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Rapid Modulation of Spinach Leaf Nitrate Reductase Activity by Photosynthesis[1]

I. Modulation *in Vivo* by CO_2 Availability

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ABSTRACT

It has been shown recently that in spinach leaves (*Spinacia oleracea*) net photosynthesis and nitrate reduction are closely linked: when net photosynthesis was low because of stomatal closure, rates of nitrate reduction decreased (WM Kaiser, J Förster [1989] Plant Physiol 91: 970–974). Here we present evidence that photosynthesis regulates nitrate reduction by modulating nitrate reductase activity (NRA, EC 1.6.6.1). When spinach leaves were exposed to low CO_2 in the light, extractable NRA declined rapidly with a half-time of 15 minutes. The inhibition was rapidly reversed when leaves were brought back to air. NRA was also inhibited when leaves were wilted in air; this inhibition was due to decreased CO_2 supply as a consequence of stomatal closure. The modulation of NRA was stable *in vitro*. It was not reversed by gel filtration. In contrast, the *in vitro* inhibition of nitrate reductase (NR) by classical inhibitors such as cyanide, hydroxylamin, or NADH disappeared after removal of free inhibitors by gel filtration. The negative modulation of NRA in $-CO_2$-treated leaves became manifest as a decrease in total enzyme activity only in the presence of free Mg^{2+} or Ca^{2+}. Mg^{2+} concentrations required for observing half-maximal inhibition were about 1 millimolar. In the presence of EDTA, the enzyme activity was always high and rather independent of the activation status of the enzyme. NRA was also independent of the pH in the range from pH 7 to pH 8, at saturating substrate and Mg^{2+} concentrations. The apparent substrate affinities of NR were hardly affected by the *in vivo* modulation of NR. Only V_{max} changed.

In addition to CO_2 assimilation, nitrate reduction in leaves is an important sink for photosynthetically produced reductands. It has to be expected, therefore, that CO_2 and NO_3^- reduction interact in various ways. A simple type of interaction would be competition for reductant. In N-depleted algae such competition has been impressively demonstrated (17), and results obtained recently with intact barley plants indicate that higher plants may respond in a similar way (1). On the other hand, in isolated chloroplasts systems, competition of CO_2 and NO_2^- for reductands has been questioned (13, 14).

Evidence for a reverse type of interaction—a regulation of nitrate reduction by photosynthesis—is less well demonstrated and understood, although it should be important in adaptation of plants to rapidly changing environmental conditions. We have shown recently that light-dependent reduction of nitrate in spinach leaves (*Spinacia oleracea*) ceased when the leaves closed their stomata (7). Preliminary experiments had indicated that the inhibition of overall nitrate reduction was paralleled by a rapid decrease in the level of extractable NR[2] (EC 1.6.6.1) activity (7). Our assumption was, therefore, that nitrate reduction in leaves is synchronized with CO_2 assimilation probably via a rapid modulation of NR. Below, and in a subsequent paper, the proposed NR enzyme modulation is described in more detail. It is shown that NR modulation has some unusual properties which have not been observed so far.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* var "Yates") was grown in a greenhouse under additional artificial illumination (HQi 400 W, Schreder, Winterbach, Germany), at a variable total photon flux density between 250 and 400 μmol m^{-2} s^{-1} (PAR) and a mean day length of 11 h. Air humidity varied from 50 to 80%; daytime temperatures were 20 to 26°C, and night temperatures were 16 to 22°C.

NRA

NRA was measured in leaf extracts by following the production of nitrite from added nitrate according to Hagema and Reed (4), but with some modifications. Leaves were rapidly frozen in liquid nitrogen and ground to a fine powder in a mortar. Buffer A (2 mL) (50 mM Hepes-KOH [pH 7.8], 50 μM leupeptin [Sigma Chemicals, St. Louis], and $MgCl_2$ as indicated) was added to the still frozen leaf powder, and vigorous grinding continued for at least 1 min. The thawed suspension was cleared by a 2 min centrifugation at 15,000*g* (Eppendorf Type 5414 S), and the supernatant was used directly or after desalting by gel filtration at 4°C (Sephadex G 25 M, Pharmacia, Uppsala, Sweden). Unless mentioned otherwise, the standard reaction medium consisted of 0.9 mL buffer B containing 20 mM Hepes-KOH (pH 7.8), 50 μM leupeptin, 0.5 mM NADH, 2 mM KNO_3, and 10 μM FAD.

