Site-specific serine phosphorylation of spinach leaf sucrose-phosphate synthase

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We recently reported [Huber, Huber & Nielsen (1989) Arch. Biochem. Biophys. **270**, 681–690] that spinach (*Spinacia oleracea* L.) sucrose-phosphate synthase (SPS; EC 2.4.1.14) was phosphorylated *in vivo* when leaves were fed [^{32}P]P_i. *In vitro* the enzyme was phosphorylated and inactivated by using [γ - ^{32}P]ATP. We now report that SPS is phosphorylated both *in vivo* and *in vitro* on serine residues. The protein is phosphorylated at multiple sites both *in vivo* and *in vitro* as indicated by two-dimensional peptide maps of the immunopurified SPS protein. After being fed with radiolabel, leaves were illuminated or given mannose (which activates the enzyme), in the presence or absence of okadaic acid. Feeding okadaic acid to leaves decreased the SPS activation state in the dark and light and in leaves fed mannose. Across all the treatments, the activation state of SPS *in situ* was inversely related to the labelling of two phosphopeptides (designated phosphopeptides 5 and 7). These two phosphopeptides are phosphorylated when SPS is inactivated *in vitro* with [γ - ^{32}P]ATP, and thus are designated as regulatory (inhibitory) sites [Huber & Huber (1991) Biochim. Biophys. Acta **1091**, 393–400]. Okadaic acid increased the total ^{32}P -labelling of SPS and in particular increased labelling of the two regulatory sites, which explains the decline in activation state. In the presence of okadaic acid, two cryptic phosphorylation sites became labelled *in vivo* that were not apparent in the absence of the inhibitor. Overall, the results suggest that light/dark regulation of SPS activity occurs as a result of regulatory serine phosphorylation. Multiple sites are phosphorylated *in vivo*, but two sites in particular appear to regulate activity and dephosphorylation of these sites *in vivo* is sensitive to okadaic acid.

INTRODUCTION

Sucrose-phosphate synthase (SPS) is a key enzyme of the biosynthetic pathway for sucrose, one of the major end products of leaf photosynthesis and the primary transport form of reduced carbon in many higher plants. The regulation of SPS activity *in situ* is complex and occurs at several levels, one being allosteric control, which is exerted by the cytoplasmic ratio of glucose 6-phosphate and P_i, an activator and inhibitor respectively [1]. On another level, SPS has been shown to exist in kinetically distinct states [2] that are interconvertible via covalent modification of the enzyme [3].

Specifically, these forms of SPS have been observed in some plant species, such as spinach, where inactivated SPS present in darkened leaves can be rapidly converted into an activated state on illumination [2]. Experimentally, SPS is most highly activated in leaves supplied with the phosphate-sequestering agent, mannose (in either dark or light) [2]. Mannose apparently acts to decrease cytoplasmic P_i pools by forming mannose phosphate, which cannot be metabolized further. The decrease in P_i phosphate concentration may play a role in signalling light/dark changes to the cell.

These dark-, light- and mannose-induced states of SPS have been stably extracted and shown to differ in substrate and effector affinities but not V_{max} . [2,3]. Recently Siegel & Stitt [4] reported that the kinetic changes involve small alterations in substrate affinity but large changes in sensitivity to the allosteric activator, glucose 6-phosphate, and inhibitor, P_i. The different kinetic forms of SPS are defined by their activation states, which are expressed as the ratio of enzyme activity measured under limiting substrate conditions (plus P_i) to the SPS activity measured under saturating substrate conditions.

The mechanisms responsible for regulatory covalent modification of SPS and exploration *in situ* of the controls involved have formed the focus for our current investigations. We recently reported that SPS is subject to protein phosphorylation with the result that SPS activity is altered [5]. These results provided the first evidence to support regulatory protein phosphorylation as a mechanism of generating the kinetically distinct forms of SPS observed in illuminated versus darkened spinach leaves. Initially, the 120 kDa subunit of SPS was shown to be phosphorylated as a result of [^{32}P]P_i feeding of detached leaves [5]. Subsequently, two lines of evidence suggested that phosphorylation resulted in inactivation of the enzyme: (i) greater labelling of the SPS subunit was observed when leaves were fed [^{32}P]P_i in the dark compared with the light; and (ii) labelling of partially purified SPS, phosphorylated *in vitro* with [γ - ^{32}P]ATP, accompanied inactivation of the enzyme [5]. These results suggested that SPS could exist in two states: the dephosphorylated (active) state and the phosphorylated (less active) state.

