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Comparative Studies of the Light Modulation of Nitrate Reductase and Sucrose-Phosphate Synthase Activities in Spinach Leaves[1]

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ABSTRACT

We recently obtained evidence that the activity of spinach (*Spinacia oleracea* L.) leaf nitrate reductase (NR) responds rapidly and reversibly to light/dark transitions by a mechanism that is strongly correlated with protein phosphorylation. Phosphorylation of the NR protein appears to increase sensitivity to Mg^{2+} inhibition, without affecting activity in the absence of Mg^{2+}. In the present study, we have compared the light/dark modulation of sucrose-phosphate synthase (SPS), also known to be regulated by protein phosphorylation, and NR activities (assayed with and without Mg^{2+}) in spinach leaves. There appears to be a physiological role for both enzymes in mature source leaves (production of sucrose and amino acids for export), whereas NR is also present and activated by light in immature sink leaves. In mature leaves, there are significant diurnal changes in SPS and NR activities (assayed under selective conditions where phosphorylation status affects enzyme activity) during a normal day/night cycle. With both enzymes, activities are highest in the morning and decline as the photoperiod progresses. For SPS, diurnal changes are largely the result of phosphorylation/dephosphorylation, whereas with NR, the covalent modification is superimposed on changes in the level of NR protein. Accumulation of end products of photosynthesis in excised illuminated leaves increased maximum NR activity, reduced its sensitivity to Mg^{2+} inhibition, and prevented the decline in activity with time in the light seen with attached leaves. In contrast, SPS was rapidly inactivated in excised leaves. Overall, NR and SPS share many common features of control but are not identical in terms of regulation in situ.

Many metabolic processes in leaves are stimulated by (or dependent upon) light as a result of a requirement for products of thylakoid electron transport (e.g. reduced Fd) or products of carbon dioxide assimilation (e.g. metabolic intermediates). For example, sucrose synthesis and nitrate assimilation are major processes in leaves that are generally coordinated with photosynthesis. Thus, light stimulates the rate of carbon flux into sucrose and the rate of nitrate assimilation and formation of amino acids. The coordination of each process with photosynthesis is achieved, in part, by regulation of the activities of one or more key enzymes in each pathway. Recently there has been progress in the identification of mechanisms for the regulation of key enzymes involved in both pathways.

With respect to the nitrate assimilation pathway, it is generally recognized that control of NR[2] activity may play a critical role in regulation of nitrogen assimilation (3). In the sucrose-formation pathway, it is thought that SPS is an important control point (11, 28, 33). Both SPS and NR are localized in the mesophyll cell cytoplasm, and the activities of both enzymes respond to light/dark signals. Light activation of SPS has been recognized for some time (12 and references therein), whereas similar regulation of NR has only recently been reported (10, 15, 22, 23). We recently suggested (10) that there may be two factors responsible for rapid changes in NR activity following light/dark transitions: (a) changes in the steady-state level of NR protein; and (b) posttranslational modification of existing NR protein that alters the sensitivity of NR to inhibition by Mg^{2+}. The posttranslational mechanism involved in altering sensitivity to Mg^{2+} inhibition (13) appears to be protein phosphorylation (10, 14). Both SPS and NR are more heavily phosphorylated in the dark, and light activation involves dephosphorylation that is sensitive to okadaic acid, suggesting involvement of type 1 or 2A protein phosphatases (10).

Phosphorylation does not affect maximum catalytic activity (V_{max} or "nonselective" assay) of either spinach SPS or NR. Rather, the kinetic effect of phosphorylation can only be observed under "selective" assay conditions. With SPS, the enzyme must be assayed with limiting substrate concentrations in the presence of Pi, an inhibitor (29), whereas with NR, the enzyme must be assayed in the presence of millimolar concentrations of Mg^{2+} (10, 13–15, 23). NR assayed in the absence of Mg^{2+} probably reflects the steady-state level of NR protein (5). Consequently, the phosphorylation status of both enzymes can be inferred from assays conducted with selective versus nonselective conditions.