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[2] Abbreviations: NR, nitrate reductase; NRA, nitrate reductase activity.

The reaction was started by addition of 50 to 100 μL of the leaf extract to the reaction medium. Aliquots (200–500 μL) were removed after 1 to 3 min and injected into 20 to 50 μL 0.5 M zincacetate solution in order to stop the reaction. Where necessary, excess unreacted NADH was removed by PMS treatment (4). Colorimetric determination of the formed nitrite followed the classical procedures (4).

RESULTS

When spinach leaves were kept under saturating light and CO_2, their NRA was at maximum. Almost instantaneously after switching to CO_2-free air, NRA decreased (Fig. 1). It increased again after readdition of CO_2. The half-time for deactivation or activation was about 15 min.

The rapid change in NRA was observed only when the leaf extracts contained free Mg^{2+} (Fig. 1). When divalent cations in the extracts and in the reaction medium were chelated by excess EDTA, the measured NRA was always very high and hardly any change in activity could be observed after a $+CO_2/-CO_2$ treatment (Fig. 1). Protein degradation should have changed the activity irrespective of the measuring conditions. We have shown recently that nitrate reduction in leaves was inhibited by water stress. In fact, we also observed a decrease in extractable NRA (measured in buffer containing free Mg^{2+}) when leaves were exposed to water stress by rapidly wilting them for 1 to 3 h at a photon flux density of 350 μE m^{-2} s^{-1} (PAR) under a stream of air (Fig. 2). A decrease of NRA of similar velocity during wilting of wheat seedlings has been reported recently (9). The decrease in NRA might be attributed to a direct dehydration effect at the cellular level. However, this was not the case: when leaves were wilted under 5% CO_2 in order to overcome their diffusional resistance which is very high under stress, a similar degree of dehydration as in air caused no decrease in NRA (Fig. 2). It is concluded that the *in situ* inactivation of NRA during wilting in air was a consequence of decreased rates of net photosynthesis due to stomatal closure. This finding complements our recent conclusions on the mode of action of water stress on nitrate reduction in leaves (7). It has to be mentioned that the applied wilting of leaves in air had no effect on their photosynthetic capacity (6).

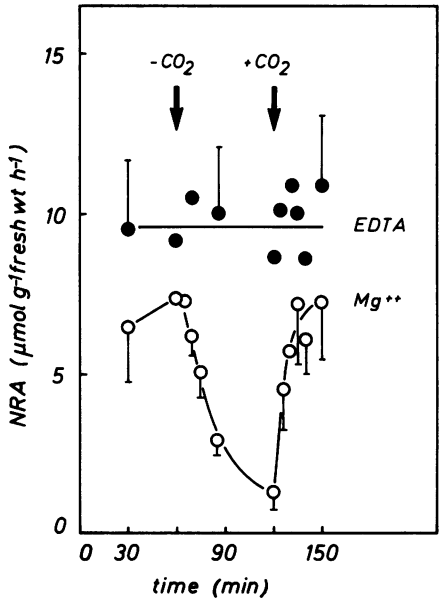

Figure 1. NRA in crude extracts from spinach leaves which were illuminated in air, then in CO_2-free air, and again in air. Gas volume in the cuvette was about 4 L, and gas flow was adjusted about 4 L/min. At each time point, a single leaf was removed from the cuvette and used for NR assay. Both the extraction and the assay were done in a buffer containing either 5 mM $MgCl_2$ (●) or 5 mM EDTA (○). Values are means from four separate experiments ±SD.

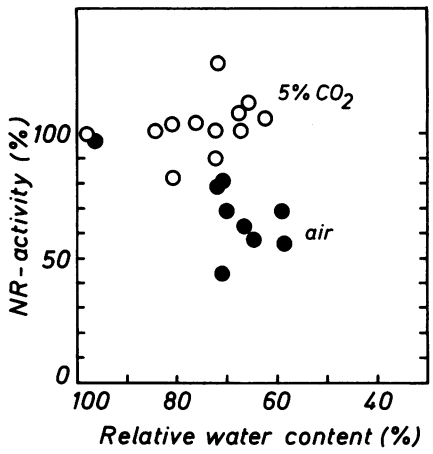

Figure 2. NRA in crude extracts from spinach leaves that were wilted to the indicated relative water content either in air or in air containing 5% CO_2. Detached leaves with the petioles in water were exposed in a cuvette at room temperature to a photon flux density of 350 μmol m^{-2} s^{-1} (PAR). Each point respresents the enzyme activity in a separate extract from one leaf. The time to reach the lowest indicated relative water content was about 3 h.

