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Regulation of sucrose-phosphate-synthase activity in spinach leaves by protein level and covalent modification*

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Abstract. A dot-blot technique was developed using monoclonal antibodies to measure, rapidly and accurately, the amount of sucrose-phosphate synthase (SPS; EC 2.4.1.14) protein present in a crude extract from spinach (*Spinacia oleracea* L. cv. Dark Green Bloomsdale) leaves; this was compared with SPS activity in this material. During leaf development, increased SPS activity followed closely the increase in enzyme-protein level, indicating de-novo synthesis or altered turn-over rates for SPS. In contrast, activation of SPS by illumination of leaves or by mannose treatment of leaf discs in the dark (M. Stitt et al. Planta *174*, 217–230) occurred without a significant change in the level of enzyme protein. Since conditions which altered SPS activity did not affect immunoprecipitation or mobility of the 120-kilodalton (kDa) subunit of the enzyme during denaturing gel electrophoresis, some form of protein modification other than proteolysis must be involved. Overall, the results indicate that regulation of SPS activity can involve changes in the level of enzyme protein and-or covalent modification.

Key words: Leaf development – Light (enzyme activation) – Photosynthate partitioning – *Spinacia* (sucrose-phosphate synthase) – Sucrose-phosphate synthase

Introduction

Sucrose is one of the major end-products of leaf photosynthesis, and also the primary transport form of reduced carbon in many higher plants. Regulation of the sucrose-formation pathway in the cytoplasm occurs at the level of several different enzymes, but cytosolic fructose 1,6-bisphosphatase and sucrose-phosphate synthase (SPS) are recognized to be key control points (for review see Stitt et al. 1988a). The control of SPS activity in situ is thought to be a complex function of 'fine' and 'coarse' controls. Fine control refers to inhibition or stimulation of enzyme activity by metabolic effectors whereas coarse control refers to changes in extractable activity as measured in vitro (ap Rees 1980). Coarse control appears to be of particular importance as it may be responsible for changes in the rate of sucrose formation that occur diurnally and in response to source-sink manipulation (Huber et al. 1986). Recently, Stitt et al. (1988b) demonstrated activation of SPS activity in intact spinach leaves by light, and also observed that activity was increased in darkness by feeding mannose to leaf discs. It was proposed that the enzyme can exist in two or more kinetically distinct forms in vivo, and that the distribution between these forms may be linked to changes in the availability of inorganic phosphate.

The mechanism(s) of coarse control of SPS activity is not known. We reasoned that a first step in elucidating the mechanism(s) may be to distinguish between covalent modification of existing SPS protein and changes in the level of enzyme protein. In order to do this, we developed an immunochemical dot-blot technique using monoclonal antibodies for quantitation of the amount of SPS protein present in crude extracts. In the present study, we have attempted to relate changes in

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Abbreviations: kDa = kilodalton; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis; SPS = sucrose-phosphate synthase.

SPS activity and enzyme protein level in response to: 1) leaf development (immature versus mature leaves); and 2) light versus darkness, and treatment with mannose versus sorbitol (all with mature leaves). The results provide strong evidence for covalent modification of SPS in mature leaves and regulation of SPS-protein level during leaf development.

Material and methods [1]

Chemicals. All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., USA. Nitrocellulose (0.45 μm) and Western-blotting-grade, affinity-purified goat anti-mouse immunoglobulin (IgG)-alkaline phosphatase conjugate were purchased from Bio-Rad Laboratories, Richmond, Cal., USA.

Plant material and experimental treatments. Spinach (*Spinacia oleracea* L. cv. Dark Green Bloomsdale; obtained from Wyatt-Quarles Seed Co., Raleigh, N.C., USA) was grown in a soil mixture in an environmental chamber under a 12-h light period daily (350 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and a temperature cycle of 22° C in light 17° C in darkness. Lights in the environmental chamber were a combination of fluorescent (High-Output, cool-white, F72T12CWHO; General Electric Co., Charlotte, N.C.) and incandescent (GE, 52W W-Miser Bulbs) lamps.