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[2] Abbreviations: NR, NADH-nitrate reductase; SPS, sucrose-phosphate synthase.

In the present study, we set out to characterize some of the factors that may affect the in vivo activation of NR and SPS by light in spinach (*Spinacia oleracea* L.) leaves. Specific objectives were to compare NR and SPS light activation with respect to: (a) leaf age; (b) diurnal changes; and (c) accumulation of end products of photosynthesis. The results obtained indicate that although NR and SPS appear to be inactivated by phosphorylation in darkness and respond similarly to many conditions, it appears that the interconverting enzymes that act on SPS and NR are quite distinct and may respond to different "signals."

MATERIALS AND METHODS

Materials

All biochemicals were purchased from Sigma Chemical Co.[3]

Plant Material

Spinach (*Spinacia oleracea* L. cv Bloomsdale) was grown in soil with a standard Hoagland solution (10 mM NO_3^-). Plants were grown either in a growth chamber with an 11-h photoperiod (350 μmol·m^{-2}·s^{-1}) and a 25/20°C (day/night) temperature regimen or in the greenhouse during the winter months. Greenhouse temperature was maintained between 25 and 20°C without supplemental lighting. In some experiments, leaf tissue was harvested in the light or dark and frozen immediately in liquid nitrogen. In other experiments, leaves were excised and the petioles were recut under degassed water and placed in degassed water as indicated in the text.

Extraction and Enzyme Assays

Frozen leaf tissue was ground in a chilled mortar with extraction buffer (1 g/2 mL) containing 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, and 0.1% (w/v) octyl phenoxy polyethoxyethanol (Triton X-100). The homogenates were centrifuged at 20,000*g* for 0.5 min in 1.5-mL microcentrifuge tubes. The supernatant fractions were desalted immediately by centrifugal filtration on Sephadex G-25 columns (1 × 5 cm) equilibrated with extraction buffer minus Triton X-100, and with the concentration of DTT reduced to 2.5 mM. SPS activity was assayed with limiting substrates plus Pi (limiting assay) and with saturating substrates (V_{max} assay). SPS activation state is defined as the limiting activity expressed as a percentage of the V_{max} activity. The composition of the reaction mixtures, and other details of the assay procedure, were as described (12). NADH:NR activity was assayed colorimetrically as described by Huber et al. (10). The 1-mL reaction mixtures contained 50 mM Mops-NaOH (pH 7.5), 5 mM $MgCl_2$, 10 mM KNO_3 (+Mg^{2+} assay); or 50 mM Mops-NaOH (pH 7.5), 1 mM EDTA, and 10 mM KNO_3 (−Mg^{2+} assay). All assays contained 0.1 mM NADH and were initiated by addition of enzyme extract and terminated by addition of zinc acetate. Other details of the product detection were as previously described (25).

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or the North Carolina Agricultural Research Service and does not imply its approval to the exclusion of other products that may also be suitable.

RESULTS AND DISCUSSION

Activation Kinetics

An initial time-course experiment was conducted to compare the activation/inactivation kinetics of NR and SPS in vivo. Both enzymes were measured under the selective conditions, where effects of covalent modification are apparent. With SPS, the activation state of the enzyme increased rapidly with illumination of leaves, reaching a maximum within about 15 min; inactivation upon darkening occurred more slowly, in about 30 min (Fig. 1). In contrast, the maximum light stimulation of NR activity (measured in the presence of Mg^{2+}) required 30 to 60 min, but the dark inactivation was complete within 10 min (Fig. 1). In other experiments, the half-time for dark inactivation of NR (assayed with Mg^{2+}) was found to be about 2.5 min (data not shown). Thus, while both enzymes are activated by light and inactivated in the dark, the time courses are significantly different, suggesting that the underlying control mechanisms are not identical.

