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Sucrose-phosphate synthase is dephosphorylated by protein phosphatase 2A in spinach leaves

Evidence from the effects of okadaic acid and microcystin

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Sucrose-phosphate synthase (SPS) purified from spinach leaves harvested in the dark, was activated by mammalian protein phosphatase 2A (PP2A). Activation of SPS in a fraction from darkened spinach leaves was largely prevented by either okadaic acid or microcystin-LR (specific inhibitors of PP1 and PP2A), while inhibitor-2 (a PP1 inhibitor) or Mg^{2+} (essential for PP2C) were ineffective. In vivo, okadaic acid and microcystin-LR prevented the light-induced activation of SPS and decreased sucrose biosynthesis and CO_2 fixation. It is concluded that PP2A is the major SPS phosphatase in spinach. This study is the first to employ microcystin-LR for modulating protein phosphorylation in vivo.

Microcystin-LR; Okadaic acid; Protein phosphatase; Photosynthesis; Sucrose-phosphate synthase

1. INTRODUCTION

In green leaves of higher plants, most of the photosynthetically-fixed carbon is ultimately directed towards cytosolic synthesis of sucrose (which is then either stored in vacuoles or transported to other parts of the plant) or towards deposition of starch in chloroplasts (reviewed in [1]). The relative distribution of carbon among these pools varies dramatically in different crop plants (e.g. [2,3]) and under different environmental conditions. Since both the rate of carbon fixation and the subsequent distribution of carbohydrate within a plant is critical to the quality and yield of the harvest [2] it is of considerable importance to understand how the participating metabolic pathways are controlled.

The cytosolic fructose-1,6-bisphosphatase and sucrose-phosphate synthase (SPS) have been pinpointed as the enzymes likely to contribute most significantly to the regulation of sucrose biosynthesis [1]. The activity of fructose-1,6-bisphosphatase, the first committed step of this pathway, is controlled by changes in the concentration of its inhibitor fructose-2,6-bisphosphate [4–6]. SPS is also regulated allosterically, Glc6P being an essential activator and P_i a feedback inhibitor [7,8]. In addition, SPS activity in spinach leaves is regulated in a diurnal manner by a covalent modification that alters the kinetic properties of the enzyme [8–10]. Thus, SPS purified from spinach leaves that have been illuminated is less sensitive to inhibition by P_i and has a lower K_m for Fru6P (high-activity form) than has SPS purified from leaves harvested in the dark (low-activity form) [8]. Feeding of mannose to leaves mimics the effect of light on the covalent modification of SPS [8,11]. The effect of mannose suggests that modification of SPS is modulated by levels of intracellular metabolites generated during photosynthesis, rather than by the light conditions per se [11]. The recent observation that incubation of partially purified spinach leaf SPS with [γ-^{32}P]ATP results in incorporation of ^{32}P and is accompanied by a parallel decrease in activity measured in the presence of limiting Fru6P and P_i [12], indicates that covalent modification of SPS may be due to reversible phosphorylation. However, the molecular details and the identity of the relevant kinase(s) and phosphatase(s) are unknown.

Recently, 3 of the 4 major protein phosphatases (PP), namely PP1, PP2A and PP2C, present in animal cells were identified in plants and their properties were found to be virtually indistinguishable from the corresponding enzymes in mammalian tissues [13,14]. Similarities between plant and animal PP1 and PP2A included substrate specificity (using mammalian phosphoprotein substrates), sensitivity to the heat stable proteins inhibitor-1 and inhibitor-2 (from rabbit

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Abbreviations: SPS, sucrose-phosphate synthase; Fru6P, fructose 6-phosphate; Glc6P, glucose 6-phosphate; UDPGlc, uridine 5-diphosphoglucose; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; P_i, inorganic phosphate; PP, protein phosphatase

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skeletal muscle) which inhibit PP1 specifically [15], inhibition by the tumor promoter okadaic acid [16] and inhibition by the hepatotoxin microcystin-LR from cyanobacteria [17]. Okadaic acid and microcystin-LR are potent and highly specific inhibitors of both PP1 and PP2A [16,17].

In this paper, we have performed experiments with purified SPS in spinach leaf extracts, and in vivo which demonstrate that PP2A is the major enzyme responsible for activating (dephosphorylating) SPS in spinach leaves.

2. MATERIALS AND METHODS

2.1. *Materials*

Spinach (*Spinacia oleracea* US Hybrid 424, Ferry Morse Seed Company, Modesto, CA) was grown hydroponically in a 9 h light/15 h dark cycle [9]. The catalytic subunits of PP1 and PP2A [18], and inhibitors-1 and -2 [15] were purified to homogeneity from rabbit skeletal muscle; okadaic acid was a gift from Dr Y. Tsukitani, Fujisawa Pharmaceutical Company, Japan, and microcystin-LR was provided by Professor G. Codd, Department of Biological Sciences, University of Dundee. PD10, Mono-Q and Superose-12 columns were from Pharmacia/LKB, Freiburg. Amino-hexyl agarose was from Sigma, Deisenhofen, and biochemicals and enzymes for assay of SPS were from Boehringer, Mannheim.

2.2. *Protein phosphatase assays*

PP1 and PP2A were assayed in the absence of divalent cations using 10 μM ^{32}P-labelled glycogen phosphorylase [18]. When assaying extracts, PP1 was the phosphorylase phosphatase activity not inhibited by 1 nM okadaic acid and inhibited by inhibitor-2, while PP2A was the activity inhibited by 1 nM okadaic acid and resistant to inhibitor-2 [19]. PP2C was the Mg^{2+}-dependent casein phosphatase activity measured in the presence of 5 μM okadaic acid [19]. One unit of protein phosphatase was that amount which catalysed the dephosphorylation of 1 μmol of substrate in 1 min.

