Identification of Factors Regulating the Phosphorylation Status of Sucrose-Phosphate Synthase in Vivo

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ABSTRACT

The purpose of this study was to identify the factors that control sucrose-phosphate synthase (SPS)-kinase and SPS-protein phosphatase (SPS-PP) activity in situ, and thereby mediate the activation of SPS by light or mannose. Feeding mannose to excised spinach (Spinacia oleracea) leaves in darkness resulted in a general sequestration of cellular phosphate (as evidenced by accumulation of mannose-6-P and depletion of glucose-6-P [Glc-6-P] and fructose-6-P [Fru-6-P]) and a relatively slow activation of SPS (maximum activation achieved within 90 min). Supplying exogenous inorganic phosphate (Pi) with mannose reduced sequestration of cellular Pi (as evidenced by mannose-6-P accumulation without depletion of hexose-P) and substantially reduced mannose activation of SPS. Thus, depletion of cytoplasmic Pi may be required for SPS activation; accumulation of mannose-6-P alone is clearly not sufficient. It was verified that Glc-6-P, but not mannose-6-P, was an inhibitor of partially purified SPS-kinase, and that Pi was an inhibitor of partially purified SPS-PP. Total extractable activity of SPS-kinase did not vary diurnally, whereas a pronounced light activation of SPS-PP activity was observed. Pretreatment of leaves in the dark with cycloheximide blocked the light activation of SPS-PP (assayed in vitro) and dramatically reduced the rate of SPS activation in situ (in saturating light and carbon dioxide). We conclude that rapid activation of SPS by light involves reduction in cytosolic Pi, an inhibitor of SPS-PP, and light activation of SPS-PP, by a novel mechanism that may involve (directly or indirectly) a protein synthesis step. An increase in cytosolic Glc-6-P, an inhibitor of SPS-kinase, would also favor SPS activation. Thus, the signal transduction pathway mediating the light activation of SPS involves elements of "fine" and "coarse" control.

In leaves of many higher plant species, the activity of SPS—a key enzyme of the sucrose biosynthetic pathway—is rapidly and reversibly modulated by light/dark signals (15, 16, 26, 27). Light activation of SPS is usually complete within 10 to 15 min, and dark inactivation is a bit slower and requires about 20 to 30 min to reach completion (15). The mechanism underlying changes in activity has been demonstrated to be protein phosphorylation (10). In the light, SPS is activated by dephosphorylation catalyzed by a type 2A protein phosphatase (11, 23). In the dark, SPS is inactivated by phosphorylation catalyzed by a protein kinase (12–14). In spinach, (Spinacia oleracea) phosphorylation of SPS has little effect on maximum enzyme activity (Vmax), but affects affinity for substrates and effectors (24). In particular, inhibition of phospho-SPS by Pi is increased relative to the dephosphorylated enzyme (13, 24). Under physiological substrate concentrations, the effect of phosphorylation/dephosphorylation would be to modulate enzyme activity substantially (21, 26).

It is not known what factors regulate the phosphorylation status of SPS in situ. Metabolites seem to play a role, because activation state of SPS is linked to photosynthetic rate (1). This, plus the demonstration that mannose can activate SPS in darkness (15, 27), suggests a critical role for either Pl and/or mannose-6-P (which accumulates to high concentrations) in regulating SPS-kinase and/or SPS-PP. In addition to regulation by one or more metabolites, there is also the possibility that the interconverting enzymes may be regulated by light/dark signals. For example, the modulation of PEPC activity in leaves of C4 and CAM plants involves phosphorylation/dephosphorylation, and primary control is at the level of the activity of the protein kinase. In the C4 plant maize, PEPC is active during the day and responds to light/dark signals (17), whereas in CAM plants, PEPC is active in darkness and is under circadian control (22). In both C4 and CAM plants, the enzyme is activated by phosphorylation, and the major means by which light (in C4) or the circadian clock (in CAM) controls the phosphorylation of PEPC is by modulation of the activity of the PEPC-kinase (3, 6, 20). Activation of PEPC-kinase has been shown to involve a protein synthesis step in the C4 plant Zea mays (18) and the CAM plant Bromylland fedtschenkoi (3). In contrast, PEPC-PP activity appears to be independent of light/dark signals.