To supply sorbitol or mannose to leaf tissue, 10–20 leaf discs (approx. 1 cm diameter) were floated on 0.2 M solutions (1 disc/ml) for 10–12 h in the dark, then rapidly rinsed, and blotted before freezing in liquid N_2.

Light activation of attached or detached spinach leaves involved transfer of either an intact spinach plant or excised leaf (with cut stem in water) from darkness to an illuminated chamber (350 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 15–30 min.

Extraction of SPS. Sucrose-phosphate synthase was extracted from the discs and whole leaves by grinding the tissue in a precooled mortar using a 1:4 tissue-to-buffer ratio in medium containing 50 mM 3-[N-morpholino]propanesulfonic acid (Mops)-NaOH (pH 7.5), 15 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 2.5 mM dithiothreitol (DTT) and 0.1% octyl phenoxy polyethoxyethanol (Triton X-100). The homogenates were squeezed through two layers of Miracloth (Behring Diagnostics, La Jolla, Cal., USA) and centrifuged at 20000 · *g* for 5 min. The supernatants were immediately desalted on Sephadex G-25 (Sigma) columns equilibrated with 50 mM Mops-NaOH (pH 7.5), 15 mM $MgCl_2$, 1 mM EDTA and 2.5 mM DTT.

Assay of SPS activity. The activity of SPS was assayed under limiting and V_{max} substrate concentrations as fructose-6-phosphate (Fru6P)-dependent formation of sucrose (plus sucrose-P) from uridinediphosphate glucose (UDPG). Under low-substrate ("limiting") conditions, 45 μl of tissue extract was incubated 15 min at 25° C with 10 mM UDPG, 3 mM Fru6P, 12 mM glucose 6-phosphate (Glc6P), 10 mM Pi, 50 mM Mops-NaOH (pH 7.5), 15 mM $MgCl_2$ and 2.5 mM dithiothreitol (DTT) in a total volume of 70 μl. Under V_{max} conditions, the tissue extracts were assayed in the presence of 10 mM Fru6P, 40 mM Glu6P and 10 mM UDPG, using identical buffer, volume and reaction time as that for the limiting assays. Reactions were terminated after 15 min by the addition of 70 μl of 30% KOH and unreacted fructose was destroyed by placing the tubes in boiling water for 10 min. After cooling, 1.0 ml of 0.14% anthrone in 13.8 N H_2SO_4 was added, and the tubes incubated at 40° C for 20 min prior to measuring color development at 620 nm with a Lambda 3A spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn., USA).

Dot-blot analysis of SPS protein in tissue extracts. The levels of SPS protein in crude extracts of spinach leaves were determined using immunochemical detection of the enzyme bound directly to nitrocellulose filters. The crude extracts were centrifuged at 25000 · *g* for 10 min to remove cellular debris. It is important for the dot-blot analysis that factors which markedly increase buffer viscosity are not included in the grinding medium. The inclusion of ethylene glycol, polyvinylpyrollidone and polyethylene glycol will slow the filtration of the samples through the nitrocellulose membrane, resulting in irregular deposition of the protein both inside and outside the well. The addition of exogenous protein, such as bovine serum albumin (BSA), to the grinding buffer may subsequently result in saturation of the membrane-binding sites with nonantigenic protein, and thus should also be avoided.

The binding capacity of the nitrocellulose membrane is limited. In our assays, the "dots" contained a total of 1–10 μg soluble protein, corresponding to a maximum of about 5 milliunits of SPS activity and 30 ng of SPS protein. After dilution of the extract with Mops buffer (pH 7.5), the samples to be applied to the nitrocellulose membrane should be essentially colorless. The samples are spotted onto the filter in 25-μl aliquots using a dot-blot apparatus (Bio-Rad) under vacuum. The wells are washed with several volumes of buffer to rinse any protein clinging to the sides of the well. The membrane is immersed in phosphate-buffered saline (PBS) buffer containing 10 mM Na_2HPO_4 (pH 8.0) and 150 mM NaCl. A test strip of the blot (usually a representative row of sample dots) is sliced from the nitrocellulose and immersed for 5 min in a protein-staining solution containing 0.1% (w/v) Amido black, 45% (v/v) methanol and 10% (v/v) acetic acid. The strip is transferred to a de-staining solution composed of 90% (v/v) methanol and 2% (v/v) acetic acid, and washed until the background color of the membrane is removed. The staining of the total protein on the test strip served to identify any sample and-or sample-application problems before immunodetection of the antigen on the remaining blot. The protein must be bound evenly in a well defined circle and on only one side of the paper. Staining of protein on the reverse side of the nitrocellulose membrane indicated that the applied protein exceeded the binding capacity of the filter. If the dots were not well defined either because of uneven distribution in the well or spreading outside the wells, transmittance measurements of color development were difficult.

