Rapid Modulation of Spinach Leaf Nitrate Reductase by Photosynthesis

II. In Vitro Modulation by ATP and AMP

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ABSTRACT

Assimilatory nitrate reductase activity (NRA) in crude spinach leaf (Spinacia oleracea) extracts undergoes rapid changes following fluctuations in photosynthesis brought about by changes in external CO₂ or by water stress (WM Kaiser, E Brendle-Behnisch [1991] Plant Physiol 96:363–367). A modulation of NRA sharing several characteristics (stability, response to Mg²⁺ or Ca²⁺, kinetic constants) with the in vivo modulation was obtained in vitro by preincubating desalted leaf extracts with physiological concentrations of Mg²⁺ and ATP (deactivating) or AMP (activating). When nitrate reductase (NR) was inactivated in vivo by illuminating leaves at the CO₂ compensation point, it could be reactivated in vitro by incubating leaf extracts with AMP. For the in vitro inactivation, ATP could be replaced by GTP or UTP. Nonhydrolyzable ATP analogs (β, γ-imido ATP, β, γ-methyl-ATP) had no effect on NR, whereas γ-S-ATP caused an irreversible inactivation. This suggests that NR modulation involves ATP hydrolysis. In contrast to NR in crude leaf extracts, partially purified NR did not respond to ATP or AMP. ATP and AMP levels in whole leaf extracts changed in the way predicted by the modulation of NRA when leaves were transferred from photosynthesizing (low ATP/AMP) to photosynthetic (high ATP/AMP) conditions. Adenine nucleotide levels in leaves could be effectively manipulated by feeding mannose through the leaf petiole. NRA followed these changes as expected from the in vitro results. This suggests that cytosolic ATP/AMP levels are indeed the central link between NRA in the cytosol and photosynthesis in the chloroplast. Phosphorylation/dephosphorylation of NR or of NR-regulating protein factors is discussed as a mechanism for a reversible modulation of NR by ATP and AMP.

In the preceding article, we (9) showed that spinach leaf (Spinacia oleracea) NADH-NR² underwent rapid changes in activity when rates of net photosynthesis were varied by altering the CO₂ supply. It is believed that this modulation of NRA controls the velocity of nitrate reduction in leaves, thus bringing about a synchronization of CO₂ and nitrate assimilation under rapidly changing environmental conditions (10).

The in vivo inactivation of NRA observed under low CO₂ was characterized by two major features: in a crude extract from -CO₂ pretreated leaves the inactive enzyme remained inhibited after removal of low molecular weight compounds, including metabolites; and the inactivated enzyme had a low catalytic activity only in the presence of free Mg²⁺ (or Ca²⁺). In contrast, the fully activated enzyme was rather insensitive to Mg²⁺ (9). To find a system for an in vitro modulation of NR with features similar to those described above for the in vivo system, we examined the role of potential effectors on NRA by preincubating a rather concentrated leaf extract with the effectors in the absence of substrates for NR; subsequently, the effector was strongly diluted or removed by gel filtration, and the remaining enzyme activity was examined in standard assay at saturating or limiting substrate concentrations. In the following, it will be shown that under these conditions MgATP caused a stable inhibition of NR in vitro, which had all the characteristics of the in vivo inactivation. We also report on an in vitro reactivation of NR by AMP. Finally, we present evidence that this system is probably identical with the in vivo regulatory system.

MATERIALS AND METHODS

Preparation of leaf extracts and nitrate reductase assay were described previously (9). The basic procedure for modulating NRA was as follows. A rather concentrated spinach leaf (Spinacia oleracea) extract (1 g fresh wt extracted in 2 mL buffer to give a total liquid volume of about 2.5 mL) was preincubated at 22°C with the potential effector (e.g. ATP) for various times, as indicated in the figure legends. The extraction and preincubation medium usually contained 50 mM Hepes-KOH (pH 7.6), 50 μM leupeptin (Sigma) and MgCl₂ as indicated, but usually no NADH and NO₃⁻. After preincubation, an aliquot (50–100 μL) of the solution was injected into 900 to 950 μL of the reaction medium (without the effector), and the reaction proceeded for various times as indicated.