In the present study, we have attempted to determine the factors that are responsible for SPS activation during dark/dark transitions, and during mannose feeding in darkness. The objectives were to: (a) determine whether mannose-6-P is an inhibitor of SPS-kinase, and if so, contributes to mannose activation; (b) partially purify SPS-PP and characterize Pi inhibition; and (c) determine whether coarse control of the

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3 Abbreviations: SPS, sucrose-phosphate synthase; PP, protein phosphatase; Glc-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; CHX, cycloheximide; PEPC, phosphoenolpyruvate carboxylase; FSBA, 5'-fluorosulfonyl-benzoyladenosine; Amax, maximum assimilation rate; UDP-Glc, UDP-glucose; IC50, concentration required to produce 50% inhibition.
interconverting enzymes is involved in regulation of SPS activation state.

MATERIALS AND METHODS

Materials

Biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and 32P radionucleotide was obtained from New England Nuclear (Boston, MA). The catalytic subunit of maize PP-1, cloned and expressed in Escherichia coli (25), was a generous gift of Dr. Robert Smith.

Plant Growth and Experimental Treatments

Spinach (Spinacia oleracea) was grown in soil in growth chambers or in a greenhouse as previously described (15). Compounds (mannose, Pi, CHX, etc.) were fed to excised leaves through the transpiration stream in the light or dark as indicated.

Leaf Extraction and Assay of SPS

Leaf extracts were prepared by grinding frozen tissue in a chilled mortar using a 1:2 tissue-to-buffer ratio, for SPS-PP experiments, or a 1:4 ratio, for all other experiments. The extraction buffer contained 50 mM Mops-NaOH (pH 7.5), 10 mM MgCl2, 1 mM EDTA, 2.5 mM DTT, and 0.1% (v/v) Triton X-100. The crude extracts were centrifuged at 15,000g for 1 min and the supernatants immediately desalted on Sephadex G-25 columns (1 x 5 cm) equilibrated with extraction buffer minus Triton X-100.

Extracts were kept at 0°C or preincubated at 25°C prior to assay as indicated in the text. SPS activity was assayed with limiting substrates plus Pi (“limiting assay”) or with saturating substrates (“Vmax assay”). The ratio of the two activities, times 100, is referred to as the activation state (percent). The compositions of the reaction mixtures (70 mM) were as follows.

The limiting assay contained 10 mM UDP-Glc, 10 mM Pi (an inhibitor), 3 mM Fru-6-P, 12 mM Glc-6-P (an activator), 50 mM Mops-NaOH (pH 7.5), 15 mM MgCl2, 2.5 mM DTT, and extract. The Vmax assay was as described above except that Pi was omitted and the concentrations of Fru-6-P and G6P were 10 and 40 mM, respectively. Reactions were typically run for 10 min at 25°C and were terminated by the addition of 70 μL of 30% (w/v) KOH, followed by a 10 min incubation in boiling water. After cooling, 1 mL of 0.14% (w/v) anethole in 13.8 mM H2SO4 was added and the tubes incubated at 40°C for 20 min prior to measuring absorbance at 620 nm.

Partial Purification and Assay of SPS-Kinase and SPS-PP

Crude spinach extracts were further fractionated by precipitation of the protein with PEG 8000. The fraction that precipitated between 5 and 12% PEG was separated by anion-exchange chromatography on a Mono-Q column using a Pharmacia FPLC system as previously described (13).

SPS-kinase and SPS-PP were assayed after desalting of the column fractions (to remove salt, which interferes with assays) in the presence of 0.5% BSA on Sephadex G-25 columns (1 x 5 cm) equilibrated in 50 mM Mops-NaOH (pH 7.5), 10 mM MgCl2, and 2.5 mM DTT. In the case of SPS-kinase, the activity was measured by the ATP-dependent decrease in SPS activation state using highly activated SPS (obtained from tissue fed mannose in the light) as previously described (13). SPS-PP was measured as the increase in SPS activity (with limiting substrates + Pi) using inactivated SPS (obtained for tissue harvested in the dark) as substrate (11). In partially purified preparations, PP activity is expressed as the increase in limiting SPS activity (units mL-1). When assayed in desalted leaf extracts, SPS-PP is expressed as the increase in SPS activity (in μmol g-1 fresh weight h-1) during a 30 min preincubation at 25°C.