The remaining portion of the blot was incubated at 37° C for 2 h in PBS containing 3% (w/v) BSA. The blocking solution was removed and the filter incubated at room temperature for 3 h in a 1:20 dilution of monoclonal-antibody supernatant. The PBS dilution buffer (pH 8.0) contained 0.1% (w/v) BSA to reduce nonspecific binding further. Unbound antibodies were subsequently removed by rocking the blots with PBS containing 0.05% (v/v) Tween 20 polyoxyethylene sorbitan monolaurate) for 30–40 min at 25° C. During this washing period, the buffer was changed three or four times. The anti-mouse alkaline-phosphatase conjugate was diluted 1:3000 using PBS (pH 8.0) containing 0.1% (w/v) BSA. The blots were incubated with the

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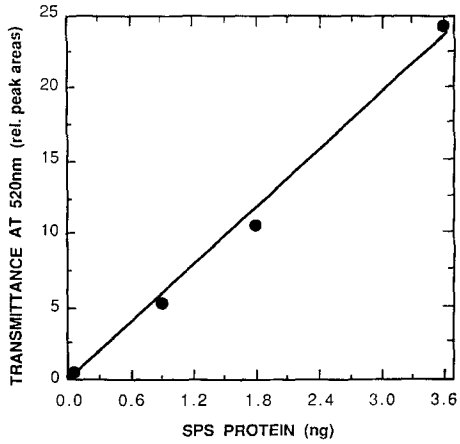

Fig. 1. Standard curve for dot-blot immunoassay measurements of spinach SPS protein levels. A leaf extract was applied in a series of dilutions to nitrocellulose and subsequently probed sequentially with SPS-specific monoclonal antibodies and an alkaline-phosphatase-conjugated indicating antibody. Incubation conditions, color development and transmittance measurements were as described in *Methods*. The amount of SPS protein per dot was determined by immunoprecipitation and quantitation of the 120-kDa subunit by gel scanning following SDS-PAGE as previously described (Walker and Huber 1988a)

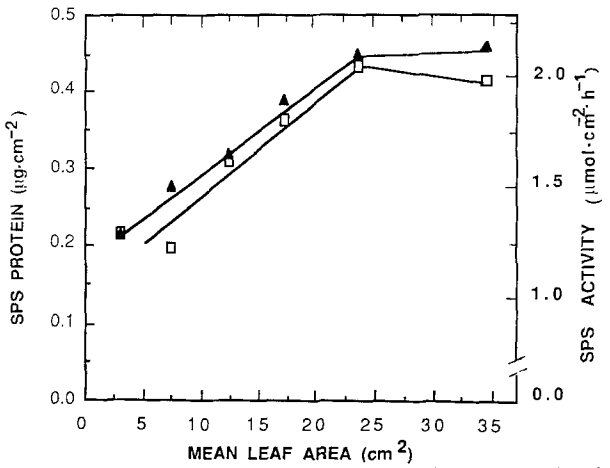

Fig. 2. Sucrose-phosphate-synthase protein levels (▲) and activity (□) in expanding spinach leaves. The tissue was harvested and separated into groups of increasing leaf area based on weight (0.045 $g \cdot cm^{-2}$). The SPS was extracted and assayed under V_{max} conditions as described in *Methods*. Equivalent volumes of extracts were applied to nitrocellulose (2–3 µg total protein/dot) and subjected to immunodetection of SPS as described in Fig. 1 and *Methods*. The level of SPS protein was calculated from the standard curve

indicating antibody for 1–2 h at 25° C; the filters were subsequently washed as previously described.