Partial purification of NR was achieved according to Sanchez and Heldt (13), with minor modifications: about 10 g of freshly harvested leaves were frozen and homogenized in liquid nitrogen, and the frozen leaf powder was suspended in 10 mL of buffer A (50 mM Hepes-KOH [pH 7.6], 10 μM flavin adenine dinucleotide). The thawed homogenate was centrifuged for 15 min at 25,000g, and the pellet was discarded. To the clear supernatant, solid ammonium sulfate

1 Abbreviations: NR, nitrate reductase; NRA, nitrate reductase activity; G6P, glucose-6-phosphate.

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**The importance of the in vivo modulation of NRA is discussed in a separate publication (Kaiser et al., 1991).
was added up to 44% saturation, and after stirring for 15 min, the suspension was centrifuged for 15 min at 15,000g. The pellet was resuspended in 4 mL buffer B (50 mM Hepes-KOH [pH 7.6], 0.1 mM EDTA, 10 μM flavin adenine dinucleotide). After another centrifugation step (5 min at 15,000g), 5 mL of the clear solution were loaded onto a Blue Sepharose CL-6B affinity column of about 6 mL bed volume, which had been equilibrated with buffer B. The column was eluted with 25 mL buffer B, containing in addition 0.1 mM KCl, and then with 20 mL of buffer B containing 0.5 mM NADH. Fractions of 2 mL were collected, with fraction two containing the maximum of about 35% of the total NRA. The preparation was kept on ice and aliquots were directly used within 3 h.

For measuring adenine nucleotide levels, detached spinach leaves were kept with their petiole either in distilled water or in 50 mM mannose. They were illuminated for 1 h in a cuvette which was either closed to bring the leaves rapidly to the CO2 compensation point, or flushed with air containing 5% CO2 in order to permit maximal rates of photosynthesis irrespective of the degree of stomatal opening. After 1 h, leaves were frozen as rapidly as possible in liquid nitrogen and ground carefully in a precooled mortar. About 5 mL of 4.5% HClO4 were added to 1 g fresh wt of leaf material. After thawing, 625 μL Tris (2 mM) were added. The debris was spun down, and 5 mL of the clear supernatant was adjusted to pH 7.4 by addition of 5 mM K2CO3. The precipitate was removed by centrifugation, and the samples were kept frozen at −70°C.

Adenine nucleotides were determined by a commercially available luciferine-luciferase assay (Boehringer, Mannheim, FRG) in combination with a phosphoenolpyruvate/pyruvate kinase/myokinase system in a Lumat LB9501 (Berthold, FRG).

RESULTS

Inactivation of NR by Nucleoside Triphosphates

When desalted (Sephadex G 25 m) leaf extracts from actively photosynthesizing fresh leaves were preincubated at room temperature with various nucleoside triphosphates (ATP, GTP, UTP), the activity of NR declined rapidly with a half-time of 3 to 5 min (Fig. 1). Because the cytosol of mesophyll cells contains nucleoside diphosphate kinase activity (EC 2.7.4.6) (3), it cannot be decided whether all nucleotides were equally effective in modulating NR or whether they were rapidly interconverted. The relative change in activity brought about by preincubation with ATP was dependent on the initial activity of the extract, which varied from 5 μmol g−1 fresh wt h−1 to 14 μmol g−1 fresh wt h−1. The higher the initial activity, the larger was the relative decrease after preincubation with ATP (not shown).

Preincubation with ADP (1 mM) also inactivated NR (not shown). However, in the presence of the myokinase inhibitor 1–5-di (adenosine-5'-pentaphosphate), preincubation of a leaf extract with ADP had no effect on NRA (not shown). It is concluded that the ADP effects in the absence of the inhibitor were actually due to a partial conversion of added ADP to ATP and AMP.