Inactivation of SPS-Kinase by FSBA

Highly activated SPS, partially purified by Mono-Q chromatography, was dialyzed against 50 mM Mops-NaOH (pH 7.5), 10 mM MgCl2, and 10% (v/v) ethylene glycol to remove salt and reductant. FSBA was added from a 100 mM stock in DMSO to a final concentration of 0.5 mM and incubated at 25°C for 1 h. The reaction was stopped by the addition of DTT to 2.5 mM. After adding BSA to 0.5% (w/v), the sample was desalted on Sephadex G-25 equilibrated in dialysis buffer plus 2.5 mM DTT. The resulting fraction was considered to be "kinase-free" because it showed no ATP-dependent inactivation of SPS.

Diurnal Changes in SPS-Kinase Activity

Aliquots of desalted crude extracts (20 and 40 μL) from spinach harvested in the dark, after 1 h of illumination (12 h sample) or after 8 h of illumination (12 h sample) were added to 300 μL of FSBA-treated, kinase-free SPS in the presence of 50 μM [γ-32P]ATP (specific activity 500 cpm/pmol). The mixtures were incubated at 25°C for 30 min, after which the SPS was immunoprecipitated and the immune complexes collected and subjected to SDS-PAGE as previously described (10). The incorporation of radioactivity into the 120-KD SPS subunit was determined by liquid scintillation counting of the excised SPS band.

Measurement of Hexose-P Pools

Hexose-P was measured enzymatically in perchloric acid extracts of frozen leaf tissue as previously described (7).

Leaf Photosynthesis Measurements

Leaf discs (10 cm2) were exposed to saturating light (1200 μmol m-2 s-1) with saturating CO2 (10% in air) for 10 min in a leaf oxygen electrode (Hansatech). Photosynthetic rates were based on oxygen evolution measured after steady-state rates were attained.
RESULTS AND DISCUSSION

Role of Metabolites in Vivo

We wanted to determine whether changes in metabolite concentrations in vivo are involved in the activation of SPS in darkness by mannose feeding to leaves. As one approach, leaves received increasing concentrations of mannose in the presence and absence of exogenous Pi (25 mM). In the absence of exogenous Pi, mannose is taken up into the mesophyll cells and phosphorylated at the expense of the cytoplasmic phosphate pools (8, 19). As a result, leaf hexose-P pools (Glc-6-P and Fru-6-P) are depleted (Fig. 1A control), and SPS is activated in darkness (Fig. 1B control; ref. 27). Supply of exogenous Pi with mannose apparently prevents the depletion of cytoplasmic Pi, as evidenced by maintenance of a substantial leaf hexose-P pool (Fig. 1A).

It is interesting that a supply of exogenous Pi almost completely prevented the mannose activation of SPS (Fig. 1B). Accumulation of mannose-6-P in leaves was similar when mannose was supplied in the presence and absence of Pi (up to 1400 μmol per mg Chl, with 25 mM mannose). These results suggest that accumulation of high concentrations of mannose-6-P alone is not sufficient to activate SPS in darkness. Rather, depletion of cytoplasmic Pi (or other phosphate esters) may be necessary for activation to occur. Choline is also a phosphate-sequestering agent (2), but, unlike mannose, is phosphorylated at the expense of the vacuolar Pi pool. Consequently, cytoplasmic Pi remains high (2), and SPS is not activated when spinach leaves are fed choline in the dark (data not shown). In order to further characterize the possible role of metabolites, the interconverting enzymes were partially purified and tested for metabolic effectors.