Effect of Leaf Age

It is well known that as leaves expand, they undergo the classic "sink to source" transition (7, 21). Generally speaking, enzymes involved in sucrose degradation and glycolytic metabolism are highest in rapidly expanding leaves and decrease

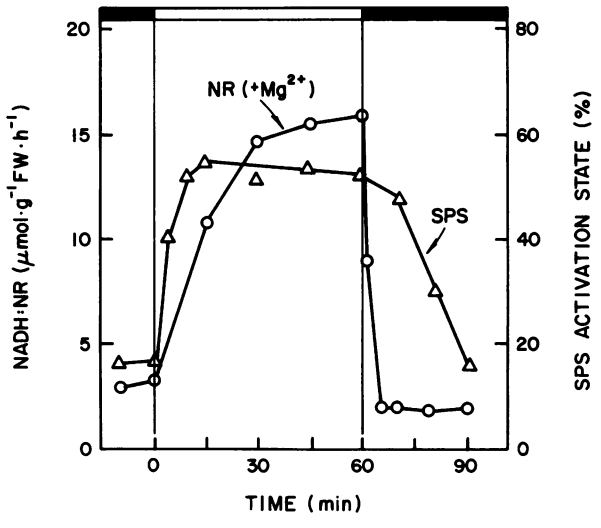

Figure 1. Time-course of the light activation/dark inactivation of NR and SPS in attached spinach leaves. Plants were preilluminated for 1 h prior to transfer to darkness for 45 min. At time zero, the lights were turned on (350 μmol·m^{-2}·s^{-1}) for 1 h followed by return to darkness. At the times indicated, leaf samples were removed, frozen in liquid nitrogen, and assayed for enzyme activities. The SPS V_{max} activity was constant at about 70 μmol·g^{-1} fresh weight·h^{-1}. Maximum NR activity (assayed without Mg^{2+}) increased from 15 to 20 μmol·g^{-1} fresh weight·h^{-1} during the dark-to-light transition. Values are means of two determinations.

in activity during expansion, whereas SPS, an enzyme involved in sucrose biosynthesis, tends to increase during expansion and parallels the onset of export. As shown in Figure 2A, the maximum activity of SPS in spinach leaves increased with leaf size until the point of full expansion was attained (about 6 g fresh weight). Previous studies have demonstrated that the increase in V_{max} correlates closely with SPS enzyme protein (31). The ability to light activate SPS also increased as leaves expanded and was generally correlated with the increase in photosynthetic activity of the leaf tissue (data not shown). In contrast, the maximum activity of NR (measured in extracts of illuminated leaves assayed without Mg^{2+}) was highest in rapidly expanding leaves and decreased approximately 20% as leaves expanded. Light/dark modulation of NR activity (assayed in the presence of Mg^{2+}) was observed both in young and mature leaves, but in absolute terms was, in fact, slightly greater in the young leaves (Fig. 2B). These results are consistent with a major role for SPS in source leaves, whereas NR appears to be highly active in both sink and source leaves. There is existing evidence that stage of tissue development affects the expression of NR. For example, in a limited study with young maize seedlings, Bowsher et al. (4) reported that shoot NR activity tended to decrease with age. Also in maize, Srivastava et al. (27) reported that NR activity was low in young leaves and reached maximum activities when leaves attained full expansion, whereas in pearl millet NR activity is very high in the youngest leaf and decreases rapidly with aging of leaves (18). The developmental patterns of NR in spinach leaves are evidently slightly different than in maize, but in both species a clear role for NR in mature leaves is apparent.