The protein kinase(s) that phosphorylates and inactivates SPS *in vitro* tends to co-purify with its protein substrate, which has been taken as an indication that the two proteins have some tendency to associate physically [6]. It was also shown that the protein kinase is inhibited by salt, which was not recognized in the original report [5], and that the presence of salt in the reaction mixture altered some characteristics such as affinity for ATP [6]. Spinach leaf SPS appears to be phosphorylated at multiple sites *in vivo*. Preliminary evidence has been obtained that exhaustive tryptic digestion of immunopurified ^{32}P -labelled SPS (obtained from leaves fed [^{32}P]P_i in the dark) yielded multiple [^{32}P]-phosphopeptides that could be separated by reverse phase chromatography or by two-dimensional t.l.c./thin-layer electrophoresis (t.l.e.) [7]. Two of the potential phosphorylation sites seemed to

Abbreviations used: SPS, sucrose-phosphate synthase; t.l.e., thin-layer electrophoresis; DTT, dithiothreitol.

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be of regulatory significance (designated in ref. [7] as phosphopeptides 5 and 7). Phosphorylation of phosphopeptides 5 and 7 occurred *in vitro* (catalysed by the endogenous protein kinase that co-purifies with SPS) and correlated with inactivation of SPS [6]. Furthermore, labelling of phosphopeptides (5 + 7) *in vivo* (after feeding of [32 P]P_i to leaves in the dark, light and with mannose-feeding) was inversely related to the SPS activation state across four different experiments conducted over a 6-month period [7]. Thus results to date indicated that SPS was phosphorylated at multiple sites and the possible regulatory significance of two sites (phosphopeptides 5 and 7) was suggested.

We have further examined the regulatory significance of phosphopeptides 5 and 7, taking advantage of okadaic acid, a specific and potent inhibitor of the type 1 and 2A protein phosphatases [8–10]. Okadaic acid has been shown to inhibit the endogenous protein phosphatase(s) that dephosphorylate and activate SPS (obtained from darkened leaves) *in vitro* [11,12]. Moreover, feeding okadaic acid to detached leaves in the dark inhibited subsequent activation of SPS by illumination of leaves [11,12]. These observations established that light activation of SPS occurs by a dephosphorylation reaction catalysed by an okadaic acid-sensitive protein phosphatase. In the present study, okadaic acid has proven to be very useful for manipulation of the phosphorylation status and activation state of SPS *in vivo* over a wide range of conditions. We have identified the amino acid phosphorylated, and provide evidence for a close correlation between the labelling *in vivo* of phosphopeptides 5 and 7 and SPS activation state from very low (< 5%) to very high (< 70%) levels. Furthermore, we show labelling of two cryptic phosphorylation sites *in vivo* in the presence of okadaic acid. These results substantially extend our understanding of the regulatory phosphorylation of SPS, a key enzyme of carbohydrate metabolism in plants.

MATERIALS AND METHODS

Materials

All biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 32 P-labelled radionucleotides were obtained from New England Nuclear (Boston, MA, U.S.A.).

Plant materials

Spinach plants were grown in a soil mixture in the greenhouse under natural lighting during the winter months (November–March) when the photoperiod was less than 12 h.

Experimental treatments

To supply [32 P]P_i to leaf tissue, the petioles of excised spinach leaves (3–4 g fresh weight) were rapidly re-cut under degassed water and the cut ends were placed in Microfuge tubes containing 1 ml of degassed water, 1 mCi of [32 P]P_i and 50 μ M unlabelled potassium phosphate (pH 6.0). The leaves were placed in a darkened environmental chamber at 20 °C and allowed to draw the liquid via the transpiration stream for about 1 h (or until the water was largely taken up). In those leaves where SPS was to be maintained in the inactivated state, degassed water was added as needed and the leaves were retained in the dark. After the initial [32 P]P_i uptake, the leaves were treated in the following manner for an additional 2 h. Light activation of SPS involved adjustment of the liquid to 1 ml with water and transfer of the leaves to an illuminated chamber (350 μ mol/m² per s). The highly activated light/mannose-treated leaves were supplied with 1 ml of 50 mM-mannose and placed in the illuminated chamber.