Regulation of SPS-Kinase

There is some evidence for a role of metabolites in regulation of the kinase. Specifically, Glc-6-P has been shown to inhibit the in vitro phosphorylation of SPS using [γ-32P]ATP and the endogenous protein kinase that copurifies with SPS (13). In the present study, we wanted to determine whether Glc-6-P inhibited the ATP-dependent inactivation of SPS. This would certainly be expected and, as shown in Figure 2, was observed. Almost complete inhibition of the ATP-dependent inactivation was achieved with 20 mM Glc-6-P, and the half-maximal effect required about 2.5 mM Glc-6-P. These concentrations of Glc-6-P are in the physiological range for spinach leaves (7, 26). In contrast, mannose-6-P had little effect on the inactivation of SPS (Fig. 2), and also did not inhibit the phosphorylation of SPS in vitro using [γ-32P]ATP (J. L. Huber and S. C. Huber, unpublished data). The Glc-6-P effect was not antagonized by Pi (data not shown), which suggests that Glc-6-P interacts with the protein kinase rather than the substrate protein, SPS. It is important to note that the partially purified SPS preparations contained the endogenous SPS-kinase but were completely free of detectable

Figure 1. Exogenous Pi blocks the sequestration of leaf Pi by mannose and prevents activation of SPS in darkness. Hexose-P pools (A) and SPS activation state in situ (B) were measured in spinach leaves supplied with different concentrations of mannose with or without 25 mM Pi. Leaves were excised after 3 h of illumination and the indicated solutions were fed via the transpiration stream for 1.5 h in the dark.

Figure 2. Inhibition of SPS-kinase by Glc-6-P but not mannose-6-P. Protein kinase activity was measured as the ATP-dependent inactivation of SPS during preincubation at 25°C in the presence of different concentrations of Glc-6-P or mannose-6-P. Highly activated SPS, obtained from leaves fed mannose, and the endogenous protein kinase were partially purified by PEG fractionation followed by Mono Q chromatography.
hexose kinase activity (data not shown). Consequently, the apparent inhibition by Glc-6-P of the protein kinase reaction is not the result of depletion of ATP in the reaction mixture by free glucose (present as a contaminant or generated during the reaction) being phosphorylated to Glc-6-P by hexose kinase. Thus, Glc-6-P is confirmed as a specific inhibitor of the phosphorylation/inactivation reaction. The striking difference between Glc-6-P and mannose-6-P shown in Figure 2 suggests that changes in the leaf concentration of Glc-6-P during light/dark transitions may play a role in regulation, whereas accumulation of mannose-6-P during mannose feeding appears to have no direct effect.

Regulation of SPS-PP

Previous studies had identified an okadaic acid-sensitive protein phosphatase in leaf extracts that could dephosphorylate and activate SPS extracted from dark leaves (11, 23). The in vitro activation of SPS in desalted crude leaf extracts was inhibited by okadaic acid, molybdate, vanadate, and Pi, but was stimulated by fluoride (11). It appears that the major phosphatase acting on SPS in vivo is of the type 2A (23), but little more is known about the nature and regulation of the SPS-PP. As a prerequisite to further characterization, we have attempted to partially purify the SPS-PP. Preliminary experiments indicated that the majority of the SPS-PP in a leaf extract tended to copurify with SPS during fractionation with PEG (5–12%), or during step elution from aminohexyl agarose (0.1–0.3 M KCl step) (data not shown). However, the two enzymes could be effectively separated by Mono Q chromatography. Figure 3 shows the separation achieved when a 5 to 12% PEG fraction was applied to the anion exchange column. With an extract prepared from leaves harvested in the afternoon, a single major peak of SPS-PP activity was observed, which was resolved from the peak of SPS activity that eluted at a slightly higher salt concentration.

Various metabolites were tested for effects on the apparent activity of SS-PP in vitro, including Glc-6-P (5 mM), Fru-6-P (5 mM), UDP-glucose (5 mM), PPI (1 mM), dihydroxyacetone-P (1 mM), Fru-2,6-P_2 (10 μM), and sucrose (100 mM). Of those tested, the only compound shown to have any significant effect was Pi. As shown in Figure 4, the IC_50 (Pi) was about 4 mM with the partially purified system. This confirms the previous observation (11), made with crude extracts, that Pi is an inhibitor of the activation/dephosphorylation of SPS. The Pi inhibition of the activation of SPS was not antagonized by Glc-6-P (up to 10 mM), which suggests that Pi is acting on the PP rather than on the protein substrate (SPS) (data not shown). The partially purified SPS-PP was slightly inhibited by fluoride (15–25% inhibition at 10 mM) but was completely inhibited by 5 mM okadaic acid, a specific inhibitor of the type 1 and 2A protein phosphatases (4). Our results are consistent with the previous report (23) that the SPS-PP is of the type 2A rather than type 1.