The inactivated state of NR was rather stable. The enzyme from $-CO_2$-treated leaves remained inhibited when low mol wt compounds were removed by gel filtration on Sephadex G-25 M (Table I). After ammonium sulfate precipitation, the inhibition was partly reversed (Table I). A number of possible mechanisms for a modulation of NRA in green cells have been reviewed recently (16). Among the most prominent low mol wt effectors of NR are cyanide (10) or hydroxylamine, or NADH in high concentrations (16). However, the observed *in vivo* inactivation of NR was certainly not caused by either of these compounds for one main reason: when cyanide or hydroxylamine or NADH in suitable concentrations were added to crude, desalted leaf extracts, NR was strongly inhibited, as expected (Table II). However, after a second desalting step on Sephadex columns equilibrated with inhibitor-free buffer, the inhibition was almost totally reversed. The high stability of the *in vivo* inhibition suggests as a regulatory mechanism a covalent modification of NR rather than a direct inhibitor action.

As pointed out above, the inactive state of NR was observed as decreased catalytic activity only in the presence of free Mg^{2+}. Most classical buffers for measuring NRA were designed to give maximal enzyme activity and contained phos-

Table I. *Inhibition of Nitrate Reductase in Vivo by Illuminating Detached Leaves for 1 h at the CO_2 Compensation Point ('$-CO_2$', Leaves in a Closed Vessel)*

Control leaves were kept in air with 5% CO_2 ('$+CO_2$') in order to permit maximum photosynthesis rates. After 1 h in the light, leaf temperature had increased from initially 20°C to about 25°C. The temperature increase itself had no effect on extractable NRA (not shown). Enzyme activity was measured in crude extracts, or after gel filtration (Sephadex G-25 M), or after ammonium sulfate precipitation (44% saturation) in order to remove low mol wt compounds ($n = 6$, ±SD).

	NR Activity + CO_2	$-CO_2$	Control
		μmol NO_2^- g^{-1} fresh wt h^{-1}	%
Crude extract	7.9 ± 1.8	3.5 ± 0.6	45
Gel filtrate	4.9 ± 1.8	2.2 ± 1.2	45
Ammonium sulfate precipitate	6.7 ± 2.4	4.3 ± 2.0	64

phate buffer at concentrations exceeding 50 mM (4). Under such conditions, Mg^{2+} activity is low, and NRA will be at its maximum catalytic activity, irrespective of its activation status. This might explain why the inhibition has not yet been observed so far. In order to determine the Mg^{2+} concentration required for the stabilization of the inactive state of NR, crude extracts from $+CO_2/-CO_2$ pretreated leaves were desalted on Sephadex G 25 M columns equilibrated with Mg^{2+}-free buffer. A small aliquot (100 μL) of the extract was then preincubated for 5 min in the reaction medium containing various concentrations of $MgCl_2$ or $CaCl_2$. Under these conditions the half-maximal Mg^{2+} concentration required for measuring the low activity state of NR was 1 to 2 mM, and this is well within a physiological concentration range (Fig. 3). Mg^+ could be replaced by Ca^{2+} at similar concentrations. Monovalent cations (K^+ or Na^+) were ineffective even at higher concentrations (not shown). The divalent cations had hardly any effect on the enzyme activity in extracts from $+CO_2$ leaves (Fig. 3). NRA, measured in the presence of 5 mM Mg^{2+} and at saturating substrate concentrations, was not affected by pH changes within the pH range from pH 7 to pH 8, both, in the fully active and the less active state (Fig. 4).

Table II. *Inhibition of NR by Cyanide, Hydroxylamine, or NADH, and Reversal of the Inhibition by Gel Filtration (Sephadex G-25 M)*

The crude, desalted leaf extract was preincubated for 5 min at room temperature with the indicated inhibitor concentrations. Aliquots (100 μL) of the preincubated extracts were added to 900 μL of the standard reaction medium containing the same final inhibitor concentrations, and nitrate reduction was allowed to proceed for 5 min. Means from two separate experiments.