The inactivation process of NR by preincubation with ATP was strictly Mg2+ dependent. Mg2+ could not be replaced by Ca2+.
Ca\(^{2+}\) (Fig. 2). Once NR had been inactivated by ATP and Mg\(^{2+}\), and both Mg\(^{2+}\) and ATP were removed by subsequent gel filtration, the catalytic activity increased. Subsequently, addition of Mg\(^{2+}\) alone was sufficient to decrease the catalytic activity to its original (inhibited) state (Fig. 2). Here then, Mg\(^{2+}\) could be replaced by Ca\(^{2+}\). This closely resembles the situation after in vivo modulation (compare to ref. 9) where ATP-inactivated NR was also fully reactivated when excess Mg\(^{2+}\) was chelated by EDTA (Fig. 3). Both the ATP concentrations and the Mg\(^{2+}\) concentration required for the inactivation process itself (not shown) or for maintaining the inactivation after preincubation with ATP (Fig. 2) were within a physiological concentration range. Half-optimal concentrations were about 2 mM Mg\(^{2+}\) (Fig. 2), and 0.2 mM ATP (Fig. 4). The inactivated state of NR following preincubation with ATP was stable. When ATP was removed by gel filtration in Mg\(^{2+}\)-containing buffer, NR remained in the inhibited state (Table I, also compare Fig. 2). The ATP-dependent inactivation was thus as stable as the inactivation of NR in vivo by \(-\text{CO}_2\) treatment (compare to ref. 9). It was also partially reversed by ammonium sulfate precipitation and subsequent resolution in Mg-containing buffer (Table I). The reason for the partial reversal of the inhibition after ammonium sulfate precipitation is as yet unclear, but it might indicate the involvement of additional protein factors in mediating the ATP-dependent inactivation of NR.

The inactivation of NR by preincubation with ATP decreased \(V_{\text{max}}\), but effects on substrate affinity were negligible (Table II). It has to be mentioned that our conventional procedure for determining the \(K_m\) NADH was not precise due to the high affinity of NR for NADH. Determination of \(K_m\) with the steady-state system described recently (13) would have been advantageous but could not be used with crude extracts, as the activity of subsidiary enzymes could not be controlled here. In spite of that, the data permit the conclusion that ATP inactivation of NR had similar characteristics as the in vivo inactivation by low \(\text{CO}_2\) conditions (9).

To find out whether the inactivation of NR by ATP involved ATP hydrolysis, we examined the effect on NR of several nonhydrolyzable ATP analogs (Fig. 5). \(\beta\), \(\gamma\)-Imido-

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure3.png}
\caption{Inactivation of NR by preincubation with 1 mM ATP in the presence of 5 mM MgCl\(_2\) and subsequent reactivation by addition of 5 mM EDTA to the preincubation medium. The reaction medium also contained 5 mM MgCl\(_2\) (up to min 12), and thereafter 5 mM EDTA. Reaction time was 1 min (\(n = 3\), ± sd).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure4.png}
\caption{Inactivation of NR during a 15 min preincubation period, at different ATP concentrations under Mg\(^{2+}\) excess (5 mM). Reaction time was 1 min. To inhibit ATP consumption by myokinase action, the latter was inhibited by addition of 50 \(\mu\)M 1-5-d(adenosine-5'-pentaphosphate). Other conditions as before. Each point represents the mean of two separate experiments.}
\end{figure}

ATP and \(\beta\), \(\gamma\)-methyl-ATP caused little or no inactivation of NR, whereas \(\gamma\)-S-ATP was almost as effective as ATP (Fig. 5). However, the inactivation of NR by preincubation with \(\gamma\)-S-ATP was not abolished by treatments which reversed the ATP-dependent inactivation (see below). Altogether, the data strongly suggest that the inactivation involved ATP hydrolysis.

\begin{table}
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{Table I. Stability of the ATP-Inactivated State of NR after Gel Filtration (Sephadex G 25 ml) or Ammonium Sulfate Precipitation (44\% Saturation)} & \\
\hline
Preincubation with ATP was 15 min at 20°C. Reaction time was 2 min. Extraction buffer and reaction medium all contained 10 mM MgCl\(_2\) and Sephadex columns were also equilibrated with MgCl\(_2\)-containing buffer (\(n = 2-4\), ± sd). & \\
\hline
\textbf{NRA} & \textbf{\(\mu\)mol g\(^{-1}\) fresh wt h\(^{-1}\)} \\
\hline
Initial activity, desalted extract & 6.7 ± 2 \\
After AS* precipitation & 7.3 ± 3 \\
Desalted extract, after preincubation with ATP & 1.1 ± 0.3 \\
After preincubation with ATP and subsequent desalting & 1.2 ± 0.3 \\
After preincubation with ATP and subsequent AS precipitation & 4.8 ± 2 \\
\hline
\hline
*AS, ammonium sulfate & \\
\end{tabular}
\end{table}