The lack of substantial inhibition by fluoride is consistent with earlier studies conducted with crude leaf extracts (9, 11). The activation of dark-SPS in desalted leaf extracts is stimulated by fluoride (9). The present results indicate that the effect of fluoride is indirect, and apparently results from inhibition of other phosphatases in the leaf extract that interfere with activation of SPS (9). We have also confirmed the observation that PP1 was ineffective in activating phosho-SPS. We incubated partially purified SPS with the catalytic subunit of maize PP1, cloned and expressed in E. coli.
irreversible inhibitor in kinase purified by SPK in vitro. There was tionally varied with shown). Thus, spinach activities of the and found (25), approximately 120-kD SPS subunit was (25 min), and the excised leaves were then kept in the dark for either 45 min, 2 h, or 5 h as indicated. Desalted leaf extracts were prepared and SPS activity was assayed (with limiting substrates plus Pi) immediately (t0) and after a 30-min preincubation (t30) at 25 °C. The increase in limiting SPS activity is taken as an estimate of endogenous SPS-PP activity.

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the end of the dark period and increased about fourfold after the first hour of illumination (Fig. 6A). Thereafter, SPS-PP activity declined progressively with time of day. After 9 h of illumination, SPS-PP activity was approaching the low level found in darkness. Consistent with previous reports (27), the activation state of SPS in situ (Fig. 6B) increased rapidly after the onset of illumination and thereafter declined throughout the photoperiod. After 9 h of illumination, the activation state of SPS had declined to almost the level found in darkness (Fig. 6B and ref. 27). The striking correspondence between the diurnal changes in SPS-PP activity (assayed in vitro) and SPS activation state in situ suggests that coarse control of the PP may contribute to the regulation of the phosphorylation state of SPS in vivo. In other experiments, we determined that the diurnal changes in SPS-PP activity and SPS activation state were in response to light/dark signals, rather than reflecting an endogenous rhythm, by extending the normal dark period by 8 h. Both SPS-PP activity and SPS activation state remained low throughout the extended dark period, and both increased dramatically upon illumination (data not shown).

Light Activation of SPS-PP Requires Protein Synthesis

The apparent light activation of SPS-PP could reflect a covalent modification, protein turnover, or a tight-binding activator/inhibitor. To begin to distinguish among these possibilities, we fed the protein synthesis inhibitor CHX to detached spinach leaves in the dark prior to illumination. As shown in Figure 7, increasing concentrations of CHX essentially prevented the activation of SPS-PP by light. The activity of SPS-PP in darkened leaves was not significantly affected by CHX. These results strongly suggest involvement of a protein synthesis step in the activation process.

