number of metabolites such as ADP and ATP (5, 17), Pi (20), NADH and cyanide (7, 26), and amino acids (18, 19) were reported to affect NR activity, a systematic study of the putative regulatory role of these and other metabolites showed that none of these substances, when added to partially purified NR at those concentrations occurring in the cytosol of illuminated and darkened spinach leaves, were able to change NR activity to such an extent that the enzyme could be switched off during sudden darkening (25).

Recently, Kaiser and Förster (11) presented evidence that NR may be altered in its activity by interconversion of the enzyme protein. These authors found that in illuminated spinach leaves the extractable NR activity assayed under standard conditions largely decreased when leaves kept under ambient $CO_2$ were flushed with $CO_2$-free air, and the deactivation was reversed when the leaves were brought back to air. Moreover, the extractable NR activity was found to increase when darkened leaves were illuminated (11). The deactivation of NR during the exposure of leaves to $CO_2$-free air occurred with a half-time of about 20 min (10, 11). To avoid an accumulation of nitrite when a leaf suddenly becomes shaded, a much higher rate of NR deactivation would be required. In the present report, from measurements of nitrite contents and of NR activity in spinach leaves performing $CO_2$ and $NO_3^-$ assimilation, it is shown that during sudden darkening NR is deactivated with a half-time of only 2 min, so rapidly that only a minor temporary increase like an overshoot of the leaf nitrite content is observed during this transition.

## MATERIALS AND METHODS

### Plant Material

Spinach (*Spinacia oleracea*, U.S. Hybrid 424, Ferry-Morse Seed Co. Mountain View, CA) was grown hydroponically in a climatized chamber with a 9-h light/15-h dark cycle with a light intensity of 400 $\mu$E $m^{-2}$ $s^{-1}$. The temperature during the light period was 22°C and during the dark 16°C. The hydroponic medium contained 14 mM $NO_3^-$ (22). Photosynthesis of these leaves was measured with an IR gas analyzer. For the experiments, leaf samples of 49-d-old plants were used.

### Assay of Nitrate and Nitrite in Leaf Samples

From leaf samples frozen in liquid nitrogen, chloroform-methanol extracts were prepared (29). In these extracts, the quantity of nitrite was determined (8). Nitrate was measured

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[2] Abbreviation: NR, nitrate reductase.

spectrophotometrically. The extract was added to 50 mM potassium phosphate (pH 7.3) containing 0.33 mM NADPH, and the reaction was started with 66 munits/mL of *Aspergillus* NR (Boehringer, Mannheim). The decrease of $A_{334}$ was monitored.

### Assay of NR Activity in Leaves

NR activity was determined by measuring the formation of $NO_2^-$. The extraction of the leaf material and the measurements of the NR activity were carried out according to the method of Kaiser and Brendle-Behnisch (10) with the following modifications. At the indicated times, leaves were frozen in liquid nitrogen, homogenized to a fine powder, and stored in liquid nitrogen until measurement. For the activity assay, 1 mL of a medium containing 50 mM Hepes-KOH (pH 7.5), 2 mM DTT, 0.2% (v/v) Triton X-100, and $MgCl_2$ at concentrations as indicated was added to the still-frozen leaf material (approximately 0.2 mg Chl). The solution was homogenized, and a 100 μL-aliquot was added to 450 μL of the reaction medium, containing 40 mM Hepes-KOH, 5 mM $KNO_3$, 0.2 mM NADH, and $MgCl_2$. The time between the addition of the medium to the frozen leaf material and the start of the activity assay was 2 min. For the enzyme assay, the incubations were carried out at 30°C for 2 min and were stopped by the addition of 100 μL of 1 M zinc acetate solution. Unreacted NADH was oxidized by the addition of 50 μL of 0.15 mM phenazine methosulfate. The formed nitrite was determined according to the method of Hageman and Reed (8).