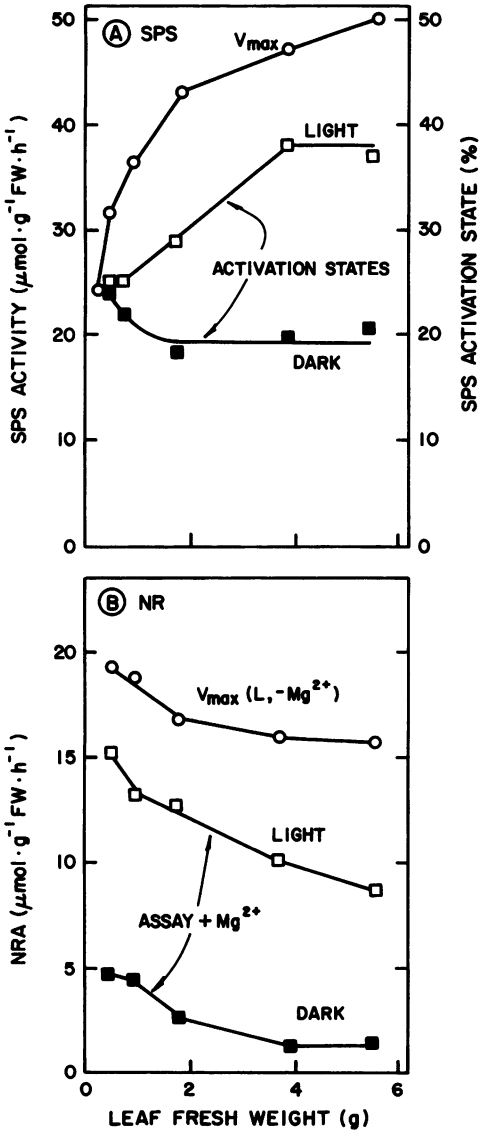

Figure 2. Effect of leaf age on light/dark modulation of SPS and NR activities in attached spinach leaves. Plants were illuminated for 1 h at the beginning of the photoperiod, and samples of leaves of different sizes were harvested into liquid nitrogen (light sample). The plants were then darkened, and after 45 min the remaining tissue from sampled leaves was harvested for the dark sample. Values are means of two determinations.

Diurnal Changes in Enzyme Activities

It is well known that the activation state of SPS in spinach leaves increases rapidly upon illumination and then declines gradually throughout the light period (24, 29). This pronounced diurnal fluctuation in SPS activation state (even though irradiance and assimilation rate remain constant; data not shown) are shown in Figure 3B.

There were significant diurnal changes in NR activity as well. As shown in Figure 3A, with the onset of illumination there was an increase in maximum NR activity (assayed in the absence of Mg^{2+}), which thereafter remained high and decreased only slightly as the photoperiod progressed (Fig. 3A, $-Mg^{2+}$ curve). After the transition to darkness, maximum NR activity decreased to the "predawn" level within about 60 min. When NR was assayed in the absence of Mg^{2+}, but with the nonspecific activator Pi (19), activities were stimulated about 35% at all times of the diurnal cycle. Thus, sensitivity to Pi activation did not vary diurnally. It is quite likely that, to a first approximation, the changes in maximum NR activity (assayed in the absence of Mg^{2+}) reflect changes in the steady-state level of NR protein (5, 10). However, it should be noted that changes in the V_{max} activity of NR have been observed in the absence of changes in NR enzyme protein (22), indicating that the two are not always exactly correlated in all circumstances.

In contrast, NR activity assayed in the presence of Mg^{2+} was very low in darkness, increased rapidly with illumination, and then decreased significantly during the photoperiod (Fig. 3A, $+Mg^{2+}$ curve). Upon transfer to darkness, NR activity ($+Mg^{2+}$) decreased rapidly to very low levels. Comparison of the NR activities measured in the presence and absence of Mg^{2+} (Fig. 3A) indicated that sensitivity of NR to Mg^{2+} inhibition varied dramatically with light/dark transitions but also changed significantly during the photoperiod. In the dark (predawn), inhibition by 5 mM Mg^{2+} was strong (about 85%). During the first several hours of illumination, there was essentially no inhibition by Mg^{2+}, but as the photoperiod progressed, inhibition by Mg^{2+} increased slowly to reach about 50% at the end of the day. Darkening of leaves rapidly