Pretreatment of leaves with CHX in the dark had a significant effect on the light activation of SPS in situ. The results presented in Figure 8 show the effect of CHX on O₂ evolution and SPS activation state during induction of photosynthesis in spinach leaves in saturating light and CO₂. Oxygen evolution exhibited characteristic oscillations followed by a slow rise to the maximum rate (A₁₅₋₅). The effect of CHX was to reduce A₁₅₋₅ by about 25%. In contrast to the rather small effect of CHX on assimilation rate, the activation of SPS was dramatically reduced. In control leaves, the activation of SPS (assayed with limiting substrates plus Pₐ) occurred during the first 10 min of illumination, generally tracking the increase in photosynthetic rate. In the CHX-treated leaves, some activation of SPS occurred, but the rate of activation was greatly reduced. These results are consistent with the effect of CHX on the extractable activity of SPS-PP and suggest that rapid light activation of SPS in situ requires a protein synthesis step. It is important to note that the CHX treatment had no significant effect on the V₁₅₋₅ activity of SPS. Thus, there was no significant turnover of the SPS enzyme protein in these short-term experiments. Therefore, it appears that the protein synthesis step is required for the activation of SPS rather than for the synthesis of SPS per se.
shown to be inhibited by Glc-6-P, but not its epimer mannose-6-P. The SPS-PP has been partially purified and inhibition by Pi (11) confirmed (Fig. 4). A decrease in cytoplasmic Pi concentration may be essential for the in situ activation of SPS. Thus, supply of exogenous Pi can block mannose activation in darkness (Fig. 1) and reduce activation by light (27). When cytoplasmic Pi concentration is high, SPS-PP is inhibited and activation by dephosphorylation is prevented. Regulation of the SPS-kinase by Glc-6-P may or may not contribute to control of SPS activation state, inasmuch as the whole leaf Glc-6-P concentration may or may not fluctuate with light/dark transitions. However, there is the potential for Glc-6-P to alter SPS activation state in vivo via inhibition of the protein kinase even if changes in cytoplasmic Glc-6-P are relatively small, as is characteristic of the signal amplification of cyclic cascade systems.

In addition to metabolite (fine) control, we have also demonstrated an apparent light activation of SPS-PP. The light activation requires a protein synthesis step and can be blocked by the inhibitor CHX. The light activation of SPS-PP appears to be essential for rapid activation of SPS in situ as pretreat-

**CONCLUSIONS**

In summary, the present study has demonstrated that the phosphorylation status of SPS in spinach leaves changes in response to light/dark transitions and time of day as a result of "fine" and "coarse" control mechanisms. SPS-kinase is
ment of leaves in the dark with CHX dramatically slowed the activation/dephosphorylation of SPS in the absence of a large effect on photosynthesis (Fig. 8). Thus, our results suggest that coarse control of SPS-PP occurs in vivo and contributes to regulation of SPS phosphorylation status. Elucidation of this level of control may help to explain why SPS activation state in spinach leaves tends to be highest early in the photoperiod and decline progressively throughout the day (26, 27). The decrease in SPS activation state late in the photoperiod has been shown to be associated with increased fructose-2,6-bisphosphate levels and altered partitioning of carbon (to favor starch relative to sucrose) (21). What has been unclear is what factor(s) are responsible for the inactivation of SPS. Typically, it has been interpreted as a response to sucrose accumulation in the leaf tissue and, thus, a component of feedback inhibition (26). However, cytoplasmic sucrose concentration is thought to be relatively constant throughout the photoperiod, with net accumulation restricted to the vacuole (7). Moreover, sucrose itself has no effect on SPS, SPS-kinase, or SPS-PP. Thus, sucrose accumulation during the photoperiod may generally correlate with deactivation of SPS but seemingly cannot be the direct causal factor. Rather, we propose that the decline in SPS-PP activity during the day restricts the enzymatic capacity to dephosphorylate and activate SPS. As SPS-PP activity declines (Fig. 6A) while SPS-kinase activity remains essentially unchanged during the day (Fig. 5), the phosphorylated (inactivated) form of SPS would be favored late in the day, as is observed (Fig. 6B). The basis for the light activation of SPS-PP, and the role of protein synthesis, is unclear at the present time and will require further study. Once the mechanism of light activation of SPS-PP is elucidated, we may be able to understand the molecular basis for the decline in SPS-PP activity during the photoperiod.

There are now two examples of cytoplasmic enzymes that are modulated by light/dark signals by a mechanism involving protein phosphorylation, and where control involves a protein synthesis step: PEPC and SPS (this work). It may be significant that, in both cases, light signals result in increased activity of the interconverting enzyme that activates the target enzyme: the protein kinase for PEPC (6, 18, 20) and the phosphatase for SPS. The interconverting enzymes that inactivate the target proteins do not appear to be regulated in this fashion. For both PEPC and SPS the next step will be to determine whether the kinase or phosphatase itself is synthesized, or whether the activity of an existing protein is being modified, perhaps by a regulatory factor.

LITERATURE CITED


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