## RESULTS AND DISCUSSION

### $CO_2$ and Nitrate Assimilation of Spinach Leaves

Our spinach plants grown in hydroponic culture under artificial light showed an average photosynthesis rate of 70 μmol $CO_2$/mg Chl·h, as measured in the growth chamber with an IR gas analyzer. The leaves of these plants contain high amounts of nitrate, which as in other plants (16) is predominantly located in the vacuoles (B. Riens, unpublished results). As shown in Figure 1, the nitrate content of spinach leaves fluctuates during the diurnal cycle. According to earlier results (28) the nitrate content having its maximum at the beginning of the illumination period decreases gradually during the day and is replenished during the following night. Because nitrate is not transported in the phloem (14), the nitrate having been transferred to the leaves by the transpiration stream has to be utilized there. For this reason, the decrease of leaf nitrate observed during the light period reflects the reduction of the nitrate that had been stored in the vacuole. On the other hand, the increase of nitrate during the dark period reflects the minimal nitrate transfer into the leaves by the transpiration stream. Although it is possible to determine the nitrate uptake rate from the solution, this rate would only reflect the maximal value for the nitrate uptake into the leaves, because some nitrate reduction occurs in the roots too. If one assumes that the transport of nitrate from the roots to the leaves occurring during the day is at least as high as during darkness, then the rate of nitrate reduction in a leaf of a spinach plant under constant growth conditions may be estimated from the sum of the rate of the decrease of vacuolar

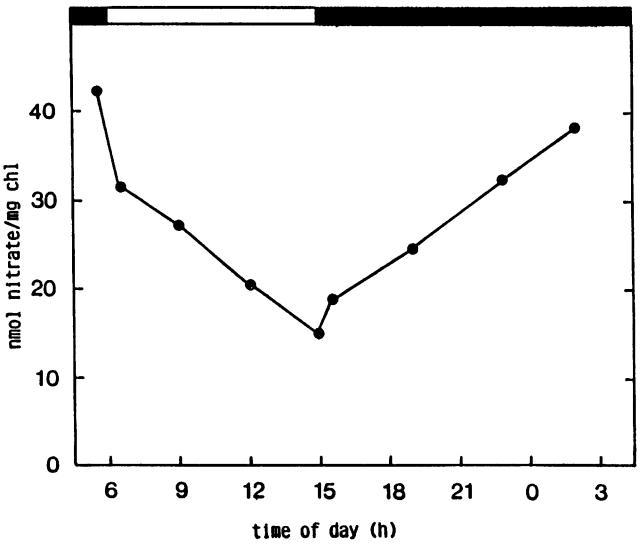


**Figure 1.** Nitrate contents of spinach leaves during a 24-h cycle. The results are mean values of three separate experiments.

nitrate and the rate of putative nitrate transfer from the roots to the leaves. From these considerations, we estimate that the minimal rate of nitrate reduction in these leaves is approximately 4 μmol/mg Chl·h (Fig. 1), which is equivalent to 6% of the rate of $CO_2$ assimilation. In earlier experiments with excised spinach leaves, the rate of nitrate assimilation was evaluated from the decrease in leaf nitrate content as 10 μmol/mg Chl·h, corresponding to 6 to 10% of the average rate of net photosynthesis in air (11).

### Nitrite Levels in Leaves during a Light-Dark Transition

In the experiment shown in Figure 2, the nitrite content of leaves was determined during a light-dark transition. In the steady state of photosynthesis, the nitrite content of the leaves is <10 nmol/mg Chl. When the leaves are placed into darkness, the leaf nitrite content increases after 2 min maximally by 18 nmol/mg Chl and then decreases again. If one takes into account that during darkening nitrite reduction is immediately brought to a halt due to lack of reduced ferredoxin and that nitrite reduction proceeding in the dark would only occur at low rates and would also require some time for the dark activation of key enzymes of the oxidative pentose phosphate pathway (4, 24) and starch degradation (13), a continuing nitrate reduction during darkness proceeding at an unaltered rate of 4 μmol/mg Chl·h would result in the accumulation of 130 nmol/mg Chl nitrite in 2 min. The maximum amount of nitrite actually accumulated after 2 min is one order of magnitude lower than this value. Using the slope of the decrease in nitrite content during the first 15 min of darkness, we calculated a minimal rate of nitrite reduction of about 100 nmol/mg Chl·h, which corresponds to 2.5% of the nitrate reduction rate estimated above. These findings indicate that after darkening the decrease of stromal nitrite reduction caused by lack of photosynthesis is accompanied by a similar rapid decrease of nitrate reduction in the cytosol.