		Relative NRA	
		Before gel filtration	After gel filtration
	mM	% of control	
KCN	0.5	5.2	88
NH_2OH	1	1.6	90
NADH	3	8.9	92

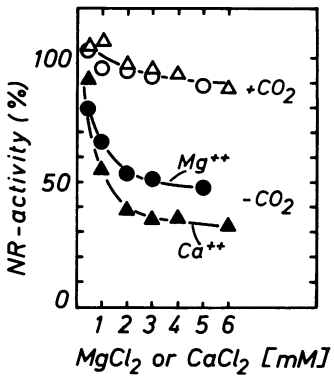

Figure 3. NRA in extracts from leaves illuminated for 1 h at the CO_2-compensation point ("$-CO_2$") or in air containing 5% CO_2 ("$+CO_2$"). Extracts (1 g fresh wt) were prepared in buffer (2 mL) containing 5 mM $MgCl_2$ and were desalted over columns equilibrated with Mg^{2+}-free buffer. Extract (100 μL) was added to 900 μL of the reaction medium containing various concentrations of $MgCl_2$ or $CaCl_2$ but no substrates. After preincubation for 5 min, the reaction was started by addition of NO_3^- and NADH and was allowed to proceed for 3 min. Means from two separate experiments.

We attempted to determine some kinetic characteristics of NR before and after inactivation by treatment of leaves with $+CO_2/-CO_2$ (Figs. 5 and 6). Due to the high affinity of NR for NADH, and due to the danger of side reactions, such measurements are difficult to be carried out with crude leaf extracts. On the other hand, systems allowing K_m determinations under steady-state flux conditions (15) cannot be used with crude extracts, because subsidiary enzyme activities are not controlled. In spite of these difficulties, in eight separate determinations which were carried out over a period of 2 years with somewhat variable plant material we observed a K_m (NADH) of 2 to 8 μM and a less variable K_m NO_3^- of 35 to 60 μM, which were within the range reported for purified enzyme preparations (3), and for a coupled enzyme system working with very low steady-state NADH-concentrations (15). Independent of the variability of the measured kinetic constants, within one experiment the *in vivo* inactivation caused hardly any change in the K_m for both substrates, whereas V_{max} was decreased. The inactivation thus produces a noncompetitive type of inhibition.

DISCUSSION

It seems obvious that photosynthesis regulates nitrate reduction in spinach leaves via a rapid reversible modulation of NRA. The maximum variation in extractable enzyme activity observed in many experiments was at most sevenfold, and a mean value was threefold. The NR modulation is obviously a rather "soft" regulation and not an on/off switch. Probably this reflects the fact that the main purpose of that regulation is to adapt the velocity of nitrate reduction to that of carbon assimilation.

According to our results, NR in leaves exists in two activation states which differ in their response to divalent cations. The inactivated state of NR was manifest as decreased catalytic activity only in the presence of free Mg^{2+} or Ca^{2+}. Under

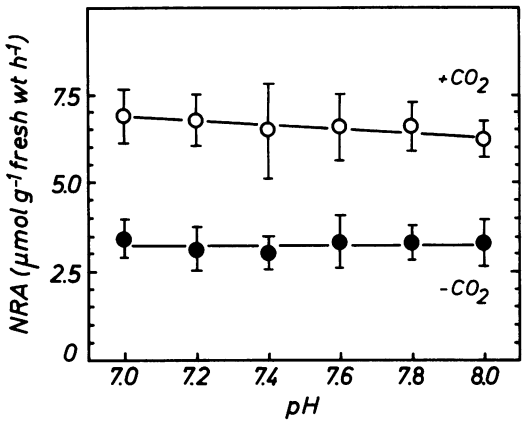

Figure 4. pH profile of NRA in desalted extracts from spinach leaves that had been illuminated for 1 h in 5% CO_2 or at the CO_2 compensation point. The Mg^{2+} concentration both in the extraction buffer and in the reaction medium was 5 mM ($n = 3$).