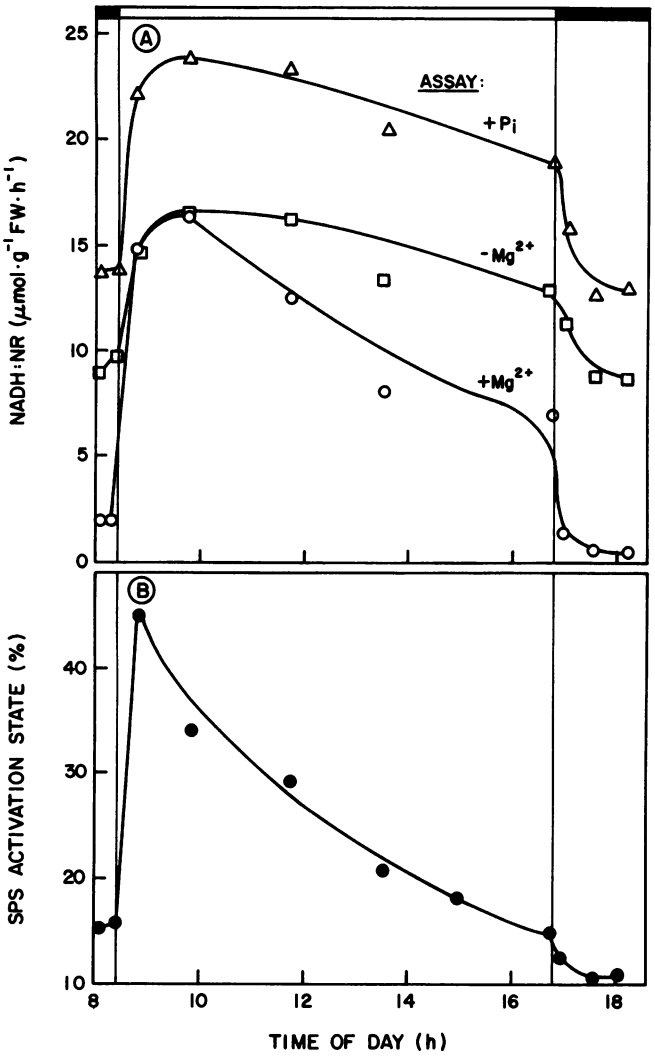

Figure 3. Diurnal changes in (A) NR activities and (B) SPS activation state in situ in attached spinach leaves. At the times indicated, leaf tissue was harvested into liquid nitrogen and assayed for (A) maximum NR activity (assayed without Mg^{2+} and with or without Pi, an activator) or NR activity assayed with Mg^{2+}; and (B) limiting and V_{max} SPS activities, from which activation state was calculated. SPS V_{max} activity was constant at about 65 μmol·g^{-1}fresh weight·h^{-1}. Values are the means of two determinations.

restored strong inhibition by Mg^{2+} (Fig. 4). Thus, there is a general similarity between NR and SPS concerning diurnal changes in kinetic properties. With time of day, both enzymes "deactivate" in situ as SPS and NR become increasingly sensitive to inhibition by Pi and Mg^{2+}, respectively. These changes in kinetic properties probably reflect changes in the phosphorylation state of the enzymes. Diurnal changes in leaf NR activity have been reported in many species (16, 22, 26 and references therein). In many, but not all, cases, NR activity tends to decrease with time of day, and in some studies, the changes in NR activity (assayed without Mg^{2+}) generally track changes in NR protein level (16, 22). Diurnal changes in sensitivity to Mg^{2+} inhibition (Fig. 4) would clearly be superimposed on changes in maximum NR activity, which has been the focus of most previous studies.

Differential Effect of End Product Accumulation in Leaves

The similar diurnal responses suggest that mechanisms exist to regulate the phosphorylation states of the SPS and NR independent of changes in photosynthesis (which remains essentially constant during the light period; data not shown). It is possible that accumulation of photosynthetic products such as soluble sugars or amino acids can influence enzyme activities. For example, sucrose accumulation may be involved in the feedback inhibition (inactivation) of SPS, whereas accumulation of amino acids may influence NR activities. There is the general notion that amino acids can be negative effectors of NR, at least in certain tissues. In tobacco leaves, Deng et al. (6) have shown that diurnal variations in NR mRNA levels were inversely related to leaf glutamine content. They suggested that glutamine, and/or other amino acids, may exert a negative control on NR gene expression.