Reversal of the ATP-Dependent Inactivation of NR

The involvement of ATP hydrolysis in the inactivation of NR suggested a covalent modification of the protein, \(e.g.\) by phosphorylation or by adenylation. In both cases, removal of ATP after inactivation should not necessarily result in a reactivation. However, results were at first contradictory. When, after inactivation of NR, excess ATP was removed by gel filtration, NR remained inactive for some time, as shown above (Table I). However, when excess ATP was removed by adding glucose + hexokinase (EC 2.7.1.1) as an ATP trapping
system, this caused an unexpected reactivation of NR (Fig. 6). Both systems (gel filtration and glucose + hexokinase addition) remove ATP. However, according to Eq. 1, the latter system produces in addition, G6P and ADP. Further, since crude leaf extracts contain considerable myokinase activity (EC 2.7.4.3), the reaction will proceed according to Eq. 2, and the major products are not G6P and ADP, but G6P and AMP, and presumably very little ADP.

\[
\begin{align*}
2 \text{ glucose} + 2 \text{ ATP} & \rightarrow 2 \text{ G6P} + 2 \text{ ADP} \quad (1) \\
2 \text{ ADP} & \Rightarrow \text{ ATP} + \text{ AMP} \quad (2)
\end{align*}
\]

Reactivation of the ATP-inactivated NR by glucose/hexokinase treatment might thus be traced back either to production of G6P or to production of AMP. G6P itself did not reverse the inhibition of NR by ATP (not shown). However, when AMP was added in excess over ATP, NR was completely reactivated and the final activity often exceeded the initial activity before inactivation (Fig. 7). Due to myokinase activity in the crude extract, excess AMP consumes ATP in a reversal of Eq. 2. But NR was also reactivated by AMP, when ATP was first removed by gel filtration (Fig. 8). The reactivating effect of AMP is thus a direct one, and cannot be traced back to a myokinase-dependent ATP removal only. Figure 8 demonstrates also that after ATP removal by gel filtration, only relatively low AMP concentrations were required (about 100 \( \mu \text{M} \)) to reactivate NR. When NR was inactivated by preincubation of a crude extract with \( \gamma\text{-S-ATP} \), neither treatment with glucose/hexokinase (Fig. 6) nor addition of excess AMP reversed the inhibition (Table III).

### Lack of Inactivation by ATP of a Partially Purified NR

The above described experiments were thus far carried out with crude leaf extracts. These systems have the disadvantage that concentrations of added metabolites are only badly controlled. This may cause problems, as shown above for the ATP/AMP effects. On the other hand, work with crude extracts is advantageous if one suspects an enzyme modulation involving secondary enzyme systems. In fact, we found that after partial purification of NR by ammonium sulfate

| Table II. Effect of ATP-Dependent Inactivation (1 mm, 15 min) on Kinetic Constants of NR |
|-----------------|-----------------|-----------------|-----------------|
| Treatment       | \( K_m \) NADH | \( K_m \) NO\(_3^-\) | \( V_{max} \) |
| Control (−ATP)  | 8.0 ± 4         | 38 ± 8          | 10.5 ± 2.7     |
| After preincubation with ATP (1 mm) | 6.2 ± 3         | 40 ± 15         | 1.8 ± 0.7      |
fractionation and affinity chromatography on blue Sepharose, the responsiveness to ATP (or AMP) was lost (Table IV). The reason is yet unclear, but it suggests again the participation of other protein factors for ATP-dependent inactivation of NR.