our experimental conditions, the required Mg^{2+} concentrations were well in the range of Mg^{2+} activities (1–3 mM) found, *e.g.* in isolated chloroplasts (8, 12). If we assume similar Mg^{2+} activities in the cytosol, then the inhibition represents certainly a natural situation. It is not known whether cytosolic free Mg^{2+} undergoes similar changes as reported for the chloroplast stroma during light-dark transition (8, 12), and assumptions on a more direct participation of cytosolic free Mg^{2+} in the modulation of NRA are therefore speculative. When Mg^{2+} was removed, *e.g.* by gel filtration, the inactivated status of NR was not abolished—it appeared again after

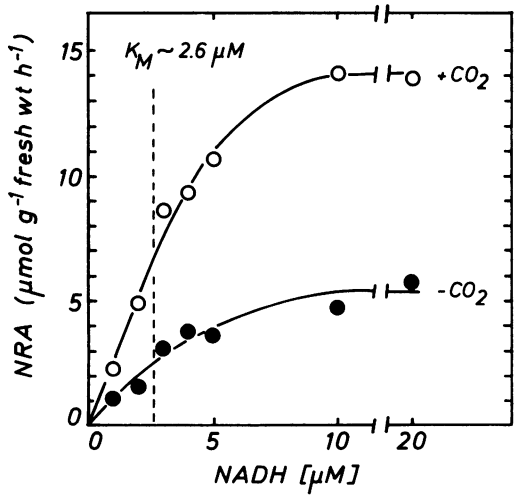

Figure 5. NRA as a function of the NADH-concentration in crude, desalted extracts from leaves illuminated for 1 h at the CO_2 compensation point or at 5% CO_2. The reaction was started by addition of an aliquot of the extract (100 μL) to 900 μL of the complete reaction medium and was terminated by addition of zincacetate after 2 min. Extinction was measured in a cuvette with 5 cm light path. The curves are from a representative experiment (two parallels) out of a series of eight similar determinations made over a period of 2 years with slightly different plant material.

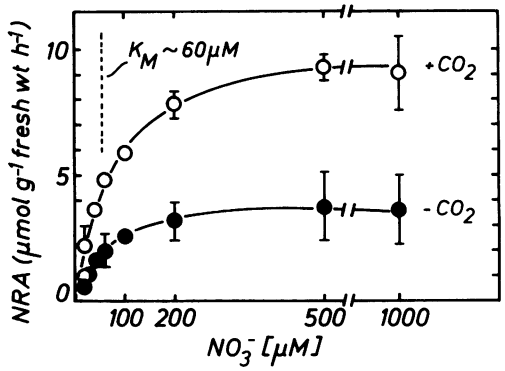

Figure 6. NRA as a function of the nitrate concentration. Conditions as before ($n = 4$, ±SD).

readdition of Mg^{2+}. Thus, Mg^{2+} itself doesn't cause an inhibition. The enzyme apparently remained in its inactivated state in the absence of Mg^{2+}, but its catalytic activity was low only in the presence of free Mg^{2+}. As NRA was almost unchanged in the pH range from 7 to 8, a regulation via cytosolic pH changes seems improbable.

Our data showed that the classical modulators of NR, such as cyanide or hydroxylamine, or "overreduction" by high NADH concentrations did not participate in NR enzyme modulation in spinach leaves. The *in vivo*-inactivated NR remained inactive after removal of low mol wt compounds by gel filtration. This observation excludes a direct inhibition by metabolites. It can be explained in two ways: either inactivation produces a stable conformational change of the NR protein, perhaps catalyzed by a second enzyme system; protein phosphorylation or adenylation might be considered as examples for this type of modulation. Or, the "inactivating factor" is a high mol wt compound, *i.e.* a protein, which is not removed by gel filtration. In fact, it has been shown that NR from various sources (roots, leaves, cell suspensions) was inhibited by a protein which was not a protease and which in leaves was apparently inhibitory only in the dark and inactivated in the light (5, 11, 18–20).

Our attempts to reactivate NR *in vitro* by addition of various metabolites or by phosphatase treatment after *in vivo* inactivation were unsuccessful, with one exception: incubation of the inhibited enzyme with AMP caused a slow but almost complete reactivation *in vitro* (not shown here). Data and conclusions about the role of AMP and of other nucleotides in the modulation of NRA *in vitro,* and evidence for their action as effectors *in vivo,* will be presented in a subsequent paper.

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