As one approach to examining this question, we studied the influence of leaf excision on NR and SPS activities. When illuminated spinach leaves are excised, normal phloem transport is disrupted and end products of photosynthesis (e.g. sucrose and amino acids) accumulate rapidly (8). As sucrose and other end products accumulate, the flux of carbon into sucrose is reduced in part as a result of inactivation of SPS (17). Thus, the light activation/dephosphorylation of SPS can be reversed in situ by accumulation of soluble sugars (29), although the specific mechanism and "signal(s)" involved remain unclear. It was of interest to compare the effects of end product accumulation on the activities of SPS and NR.

To examine this aspect in more detail, enzyme activities were compared in excised versus attached leaves. With SPS, activation state in situ declined with time of day in attached leaves (Fig. 5C; ref. 29), and excision of leaves resulted in a

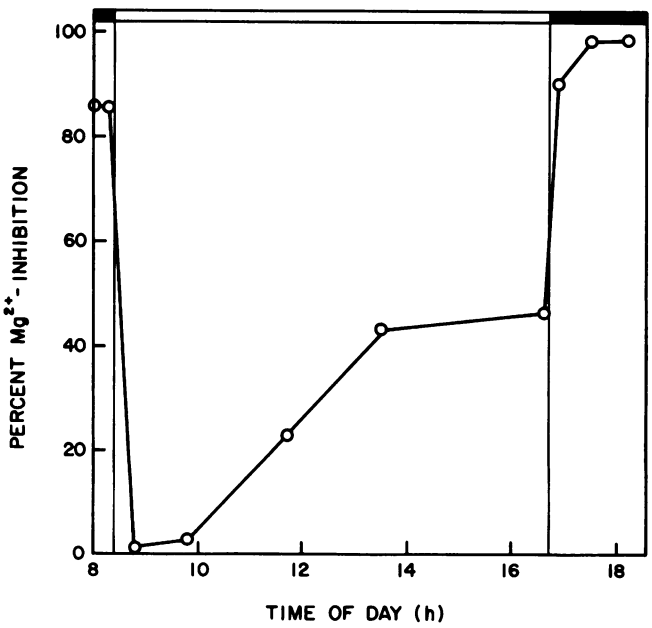

Figure 4. Diurnal changes in sensitivity of spinach leaf NR to Mg^{2+} inhibition. The percentage of Mg^{2+} inhibition was calculated from the values plotted in Figure 3A.

larger decrease in activation state relative to attached leaves (Fig. 5C). The inactivation of SPS in response to sucrose accumulation represents feedback regulation of the sucrose formation pathway, and presumably involves protein phosphorylation (9).

In contrast, NR activity responded differently when leaves were attached or detached in the light. In attached leaves, maximum NR activity (assayed in the absence of Mg^{2+}), increased upon illumination and declined approximately 15% during the first 4 h of the photoperiod (Fig. 5A; $-Mg^{2+}$ curve). NR activity assayed with Mg^{2+} also increased upon illumination, but then decreased with time to a greater extent (approximately 36%) than did maximum NR activity (Fig. 5A). Thus, sensitivity to Mg^{2+} inhibition tended to increase with time in the light. These results were expected based on earlier experiments (Figs. 3 and 4). It is important that when leaves were excised from illuminated plants 1 h into the photoperiod, there was some additional increase in NR activity (assayed both in the presence and absence of Mg^{2+}). In addition, the decline in NR activity observed in attached leaves (Fig. 5A) was prevented (Fig. 5B). In contrast, excision of leaves apparently accelerates the inactivation of SPS that occurs with time of day (Fig. 5C). Thus, accumulation of end products of photosynthesis was correlated with the activation of NR and the inactivation of SPS. These results are in agreement with the notion that high carbohydrate levels enhance the rate of nitrate reduction, especially in leaves (1, 2, 30), and emphasize that NR is not sensitive to short-term accumulation of amino acids.