**Evidence for the Involvement of the ATP/AMP System in the Modulation of NR in Vivo**

Nitrate reductase is located in the cytosol (for review see ref. 15). Accordingly, knowledge about cytosolic concentrations of ATP and ADP is required in order to judge whether the observed in vitro modulation of NR occurs in vivo. Cytosolic ATP levels in barley leaf protoplasts have been found to be higher under photorespiratory (low CO2) than under nonphotorespiratory (high CO2) conditions (5). Unfortunately, Gardeström and Wigge (5) did not measure AMP. To obtain an impression of the response of adenine nucleotide levels in leaves under the experimental conditions employed for the modulation of NR, we determined concentrations of all adenine nucleotides in total leaf extracts after leaves had been illuminated for 1 h at the CO2 compensation point or in 5% CO2 for obtaining maximum rates of photosynthesis. As predicted by the response of NR to ATP and AMP in vitro, we found that ATP levels in leaves with maximum photosynthesis rates were lower and AMP levels were much higher than in leaves at the CO2 compensation point (Table V). We are aware, however, that these measurements indicate trends only. Absolute adenine nucleotide levels in the cytosol are probably much higher (5), and ATP/AMP activity ratios must be lower than indicated by our data. Otherwise, NR would always be totally inactivated. Alternatively, the cytosol might contain metabolites acting as competitive inhibitors for ATP.

In an attempt to manipulate the adenine nucleotide levels in leaves and to study the effect of such manipulations on NR, detached leaves were fed with mannitol through their petiole, while illuminating them in high CO2 or at the CO2 compensation point. Mannitol at high external concentrations slowly penetrates the plasma membrane and enters the cells. There, it is phosphorylated by hexokinase/ATP, but the resulting mannose-6-phosphate is not metabolized further. Thus, mannose acts primarily as a trap for phosphate and ATP, and rates of photosynthesis are decreased (2, 6, 7). In fact, mannose feeding had drastic effects on total adenine nucleotide levels and on the concentration ratios of nucleotides in whole leaf extracts (Table V). Under all conditions, ATP levels decreased and AMP levels remained constant or increased slightly after mannose feeding, resulting in lower ATP/AMP ratios. As expected, mannose feeding inhibited the photosynthetic capacity of leaves by about 60%. In spite of that, mannose feeding had little or no effect on NRA under +CO2 conditions (Table V). Most important, however, was the effect of mannose on ATP/AMP and on nitrate reductase under photorespiratory (-CO2) conditions: under such conditions, NR was usually inactivated, and ATP/AMP ratios were high when mannose was absent (Table V). But in mannose-fed leaves, ATP/AMP was low (even lower than in control leaves under photosynthesizing conditions), and NR remained high (Table V). Obviously, NR is fully active when ATP/AMP ratios are low enough, independent of the actual rate of net CO2 fixation. Thus, mannose feeding in a certain sense “uncoupled” NR from photosynthesis.

As shown above, the ATP-inactivated NR was reactivated by addition of AMP. If adenine nucleotides were responsible

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**Table III. Lack of Reactivation by AMP of NR after Inactivation by Preincubation with γ-S-ATP**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>μmol g⁻¹ fresh wt h⁻¹</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.1 ± 0.6</td>
<td>100</td>
</tr>
<tr>
<td>+ATP</td>
<td>1.3 ± 0.1</td>
<td>18</td>
</tr>
<tr>
<td>+γ-S-ATP</td>
<td>1.5 ± 0.6</td>
<td>21</td>
</tr>
<tr>
<td>+ATP, then + AMP</td>
<td>8.1 ± 0.8</td>
<td>125</td>
</tr>
<tr>
<td>+γ-S-ATP, then + AMP</td>
<td>1.9 ± 0.3</td>
<td>27</td>
</tr>
</tbody>
</table>

**Table IV. Response to a Preincubation with ATP (1 mM, 12 min) of NRA (a) in a Crude, Desalted Leaf Extract, and (b) after Partial Purification by Affinity Chromatography**

<table>
<thead>
<tr>
<th>Inactivation</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>units/mg protein</td>
</tr>
<tr>
<td>Crude extract</td>
<td>62</td>
</tr>
<tr>
<td>Partially purified</td>
<td>12</td>
</tr>
</tbody>
</table>

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Figure 8. Reactivation of NR by various AMP concentrations, after inactivation with 1 mM ATP (15 min), and subsequent ATP removal by desalting. Incubation time with AMP was 30 min. Reaction time was 1 min. The closed circle indicates NRA immediately after preincubation with ATP, open circles NRA after subsequent preincubation with AMP. Each point represents the mean from two independent experiments, out of a total of four similar experiments with slightly different experimental conditions.
for the modulation of NR in the leaf, the in vivo inactivation of NR by \(-\text{CO}_2\) treatment of leaves should be reversed in vitro by AMP. Leaves were first illuminated for 1 h at the \(\text{CO}_2\) compensation point in order to inactivate NR in vivo. The crude, desalted extract of these leaves was then preincubated with 1 mM AMP. As expected, the in vitro AMP treatment caused a slow but significant reactivation of NR (Fig. 9). Although these experiments are no final proof for a causal connection of NRA and photosynthesis via cytosolic ATP/AMP ratios, they are certainly very suggestive.