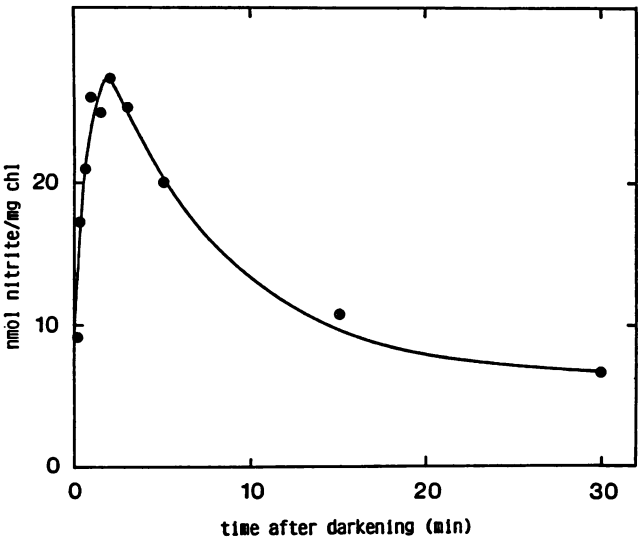


**Figure 2.** Nitrite contents in spinach leaves during a light-dark transition. The results are mean values of four separate experiments.

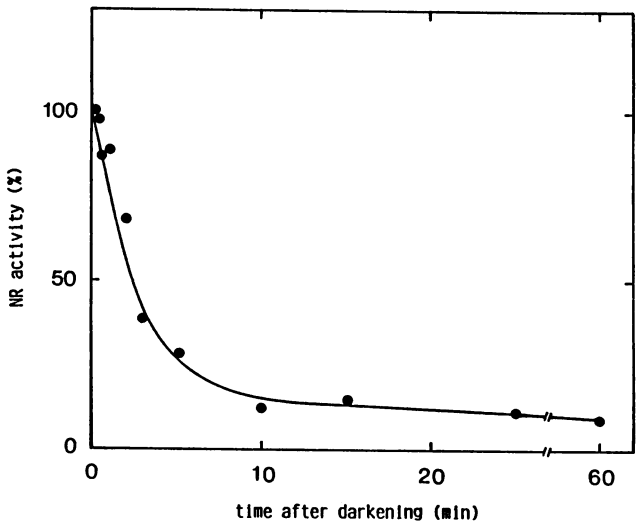


**Figure 3.** Inactivation of NR in crude spinach leaf extracts during a light-dark transition. The extraction and the assay were performed as described in "Materials and Methods" in the presence of 5 mM $MgCl_2$. The results are mean values of six separate experiments.

### Inactivation of NR during Darkening

In the experiment shown in Figure 3 from spinach plants kept under normal growing conditions in the growth chamber, at the indicated times leaf samples were immediately frozen in liquid nitrogen and subsequently analyzed for NR activity. Immediately after the end of illumination, the activity of the extractable NR assayed under standard conditions decreases very rapidly to about 15% of the control rate with a half-time of only 2 min (Fig. 3). The very rapid inactivation of NR explains why the overshoot in nitrite formation observed after darkening (Fig. 2) is so small. Remmler and Campbell (23) showed with corn leaves a decrease of NR activity by 30% within 1 h which was not accompanied by a degradation of the NR protein.

The inactivation of NR after darkening is reversible. This is shown in the experiment of Figure 4. Leaves were quenched by freezing in the light and 25 min after darkening. The extracts from these leaves were incubated for various times at 30°C before NR activity was measured. After 40 min of incubation, the inactivated enzyme from the leaves frozen in darkness was reactivated to an activity level even higher than in the untreated extracts from illuminated leaves. Incubation at 30°C further increased the activity extracted from illuminated leaves by 50%. These data indicate that the differences of enzyme activity of light- and dark-harvested leaves are not due to protein degradation but, rather, to a reversible modification of the enzyme.

For *Chlorella*, as well as for higher plants, changes of NR activity in response to dark-light shift have been described (15, 30). But here it is shown for the first time that the inactivation of the enzyme during darkening occurs with a half-time as short as 2 min to a value of only 15% of the activity found in illuminated leaves (Fig. 4). These changes can only be detected in the presence of $Mg^{2+}$ ions. As shown in Table I, NR extracted in the dark shows a different response to $Mg^{2+}$ compared with the enzyme from illuminated leaves. The addition of 5 mM $Mg^{2+}$ to the enzyme extracted from darkened leaves caused an inhibition of 76%, whereas this addition inhibited the enzyme from illuminated leaves only by 18%. As shown by Kaiser and Brendle-Behnisch (10) also, deactivation of NR by lack of $CO_2$ is only noticeable in the presence of $Mg^{2+}$ ions.