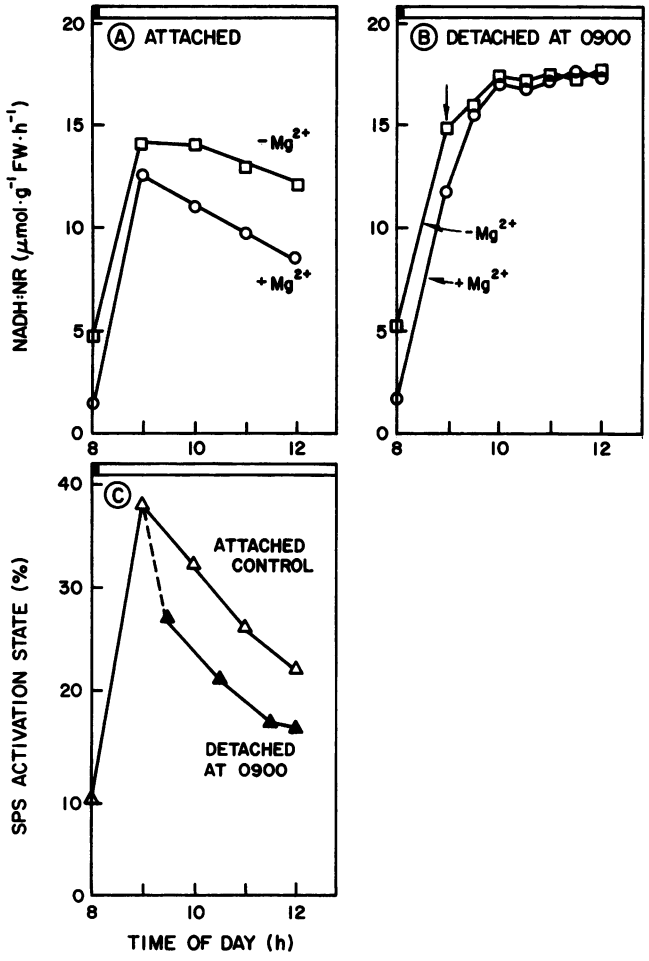

Figure 5. Effect of detaching illuminated spinach leaves on NR activity and SPS activation state. Plants were illuminated (350 μmol·m^{-2}·s^{-1}) for 1 h at the beginning of the photoperiod. Thereafter (at 0900 h), some leaves were excised and placed in water. Illumination was continued with both attached and detached leaves. At various times, samples were harvested (into liquid nitrogen) from either attached control leaves or detached leaves as indicated. In panels A and B, NR was assayed in the absence of Mg^{2+} (□) and the presence of 5 mM Mg^{2+} (○). SPS activation state in situ is compared in attached and detached spinach leaves in panel C. Values are the means of two determinations.

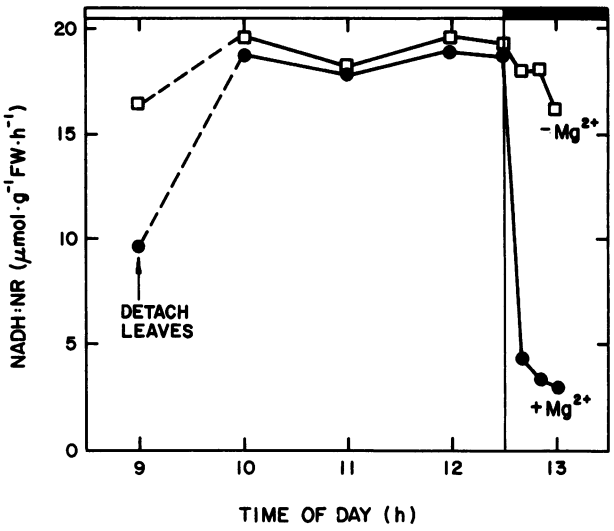

Figure 6. Light activation and dark inactivation of NR activity in spinach leaves that were excised from illuminated plants. Intact plants were illuminated (350 μmol·m^{-2}·s^{-1}) for 1 h at the beginning of the photoperiod; at 0900 h, leaves were excised and kept in the light for an additional 3.5 h, prior to darkening the leaves for 0.5 h. At various times, samples were harvested and NR was assayed in the absence (□) and presence (●) of Mg^{2+}.