To treat tissue with okadaic acid, excised leaves were first fed [32 P]P_i for 1 h as described above, then supplied with 1 ml of 5 μ M-okadaic acid (diluted in water from a 5 mM stock prepared in dimethyl sulphoxide) for an additional 1.5 h in the dark. Subsequently, the leaves were either retained in the dark or transferred to the light in the presence or absence of mannose.

Extraction and assay of SPS

SPS was extracted from the leaves by grinding the tissue in a precooled mortar using a 1:4 tissue/buffer ratio in extraction buffer containing 50 mM-Mops/NaOH (pH 7.5), 10 mM-MgCl₂, 1 mM-EDTA, 2.5 mM-dithiothreitol (DTT) and 0.1% (v/v) Triton X-100. The homogenates were centrifuged at 15000 g for 5 min and the supernatants were immediately desalted on Sephadex G-25 equilibrated with extraction buffer minus Triton X-100. The SPS activity was assayed with limiting substrates plus P_i ('limiting assay') or with saturating substrates in the absence of P_i ('V_{max} assay'). SPS activation state is defined as velocity in the limiting assay expressed as a percentage of V_{max}. The limiting assay contained 10 mM-UDP-glucose, 10 mM-P_i, 3 mM-fructose 6-phosphate, 12 mM-glucose 6-phosphate, 50 mM-Mops/NaOH (pH 7.5), 15 mM-MgCl₂, 2.5 mM-DTT and extract (total volume 70 μ l). The reaction mixtures for saturating substrates were as above except that P_i was omitted and the concentrations of fructose 6-phosphate and glucose 6-phosphate were 10 and 40 mM respectively. Assays were typically run for 10 min at 25 °C, terminated by the addition of 70 μ l of 30% KOH and the tubes were placed in boiling water for 10 min. After cooling, 1 ml of 0.14% anthrone in 6.9 M-H₂SO₄ was added, and the tubes were incubated at 40 °C for 20 min before absorbance at 620 nm was measured.

Immunoprecipitation of SPS

The 120 kDa subunit of SPS was immunopurified from crude extracts or partially purified preparations of the enzyme in the presence of 10 mM-P_i and 10 mM-KF using monoclonal antibodies [13] as described previously [5].

Partial purification of highly activated SPS

The SPS was partially purified from illuminated detached spinach leaves that were fed 50 mM-mannose (via the transpiration stream) for a period of 3 h. Mannose feeding in the light yielded SPS preparations with the highest activation state. Leaf tissue was frozen in liquid N₂ and homogenized in a cold mortar and pestle in extraction buffer. The protein fractionated between 5 and 12% poly(ethylene glycol) 8000 was obtained as described previously [5]. The poly(ethylene glycol) fraction was then subjected to anion-exchange chromatography with a Pharmacia f.p.l.c. system with a Mono Q column [5]. The enzyme was eluted at approx. 150 mM-KCl. Active fractions were pooled and dialysed for 2 h at 4 °C against 50 mM-Mops/NaOH (pH 7.5)/5 mM-MgCl₂/2.5 mM-DTT. The activated enzyme could be stored for 2–3 days at –80 °C.

Inactivation and phosphorylation of SPS *in vitro*

ATP-dependent inactivation of SPS was performed by pre-incubating partially purified enzyme at 25 °C in the absence (control) or presence of MgATP. The partially purified enzyme was preincubated in mixtures containing 50 mM-Mops/NaOH (pH 7.5), 5 mM-MgCl₂, 2.5 mM-DTT and 10–50 μ M-MgATP. At zero time, and at various times thereafter, samples were removed and assayed for SPS activity in the limiting and V_{max} assays. The decrease in activity between zero time and intervals after ATP addition was a measure of ATP-dependent SPS inactivation or apparent SPS kinase activity. Typically, SPS preincubated in the absence of ATP maintained constant enzyme activity at 25 °C.

Phosphorylation of SPS *in vitro* was performed by incubating the partially purified enzyme at 25 °C in reaction mixtures (300 µl, total volume) containing 50 mM-Mops/NaOH (pH 7.5), 5 mM-MgCl₂, 2 mM-DTT, 50 µM-ATP (approx. 200 µCi) and enzyme (approx. 0.5 µg of SPS holoenzyme). The amount of SPS protein in each reaction was estimated using the measured specific activity of 150 units/mg of SPS protein [13]. At various time intervals, the entire incubation mixture (200 µl) was placed on ice, adjusted to 1 mM-ATP, 10 mM-KF and 5 mM-P_i, and SPS was immediately immunoprecipitated by addition of monoclonal antibodies as described [5].