Although the NR deactivation in spinach leaves during a light-dark transition shown above is almost 10 times faster than the decrease in NR activity when $CO_2$ is eliminated in the gas phase, as reported by Kaiser *et al.* (10, 11), in view of

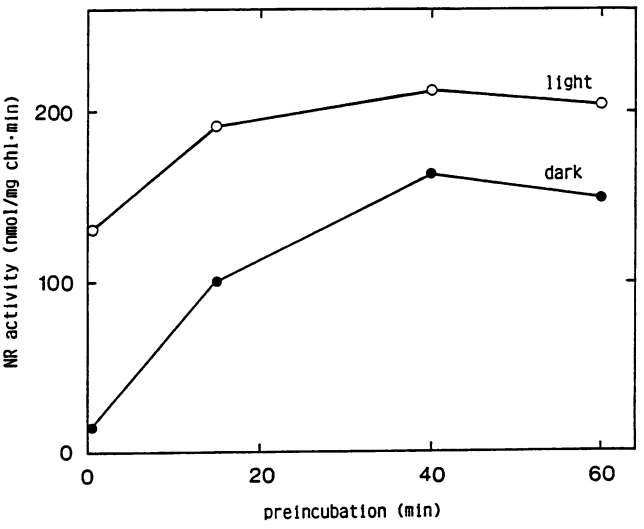


**Figure 4.** Activation of nitrate reductase in crude spinach leaf extracts after preincubation at 30°C. The leaves were harvested at the end of the light period (light) and 25 min during darkening (dark). The conditions of the activity measurements are the same as described in Figure 3. Each point represents the mean value of two separate experiments, each carried out with three parallel extracts.

**Table I.** *Dependence of the NR Activity in Crude Spinach Leaf Extracts on $Mg^{2+}$ Ions*

Leaves were harvested at the end of the light period (light) and 25 min after darkening (dark). After enzyme extraction in the absence of $MgCl_2$, the activities were measured in the presence of different $MgCl_2$ concentrations. Results are mean values ± SD from six extracts of two separate experiments.

| $MgCl_2$ | NR Activity | | |
|---|---|---|---|
| | Light | Dark | Dark/light |
| mM | nmol·mg$^{-1}$ Chl·min$^{-1}$ (%) | | |
| 0 | 246 ± 59 (100) | 103 ± 19 (100) | 0.42 |
| 0.5 | 240 ± 56 (98) | 81 ± 13 (79) | 0.34 |
| 5 | 201 ± 47 (82) | 25 ± 5 (24) | 0.12 |
| 10 | 182 ± 42 (74) | 20 ± 4 (19) | 0.11 |

the similar $Mg^{2+}$ effects, it is feasible that the modification of NR follows the same mechanism. By preincubation of a desalted crude protein extract from spinach leaves with $Mg^{2+}$ and ATP, Kaiser and Spill (12) achieved an inhibition of NR activity with similar characteristics as the *in vivo* modulation of the enzyme observed after flushing leaves with $CO_2$-free air. This deactivation by ATP could be reversed by the subsequent addition of AMP. The modulation of NR activity apparently requires another protein, because it could not be demonstrated with purified NR preparations (12). To investigate whether ATP hydrolysis is required for the modulation of NR activity in the *in vitro* system, Kaiser and Spill (12) tested whether the modification of NR activity by ATP could also be achieved with nonhydrolyzable ATP analogs. The results were ambiguous. γ-S-ATP decreased the NR activity of leaf extracts similarly to ATP, although the inhibitory effect of this analog, contrary to the effect of ATP, could not be reversed by AMP. The nonhydrolizable ATP analogs β-imido-ATP and β-methyl-ATP, on the other hand, did not inhibit the enzyme (12). Although a modulation of NR activity by protein phosphorylation is a suggested possibility, there is as yet no stringent evidence available for this notion.

In view of the opposing effects of ATP and AMP on the modulation of NR activity *in vitro* and an observed inverse correlation between the ATP/AMP ratio and the activation state of NR in spinach leaves, it has been suggested that the cytosolic ATP/AMP levels are the central link between NR activity in the cytosol and photosynthesis in the chloroplasts (12). The very rapid deactivation of NR observed in our experiments after darkening of leaves cannot be explained in these terms, because it is most unlikely that in the leaves the light-dark transition is accompanied by a dramatic increase of the cytosolic ATP/AMP ratio, as required *in vitro* for NR deactivation. It is, therefore, to be expected that another signal of unknown nature may coordinate the activation state of NR present in the cytosol with photosynthesis occurring in the chloroplasts.

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