The monoclonal antibodies bound to the blots were detected by incubating the filters in alkaline-phosphatase substrate medium containing 3.0 mM nitroblue tetrazolium, 50 µl (1.7 mg) of stock solution of 34 mg/ml 5-bromo-4-choro-3-indolyl-PO_4 (toluidine salt) in dimethylformamide, 10 ml of 100 mM 3-amino-2(hydroxymethyl)-1,3-propanediol (Tris)-HCl (pH 9.5), 100 mM NaCl and 2 mM $MgCl_2$. The reaction was allowed to proceed until optimal color development had occurred, and the color development was terminated by washing the blots with 10 mM EDTA.

Quantitation of dot-color development. Transmittance measurements of color development on the dot blots were best performed while the blots were still wet. Transmittance measurements of spinach SPS in dot-blots of crude extracts were performed on a CAMAG TLC Scanner-II (Camag Scientific, Wrightsville Beach, N.C.) at 520 nm.

Results and discussion

Immunochemical quantification of SPS protein. An enzyme-linked immunoassay was developed where the level of SPS protein present in aliquots of leaf extracts bound to nitrocellulose paper was detected by sequential incubation with SPS-specific antibodies (Walker and Huber 1988a) and goat anti-mouse antibody conjugated to alkaline phosphatase. Color development (measured in arbitrary transmittance units) was proportional to the amount of SPS protein present in the sample (Fig. 1). The standard curve shown was derived using partially purified spinach-leaf SPS; the amount of SPS protein present in each sample dot was determined by quantitative immunoprecipitation followed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and measurement of the 120-kilodalton (kDa) subunit of SPS by gel densitometric scanning (Walker and Huber 1988a). The dot-blot assay allowed the reproducible quantitation of as little as 1–2 ng of SPS protein in the tissue extracts.

Changes in SPS protein and enzyme activity during leaf development. As leaves expand and develop they undergo a progressive shift in metabolism from heterotrophic sink tissues to photosynthetically competent source tissues. Associated with the sink-to-source transistion is the development of the capacity for sucrose formation and export. As in plants such as soybean (Huber et al. 1986) and sugarbeet (Giaquinta 1978), leaf expansion in spinach (Fig. 2) was associated with increased activities of SPS per unit leaf area or weight, and this increase was in turn closely associated with increased levels of SPS protein, measured immunochemically using the dot-blot technique (Fig. 2). These results indicate that changes in SPS activity are the result of increased steady-state levels of the enzyme protein (as opposed to activation of pre-existing enzyme) and confirm the suitability of the immunoassay for application to crude leaf extracts.

Table 1. Light activation of spinach-leaf SPS

Treatment of leaf	SPS activity (μmol sucrose·h^{-1}·(g FW)$^{-1}$)		SPS protein[a]	
	Limiting assay	V_{max}	Relative amount	Tissue level (μg·(g FW)$^{-1}$)
Dark	11.5±0.8	60.2±1.5	0.90	10.8
Light (30 min)	24.1±0.2	59.5±1.0	1.00	12.0

[a] The tissue concentration of SPS protein was determined by applying equivalent volumes of each extract (approx. 2 μg/dot) to a nitrocellulose filter, followed by immunochemical analysis of SPS protein. The relative amountof SPS protein was determined by gel scanning of quantitative immunoprecipitates as described in Walker and Huber (1988a).

Table 2. Mannose activation of spinach-leaf SPS in darkness[a]

Treatment	SPS activity (μmol·h^{-1}·(g FW)$^{-1}$)		SPS protein (μg·(g FW)$^{-1}$)
	Limiting assay	V_{max}	
Sorbitol	9.6	20.1	3.2
Mannose	16.2	20.7	4.2

[a] Spinach-leaf discs were floated on 0.2 M sorbitol or mannose for 10–12 h in the dark. Tissue was frozen in liquid N_2 and extracted for SPS activity. The tissue level of SPS protein was determined using the dot-blot technique.