Phosphoamino acid analysis

SPS, radiolabelled *in vitro* or *in vivo*, was purified by immunoprecipitation and SDS/PAGE [5,6]. The 120 kDa subunit of SPS was electrophoretically transferred to Immobilon-P membrane (Millipore) using conditions specified by the manufacturer. Specifically, the membrane was wetted in methanol for 10 min and then equilibrated in transfer buffer [192 mM-glycine/25 mM-Tris base/20 % (v/v) methanol]. The gel was equilibrated for 30 min in the same buffer and the protein transfer carried out for 2 h at 0.5 A. The SPS-containing protein band was located by allowing the membrane to dry slightly (proteins appear as translucent bands), then excised and rinsed thoroughly with water. Partial acid hydrolysis was performed on the membrane in 5.7 M-HCl for 1 h at 110 °C [14]. The liquid was aspirated and dried down completely in a vacuum rotator (Hetovac). The freeze-dried powder was resuspended in 3 µl of t.l.c. buffer [1 % (v/v) formic acid/10 % (v/v) acetic acid] plus 2 µl of a mixture of phosphoserine, phosphothreonine and phosphotyrosine (1 mg/ml each) and applied in a single spot to Kodak t.l.c. cellulose chromatogram sheets. The phosphoamino acids were resolved by two-dimensional t.l.c. The first dimension was carried out at pH 1.9 [1 % (v/v) formic acid and 10 % (v/v) acetic acid] at 1000 V for 30 min. The second dimension was performed at pH 3.5 [19 % (v/v) acetic acid and 1 % (v/v) pyridine] at 1000 V for 25 min. Phosphoserine, phosphothreonine and phosphotyrosine standards were located by spraying the dried chromatograms with 0.2 % (w/v) ninhydrin [prepared in 5 % (v/v) pyridine/95 % (v/v) ethanol] and heating with a hair dryer. The positions of the standards were marked and autoradiography was performed.

Phosphopeptide analysis

SPS, radiolabelled *in vitro* or *in vivo*, was immunoprecipitated and resolved by SDS/PAGE as described previously [5,6]. After transfer to Immobilon-P, the paper strip containing the 120 kDa subunit of SPS was excised and incubated in 0.5 % (w/v) polyvinylpyrrolidone-40 in 100 mM-acetic acid for 1 h at 37 °C. The membrane was washed five times in water. SPS was then digested with trypsin [Sigma type XIII, Tos-Phe-CH₂Cl-treated] in a buffer containing 10 mM-NH₄HCO₃ (pH 8.3) and 5 % (v/v) acetonitrile using a 10:1 (w/w) SPS/trypsin ratio [15]. Digestion was carried out at 37 °C for 20 h with the same amounts of fresh trypsin being added at 6 and 18 h. After subsequent freeze-drying, the [³²P]phosphopeptides were dissolved in 5 µl of t.l.c. buffer (pyridine/acetic acid/water, 1:10:89, by vol., pH 3.5) and the sample was applied to Kodak t.l.c. chromatogram sheets. Electrophoresis was carried out in the first dimension at 1500 V for 20 min, followed by ascending chromatography in the second dimension (pyridine/butanol/acetic acid/water, 15:10:3:12, by vol.) for 3 h. Autoradiography was performed with Kodak X-Omat AR film (XAR-5) and intensifying screens at -80 °C. After identifying the location of [³²P]phosphopeptides by autoradiography, the individual spots were scraped from the t.l.c.

plates, and radioactivity was determined by liquid-scintillation counting [5].

Replication of experiments

The values presented in the Tables and Figures are typical results obtained within a given experiment. Because of the nature of the labelling *in vivo*, it was not practical to replicate treatments fully within an experiment. However, in all cases, the effects reported have been documented in at least two separate experiments.