**DISCUSSION**

Among the various compounds which have been found to affect NRA in vitro, only the adenine nucleotides ATP and AMP caused a modulation that resembled that observed after extraction of NR from photosynthesizing or photorespiring leaves. A main characteristic of the modulation was its stability after ATP removal, and its obligatory dependence on divalent cations (Mg\(^{2+}\) or Ca\(^{2+}\)). There are a number of earlier reports of adenine nucleotide effects on NR, but reported results are equivocal and some are in marked contrast to our above described results. Eaglesham and Hewitt (4) report an inhibition of NR by virtually all three adenine nucleotides (ATP, ADP, and AMP). An inhibition by ADP, but no effect of ATP and AMP was observed by other authors (11). More recently, Sanchez and Heldt (13), using a partially purified NR preparation, found an inhibition of NR by physiological ADP concentrations or ATP/ADP ratios, respectively, and at low (more natural) substrate concentrations. In this report, AMP had no effect.

The discrepancy in literature data and our own results is probably due to basic methodical differences. All the authors cited above measured NRA directly in the presence of the nucleotides without preincubation, whereas in our experiments, enzyme activity was measured after preincubation and subsequent removal or at least strong dilution of the nucleotides. Thus, a stable modulation could be detected only under our conditions. Second, with the exception of Nelson and Ilan (11), partially purified enzyme preparations were used in the other studies. As shown above, partially purified NR from spinach leaves did not respond to a preincubation with ATP. The role of divalent cations for the in vitro modulation of partially purified NR was studied only recently (13). Here, Mg\(^{2+}\) was found to counteract the inhibitory effect of high ATP/ADP ratios. The finding is again in obvious contrast to our above described data, and we are at present not able to offer a solution to the discrepancy. But our experiments have shown that divalent cations serve two different functions: (a) the presence of free Mg\(^{2+}\), was required for the inactivation process itself, and this reaction was magnesium specific; (b) the modification of NR was expressed as a decrease in catalytic activity only in the presence of divalent cations, but Mg\(^{2+}\) could be replaced by free Ca\(^{2+}\). This clear cut difference in the inactivation process and the expression of catalytic activity points to the possibility that NR is covalently modified by a second enzyme system in the presence of ATP and Mg\(^{2+}\), e.g., by protein phosphorylation. This idea is further supported by the fact that ATP analogs which are nonhydrolyzable due to modification of the phosphate bond, had no effect on NR. In contrast, \(\gamma\)-S-ATP, which has an intact bond between the \(\beta\)- and \(\gamma\)-phosphate group, is apparently hydrolyzed by some enzymes but not by others. We found that it inactivated

![Figure 9. Reactivation in vitro by preincubation with AMP (1 mM) of NR that was previously inactivated in vivo by illuminating leaves for 1 h in a closed system at the \(\text{CO}_2\) compensation point. Mg\(^{2+}\) concentration in the preincubation and reaction medium was 5 mM (n = 2).](image-url)

| Table V | **NRA, Adenine Nucleotide Levels, and Photosynthetic Capacity and in Spinach Leaf Extracts Obtained from Detached Leaves with their Petiole in Distilled Water, or in 50 Mannose Solution**

Leaves were illuminated for 1 h either in 5\% \(\text{CO}_2\) to achieve maximum photosynthetic rates (+\(\text{CO}_2\)) or in a closed system at the \(\text{CO}_2\) compensation point (−\(\text{CO}_2\)). Means from six independent experiments ±SD. Nucleotide levels are in nmol g\(^{-1}\) fresh wt. NRA is given in \(\mu\)mol NO\(_2\) g\(^{-1}\) fresh wt h\(^{-1}\), photosynthesis as \(\mu\)mol O\(_2\) evolved mg\(^{-1}\) Chl h\(^{-1}\). (n.m. = not measured).