Modulation of SPS activity in mature leaves. By means of immunochemical techniques it could be shown that light treatment of leaves increased the extractable SPS activity almost twofold relative to leaves kept in darkness (Table 1) while the SPS-protein level remained constant. Our results confirm those of Stitt et al. (1988b) that activity differences were most apparent when assays were conducted under "limiting" conditions (low substrates plus inorganic phosphate) relative to V_{max} or saturating substrate conditions. The monoclonal antibodies equally recognized the enzyme extracted from both light- and dark-treated tissue, as indicated by antibody inhibition and precipitation dilution curves for the SPS, nor were differences observed in the mobility of the SPS subunit during fractionation by SDS-PAGE as a result of light-dark treatments (data not shown). As discussed by Dennis et al. (1985) it is important to document the relative molecular mass of the protein detected immunochemically to verify that the native protein (rather than fragments) is being detected. Immunochemical dot-blot analysis of extracts prepared from light- and dark-treated leaves did not indicate any significant change in SPS-protein levels. Two independent immunochemical analyses were performed. In the first, SPS activity was quantitatively immunoprecipitated from light and dark extracts. The immunoprecipitates were then subjected to SDS-PAGE and the 120-kDa subunit of SPS quantitated by densitometric scanning of the Coomassie-stained gels; relative peak size is directly related to the amount of SPS protein and was essentially identical in the two extracts (Table 1). The amount of SPS protein was also estimated using the dot-blot technique; with this method, both light and dark samples contained approx. 10 μg SPS protein/1 g fresh weight. Thus, the light-induced increase in enzyme activity cannot be attributed to changes in steady-state levels of SPS protein in leaves.

Light activation of SPS appears to be indirect in maize (Huber et al. 1987) and spinach (Stitt et al. 1988b), because in addition to light, CO_2 is also required. Furthermore, Stitt et al. (1988b) reported that spinach-leaf SPS could be activated in darkness as a result of mannose feeding. As shown in Table 2, spinach-leaf tissue treated with 0.2 M mannose for 8 h in the dark had higher SPS activities than did control discs floated on 0.2 M sorbitol. Similar to the light-dark results, the mannose effect was pronounced when the assays were conducted under limiting conditions and was essentially absent when assays were conducted under V_{max} conditions. The SPS-specific antibodies were able to facilitate complete precipitation of the enzyme from both the mannose- and sorbitol-treated discs and the composition of immunecomplexes analyzed by SDS-PAGE demonstrated no differences in SPS mobility between the two treatments (data not shown). Immunochemical analysis of the same tissue extracts showed the presence of approximately equivalent levels of SPS protein in both the mannose- and sorbitol-treated discs, indicating covalent modification of the enzyme rather than modulation of subunit levels during mannose-induced coarse control of SPS activity.

Concluding remarks

We have obtained new insights regarding the coarse control of spinach-leaf SPS activity. Specifically, two distinct types of coarse control have been identified. First, regulation of the amount of SPS protein has been shown to occur during leaf development; SPS protein and activity increase as leaves undergo the sink-to-source transition. This indi-

cates de-novo synthesis of SPS protein and implies regulation of transcription and-or translation. The second type of coarse control involves post-translational regulation. In mature leaves, SPS activity is clearly regulated by covalent modification because activity can change while enzyme protein remains constant. This is consistent with the observation that changes in SPS activity are largely the result of a change in the regulatory properties rather than the V_{max} (Stitt et al. 1988b).

The demonstration of covalent modification implies that the enzyme exists in at least two interconvertible forms in vivo. The interconversion of these two forms of SPS may be controlled by a number of factors, including cytosolic phosphate status and sucrose accumulation (Stitt et al. 1988b). However, the *mechanism* of interconversion remains unclear. Preliminary results point to protein phosphorylation as a possible mechanism of the covalent modification. Phosphorylation of the SPS protein has been documented to occur (Walker and Huber 1988b) but the relationship to coarse control of enzyme activity remains to be established.

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