RESULTS

Phosphoamino acid analysis of SPS

Excised spinach leaves were fed [³²P]P_i in the dark and subsequently kept for an additional period in either darkness or light. SPS was extracted from these tissues, immunoprecipitated and purified by SDS/PAGE. The 120 kDa subunit of SPS was transferred to Immobilon-P and subjected to limited acid hydrolysis. This procedure has been reported to yield recoveries of phosphorylated amino acids as high as 88–96 % and is highly reproducible [14]. The radiolabelled phospho amino acids were resolved by two-dimensional t.l.c. and identified by using unlabelled phosphotyrosine, phosphothreonine and phosphoserine standards. The autoradiograms shown in Fig. 1 indicate that SPS was phosphorylated almost exclusively on serine residues in both the light and the dark. The observed phosphorylation of threonine and tyrosine residues accounted for less than 1 % of the radiolabel recovered in phosphoserine. The partially purified SPS was labelled *in vitro* exclusively on serine residues in the presence of [γ-³²P]ATP (Fig. 2). Thus the protein kinase(s) that phosphorylate SPS both *in vivo* and *in vitro* are specific for serine residues. It is possible that the same protein kinase(s) might phosphorylate the residues in other substrates.

Phosphopeptide mapping and identification of regulatory sites *in vivo*

We pursued the question of how many serine residues on SPS are phosphorylated by analysing tryptic digests of immunopurified SPS labelled *in vivo* with [³²P]P_i. Preliminary results suggested that SPS was phosphorylated *in vivo* (in the dark) at multiple sites [7]. In the present study, we used two approaches to identify which of the phosphorylation sites on SPS may be involved in light/dark regulation of activity. First, SPS was labelled *in vivo* in the dark and in the light and in the light with

Fig. 1. Two-dimensional separation of phosphorylated amino acids from SPS labelled *in vivo* with ³²P

SPS was labelled *in vivo* by feeding [³²P]P_i to detached spinach leaves in either the dark (a) or the light (b). Labelling, partial acid hydrolysis and two-dimensional t.l.c. are described in the Materials and methods section. Samples were applied to the origin in the lower right-hand corner of each panel.

Fig. 2. Identification of SPS amino acid residues phosphorylated *in vitro*

Partially purified SPS was labelled *in vitro* with an endogenous protein kinase and [γ - ^{32}P]ATP. Details of the phosphorylation, partial acid hydrolysis and two-dimensional t.l.c. are described in the Materials and methods section. The origin is indicated in the lower right-hand corner.

Table 1. Effect of okadaic acid on activation state and ^{32}P labelling of SPS *in vivo*

SPS was labelled by feeding [^{32}P]P_i to detached leaves (about 3.5 g fresh weight each) followed by uptake of okadaic acid and treatment with either darkness, light or light + mannose as detailed in the Materials and methods section. SPS was extracted from 3 g fresh weight of treated tissue and assayed for ^{32}P incorporation and enzyme activity under limiting and V_{max} conditions as described in the text. A sample of the immunoprecipitated SPS was resolved by SDS/PAGE, and radioactivity in the gel slice containing the SPS subunit was determined by liquid-scintillation counting [5]. The remainder of the sample was used for phosphopeptide analysis (see Fig. 3).

Leaf treatment	Okadaic acid	SPS activity ($\mu\text{mol/h per g}$)		Activation state (%)	^{32}P -labelled SPS (d.p.m./25 μg of SPS protein)
		Limiting assay	V_{max} assay		
Dark	—	13.7	80.0	17.1	5300
	+	2.1	50.7	4.1	6400
Light	—	25.5	86.1	29.6	3800
	+	16.7	84.0	19.9	5000
Light + mannose	—	69.6	96.6	71.2	2600
	+	42.8	100.9	42.4	3000

mannose to alter the activation state of the enzyme. Secondly, these treatments were also performed after feeding leaves okadaic acid, a type 1 and 2A phosphoprotein phosphatase inhibitor [8–10] that has been shown to inhibit the SPS phosphatase [11,12]. We then compared the phosphorylation state of specific peptides *in vivo* in relation to the activation state of SPS.