<table>
<thead>
<tr>
<th></th>
<th>+(\text{CO}_2)</th>
<th>−(\text{CO}_2)</th>
<th>+(\text{CO}_2) + Mannose</th>
<th>−(\text{CO}_2) + Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRA</td>
<td>7.6 ± 2.4</td>
<td>3.9 ± 0.1</td>
<td>6.9 ± 1.7</td>
<td>7.8 ± 1.2</td>
</tr>
<tr>
<td>ATP</td>
<td>110 ± 22</td>
<td>179 ± 44</td>
<td>54 ± 11</td>
<td>80 ± 8</td>
</tr>
<tr>
<td>ADP</td>
<td>33 ± 6</td>
<td>42 ± 6</td>
<td>31 ± 3</td>
<td>24 ± 8</td>
</tr>
<tr>
<td>AMP</td>
<td>9 ± 2</td>
<td>6 ± 1</td>
<td>10 ± 2</td>
<td>9 ± 6</td>
</tr>
<tr>
<td>ATP/AMP</td>
<td>12</td>
<td>30</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Photosynthesis</td>
<td>340 ± 10</td>
<td>n.m.</td>
<td>155 ± 7</td>
<td>n.m.</td>
</tr>
</tbody>
</table>
NR, although it could not serve as a substrate for hexokinase (data not shown). Therefore, it was not unexpected that the γ-S-ATP inactivated NR was not reactivated by glucose + hexokinase treatment. The mechanism for the reactivation of NR by AMP is as yet obscure. The effective AMP concentrations were relatively high but still in a physiological range. To our present knowledge, participation of AMP in protein dephosphorylation reactions has not yet been described (for review see ref. 1). It is also as yet unclear whether inhibitory protein factors which have been found in various plants (8, 12, 16–18) are somehow involved, e.g. instead of a direct phosphorylation of NR, one might assume a regulatory protein factor to be modulated by phosphorylation/dephosphorylation.

We have shown that γ-S-ATP caused an irreversible inactivation of NR, which might be explained by an irreversible binding of the γ-S-phosphate group to the protein. Binding of the normal ADP residue of γ-S-ATP by protein adenylation should lead to the usual reversible inactivation. This was not the case. Thus, protein adenylation as a regulatory mechanism can be excluded.

A different mode of action of adenine nucleotides on NR has been proposed earlier by Solomonson and Spehar (14). They reported on a stimulation of cyanide formation by ADP in Chlorella extracts. Cyanide itself in the presence of NADH is a potent inhibitor of NR. However, we have shown in the preceding paper (9) that under our experimental conditions, the cyanide inhibition of NR was removed by gel filtration, whereas both the in vivo inhibition of NR by -CO2 treatment of leaves, and the in vitro inactivation by ATP were not reversed.

As a working hypothesis, we propose the following model for a modification of NR (Fig. 10). Under photosynthetic (high CO2) conditions, the cytosolic ATP/AMP ratio is low and NR is active (state A). When stomata close, CO2 decreases and cytosolic ATP/AMP increases. NR (or a regulatory protein) is modified by phosphorylation, with MgATP (or MgUTP) as substrate. In the presence of free Ca2+ or Mg2+, the modified NR changes its conformation (or binds a regulatory protein) and loses its catalytic properties (state B). If divergent cations are removed, catalytic activity is fully reversed, but the NR enzyme remains sensitive to divergent cations, i.e. the anionic groups (e.g. Pi or a phosphorylated protein) remain bound (state C). When ATP/AMP ratios decrease, the anionic groups are removed and NR is transferred to state A, where it is again insensitive to divergent cations. Whether AMP acts as a substrate or as a modulator is not yet known. Also, the type and number of bound anionic groups and of participating enzymes is as yet a matter of speculation.

**ACKNOWLEDGMENTS**

The authors gratefully acknowledge the skilled technical assistance of E. Brendle-Behnisch and A. Wirth. Further, we wish to thank E. Neimanis for help with the determination of adenine nucleotides, and our colleagues R. Tischner (Göttingen) and M. Guerrero (Sevilla) for helpful discussions and advice.

**LITERATURE CITED**


*Figure 10. A hypothetical model for the modulation of NR by ATP/AMP and for the role of divalent cations. Explanations in the text.*
MODULATION OF ASSIMILATORY NITRATE REDUCTASE BY ATP AND AMP IN VITRO