Because sensitivity of NR to Mg^{2+} inhibition is correlated with the phosphorylation status of the protein (10, 15), it is possible that one or more end products of photosynthesis may either inhibit NR-kinase and/or activate NR-protein phosphatase. Thus, it was of interest to determine whether leaf detachment in the light affected the subsequent increase in NR sensitivity to Mg^{2+} inhibition that normally occurs in the dark. This was not the case, because darkening of detached leaves resulted in a rapid increase in sensitivity to Mg^{2+} inhibition (Fig. 6). Thus, inactivation of the NR-kinase(s) appears not to be a major factor involved in the hyperactivation of NR that is observed in illuminated, excised leaves.

CONCLUDING REMARKS

SPS and NR are soluble cytoplasmic enzymes that are important control points in the pathways involved in sucrose

biosynthesis and nitrate assimilation, respectively. It has been recognized for some time that NR is regulated by synthesis/degradation of the enzyme protein, and evidence has also been obtained that NR is activated by light and inactivated by darkness (22). Recent results suggest that the activities of NR and SPS both respond rapidly and reversibly to light/dark transitions by mechanisms that involve, in part, protein phosphorylation. Because both sucrose biosynthesis and nitrate assimilation must be generally coordinated with photosynthesis, it is of interest to compare the physiological conditions that affect the enzymes. Both SPS and NR can be conveniently assayed under conditions that distinguish between changes in enzyme protein level (V_{max} or nonselective assay) and covalent modification (selective assay).

In vivo, the covalent modification of SPS (assayed with Pi) and NR (assayed with Mg^{2+}) respond similarly in several regards. First, both are light- and mannose-activated (in darkness), and the mechanism involves protein phosphorylation. With both SPS and NR, phosphorylation increases sensitivity of the enzyme to an inhibitor (either Pi or Mg^{2+}, respectively). Second, both enzymes tend to "inactivate" toward the end of the photoperiod even though irradiance and assimilation rate remain constant, suggesting decreased enzymic capacity for sucrose formation and nitrate assimilation in the afternoon hours relative to the morning hours. It may be significant that, later in the photoperiod, supply of nitrate to the leaf would be expected to be lower than in the morning (20, 32). Third, both the target enzymes (SPS and NR) and their interconverting enzymes are highly active in source leaves, suggesting a physiological role of both enzymes in the synthesis of sucrose and amino acids for export.

Although many aspects of the in vivo regulation of SPS and NR are similar, there are also several fundamental differences. First, the two enzymes differ in the kinetics of light activation and dark inactivation in vivo. In general, both enzymes activate relatively slowly but NR requires a longer period of time for full activation. Conversely, the dark inactivation of SPS is much slower compared to NR, which inactivates with a half-time of about 2.5 min. The rapid inactivation of NR in darkness, which was also recently reported by Riens and Heldt (23), may serve to reduce excessive nitrite accumulation when nitrite reductase activity is restricted. Second, diurnal changes in NR activity are the result of two components: alteration of the steady-state level of enzyme protein and covalent modification (i.e. protein phosphorylation), whereas with SPS, the enzyme protein appears to be relatively stable and fluctuations in activation state are, for the most part, the result of phosphorylation (inactivation)/dephosphorylation (activation) (9, 29). Third, short-term accumulation of end products of photosynthesis in leaves (sucrose, amino acids, etc.) results in increased NR activities but decreased SPS activity. The exact nature of the "signal" metabolite(s) remains to be established, as does the mechanism involved (i.e. regulation of the kinase and protein phosphatase). Last, whereas SPS functions primarily in source leaves, NR and the NR-interconverting enzymes also occur in expanding sink leaves and presumably play an important role in the production of amino acids for use during leaf growth, at least in spinach.

Thus, although the activities of SPS and NR are both generally coordinated with light/dark signals (and photosynthesis), it is clear that the two enzymes respond in several fundamentally different ways. We are currently trying to determine whether the interconverting enzymes are distinct, or whether the action of the interconverting enzymes on the two target enzymes is differentially modulated by specific metabolites. Work is in progress to purify the protein phosphatase(s) and protein kinase(s) that act on NR.

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