The typical results presented in Table 1 demonstrate the effect of dark, light and light plus mannose leaf treatments on the activation state of SPS. The enzyme isolated from dark-treated tissue was largely deactivated (activation state of 17%) whereas the light/mannose-treated tissue exhibited the highest activation state (72%). Pretreatment of the leaves with okadaic acid prevented full light and light/mannose activation of the enzyme

Fig. 3. Evidence for multisite phosphorylation of spinach leaf SPS

Two-dimensional resolution of tryptic phosphopeptides. SPS was labelled *in vivo* by feeding [^{32}P]P_i to detached spinach leaves (about 3.5 g fresh weight) under the conditions of dark (a and b), light (c and d) and light + mannose (e and f). Each treatment was performed in the absence (a, c and e) and in the presence (b, d and f) of okadaic acid. Peptides derived from approx. 25 μg of SPS protein were applied to the origin in the lower right-hand corner of each panel. The map (g) identifies the major tryptic phosphopeptides observed (open spots). The shaded spots indicate the sites uncovered by okadaic acid treatment of the leaves (phosphopeptides 14 and 15). The dashed lines identify the location of two peptides that have been observed to label more heavily in previous experiments. Individual spots were scraped from the t.l.c. plates, and radioactivity was determined by liquid-scintillation counting [5].

as well as inducing further deactivation of the dark-treated enzyme by blocking dephosphorylation (Table 1). Overall, the phosphorylation status of SPS was highest in deactivated (dark-treated) SPS and lowest in highly activated (light plus mannose-treated) enzyme. Consistent with the inhibitory function of okadaic acid, the total ^{32}P incorporation into SPS was higher under all treatments (dark, light or mannose) in the presence of the inhibitor.

The [^{32}P]phosphopeptide patterns obtained for the dark-, light- and light/mannose-treated tissues were qualitatively similar (Figs. 3a, 3c and 3e). Consistent with previous results [5] and Table 1, deactivated SPS extracted from dark-treated tissue was most heavily phosphorylated whereas the highly activated SPS, isolated from the light/mannose-treated leaves, had incorporated the least amount of ^{32}P . On the basis of analysis of several

Fig. 4. Phosphorylation status of specific SPS phosphopeptides versus SPS activation state

(a) Phosphopeptides (Pp) 5 + 7 and (b) phosphopeptides (Pp) 10 + (Pp) 6. ○, □ and △, Minus okadaic acid; ●, ■ and ▲, plus okadaic acid. ○, and ●, Dark; □, and ■, light; △, and ▲, light + mannose. Values shown correspond to radioactivity recovered in the indicated [^{32}P]phosphopeptides that were resolved from 25 μg of SPS protein.

Fig. 5. Two-dimensional tryptic peptide map of SPS phosphorylated *in vitro*

Highly activated SPS was labelled *in vitro* in the presence of [γ - ^{32}P]ATP and an endogenous protein kinase. Details of phosphorylation, trypsin digestion and peptide mapping are described in the Materials and methods section.

experiments of this type, the ^{32}P -labelling of phosphopeptides 5 and 7 was consistently correlated with changes in SPS activation state (Fig. 4a). These results indicate that phosphorylation of the serine residues on these two tryptic peptides has an inhibitory effect and results in the inactivation of SPS. The correlation of SPS activation state with phosphorylation of the two phosphopeptides is demonstrated quantitatively in Fig. 4(a), where ^{32}P incorporation into these sites is inversely related to activation state of the enzyme. For comparison, total incorporation of ^{32}P into SPS in the different treatments is also presented (Fig. 4a). As SPS activation state decreased from 72 to 4%, total radioactivity associated with SPS was only decreased by slightly more than half. This is consistent with the postulate that some phosphorylation sites are not involved in regulation of activity (and see below). At the lowest activation state, labelling of phosphopeptides (5 and 7) accounted for 39% of the total ^{32}P incorporated, and that value decreased to 13% of total in the highest activation state sample.

Two phosphopeptides (phosphopeptides 6 and 10) whose phosphorylation state was not correlated with activation state of SPS are shown in Fig. 4(b). Clearly, the phosphorylation status of these two phosphopeptides is not directly involved in regulation of SPS activity. Interpretation of the labelling experiments shown in Figs. 4(a) and (b) must be tentative because the specific

radioactivity of the γ -phosphate of cytoplasmic ATP was not measured and could vary among treatments.

Throughout these experiments, okadaic acid consistently inhibited the dephosphorylation of phosphopeptides 5 and 7 under dark, light and mannose treatments (Figs. 3b, 3d and 3f and Fig. 4a). In addition, the inhibitor induced ^{32}P labelling of two unique sites (designated phosphopeptides 14 and 15; Fig. 3). Thus okadaic acid treatment may uncover certain phosphorylation sites on SPS that otherwise turn over rapidly, providing the first plant example of a phenomenon observed in animals [8].

Identification of phosphorylation sites labelled *in vitro*

The tryptic phosphopeptides obtained from SPS labelled *in vitro* by using [γ - ^{32}P]ATP and the Mono Q-purified light/mannose-activated enzyme were analysed by two-dimensional t.l.c./t.l.c. SPS has been previously shown to be inactivated *in vitro* in the presence of MgATP and an endogenous protein kinase [5]. The results in Fig. 5 show that, during labelling, *in vitro* SPS is labelled at two sites. The tryptic phosphopeptides co-chromatograph with phosphopeptides 5 and 7, which have been demonstrated to be regulatory sites *in vivo*. It should be noted that phosphopeptides 5 and 7 are each composed of multiple spots, which have the same mobilities during electrophoresis but separate slightly during chromatography. *In vitro*, phosphopeptides 5 and 7 are both phosphorylated following the same time course [6] and thus both appear to be of regulatory significance.

The observation that SPS is phosphorylated at only two sites *in vitro* indicates that the multiple spots observed as a result of labelling *in vivo* are not the result of procedural artifacts. Further, these results support the earlier observations that the partially purified SPS preparations retained the protein kinase activity capable of inactivating the enzyme in the presence of MgATP. Although other endogenous protein kinases not present in these preparations of SPS may play a role in regulation of the enzyme, these results confirm the presence of at least one kinase that inactivates the enzyme by phosphorylation of specific sites. Other protein kinases that presumably phosphorylate SPS *in vivo* are most probably removed during purification.

DISCUSSION

In this paper we provide evidence for phosphorylation of SPS at multiple serine residues and further provide evidence that two phosphorylation sites (phosphopeptides 5 and 7) on the enzyme appear to be largely responsible for the modulation of SPS activity by light or mannose feeding. This conclusion is based on the observation that phosphorylation of phosphopeptides 5 and 7 (by an endogenous protein kinase) results in inactivation *in vitro* and that the phosphorylation status of these two sites *in vivo* is inversely related to SPS activation state under a wide range of conditions. The exact number of phosphorylation sites on SPS will only be established when the phosphopeptides have been sequenced.

Further support for the regulatory role of phosphopeptides 5 and 7 was obtained through studies with the protein phosphatase inhibitor okadaic acid. In mammalian tissues, okadaic acid is a selective inhibitor of type 1 and 2A protein phosphatases [8–10], which play important regulatory roles in various processes *in vivo*, including regulation of muscle glycogen metabolism and contractility as well as protein synthesis [16]. The light activation of SPS *in situ* has been shown to be inhibited by various phosphatase inhibitors including okadaic acid [11,12]. In the present study, we have shown that okadaic acid can specifically inhibit the dephosphorylation of phosphopeptides 5 and 7 during light and mannose activation of the enzyme, and thereby decrease

the activation state of the enzyme (consistent with the overall striking correlation between phosphopeptides 5 and 7 and inhibition of SPS activity) (Table 1 and Fig. 4a).

The relative phosphorylation status of other phosphopeptides (identified on peptide maps of SPS labelled with ^{32}P *in vivo*) did not correlate with light/dark modulation of SPS activity (Fig. 4b). It is possible that certain sites may be phosphorylated or dephosphorylated in response to environmental signals other than light. As well, phosphorylation of some serine residues may simply be constitutive or without apparent effect on SPS activity or function. Further studies of these sites as well as sequencing and localization of the key regulatory phosphorylation regions on the enzyme subunit are clearly warranted and will provide important information about the complex role of protein phosphorylation in control of SPS activity.

The phenomenon of 'multisite phosphorylation' in which proteins are phosphorylated at multiple sites by more than one kinase is turning out to be common. Several enzymes and proteins have been identified in animal systems, including glycogen synthase [17], acetyl-CoA carboxylase [18] and histone H1 [19], which are phosphorylated on more than one amino acid residue. However, in the case of SPS it is not yet certain that each phosphopeptide resolved by two-dimension t.l.e./t.l.c. represents a distinct phosphorylation site. For example, it is well known that phosphorylation of certain sites on a protein can dramatically alter the kinetics of trypsin digestion at proximal sites [20], perhaps resulting in incomplete digestion of the substrate and overestimation of the total number of phosphorylated sites. In addition, the t.l.e. system may resolve tryptic peptides containing more than one potential phosphorylation site [21].

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