2.3. *SPS assays*

SPS was assayed as in [8]. Maximal activity was determined in the presence of 100 mM Hepes-KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 3 mM UDPGlc, 4 mM Fru6P and 20 mM Glc6P (assay volume 200 μl). The phosphate-inhibited activity was determined in the presence of 100 mM Hepes-KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 3 mM UDPGlc, 2 mM Fru6P, 10 mM Glc6P and 2.5 mM P_i. After 10 min at 25°C, the incubation was heated to 95°C for 3 min, cooled, centrifuged and assayed for UDP. No hydrolysis of UDP occurred during the incubation or in subsequent processing of the assays.

2.4. *Pretreatment of spinach plants to provide low-activity and high-activity forms of SPS*

Spinach plants were illuminated for 9 h to generate a high sucrose concentration and then darkened. This treatment converted SPS into a low-activity form that had a high K_m for Fru6P and was sensitive to inhibition by P_i. A parallel set of plants were darkened for 15 h to deplete their sucrose pools and were then illuminated for 30 min. This treatment converted SPS into a high-activity form which had a lower K_m for Fru6P and was less sensitive to inhibition by P_i. The leaves were ground finely in liquid N_2 and stored at −80°C.

2.5. *Purification of low-activity and high-activity forms of SPS from spinach leaves*

Frozen spinach leaf powder was suspended in 6 vols of 50 mM Hepes-KOH pH 7.4, 100 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 2 mM benzamidine, 2 mM ϵ-amino-n-caproic acid, 0.5 mM phenylmethylsulfonyl fluoride, centrifuged and filtered to give the extract which was immediately applied to an amino-hexyl agarose column. A step gradient of increasing NaCl concentrations eluted SPS into the 100 mM NaCl fraction, which was then chromatographed on FPLC Mono-Q and Superose-12 as described [8]. The partially-purified low-activity form was inhibited by 80% at 2.5 mM P_i whereas the high-activity form was only inhibited 25% at that P_i concentration (data not shown).

2.6. *Preparation of low-activity and high-activity ammonium sulfate fractions from spinach leaves*

All operations were performed at 0–2°C and the entire procedure was completed within 25 min. Frozen spinach leaf powder was resuspended in extraction medium and centrifuged for 5 min at 40000 × g. The supernatant was decanted and 1.25 vols of 90% saturated ammonium sulphate added, maintaining the pH at 7.5 by addition of a few drops of concentrated NH_4OH. The suspension was centrifuged for 10 min at 40000 × g, the supernatant discarded, the pellet redissolved in 1 ml of extraction buffer minus KCl and phenylmethylsulphonyl fluoride, and desalted by a passage through a PD10 column pre-equilibrated with the same buffer. The protein-containing fractions (2 ml) were pooled and aliquots frozen in liquid N_2 and stored at −80°C.

2.7. *Treatment of spinach leaf discs with okadaic acid or microcystin-LR*

Discs were cut from spinach plants 2 h into the dark period and were incubated with various concentrations of either okadaic acid (diameter 0.5 cm, 500 μl incubations) or microcystin-LR (diameter 1 cm, 1000 μl incubations) for 12 h before transferring them to a leaf-disc O_2 electrode (Hansatech, Kings Lynn, UK) containing 400 μl of 2 M $KHCO_3/K_2CO_3$ buffer as CO_2 source. The leaf discs were illuminated for 15 min (576 μmol photons $\cdot m^{-2} \cdot s^{-1}$), frozen in liquid N_2 under continued illumination, extracted and assayed for SPS. For assay of sucrose, starch, organic acids and amino acids, ^{14}C-labeled CO_2 (5 × 10 5 Bq $\cdot$ nmol^{-1}) was included in the CO_2 buffer and the leaf discs were illuminated (150 μmol photons $\cdot m^{-2} \cdot s^{-1}$) for 25 min before freezing in liquid N_2, extracting in 10 ml of 80% ethanol to separate soluble and insoluble (starch) components, and separating neutral (sucrose), basic and acidic components by microion exchange chromatography [20].

3. RESULTS AND DISCUSSION

3.1. *Activation of partially purified low-activity SPS by incubation with mammalian protein phosphatases*

Partially-purified SPS contained no detectable contaminating protein phosphatase activity, because PP1 and PP2A both bind to amino-hexyl groups at salt concentrations where SPS is eluted (Methods 2.5., [18]). Incubation of low-activity SPS with 60 mU $\cdot$ ml^{-1} PP2A caused a small increase in maximal activity and a 3-fold increase in activity measured in the presence of 2.5 mM P_i (Table I). This increase in phosphate sensitivity due to PP2A was completely abolished by the inclusion of 5 μM okadaic acid (Table I). Addition of 65 mU $\cdot$ ml^{-1} of PP1 led to a much smaller activation of SPS than that seen with PP2A and again this effect was abolished by 5 μM okadaic acid (Table I). When partially purified high-activity form SPS was incubated with PP1, PP2A or okadaic acid, no changes in activity were observed (Table I). These experiments confirm

Table I

Activation of partially-purified spinach leaf SPS by incubation with mammalian protein phosphatases (s_e for $n = 3$)

Pretreatment of SPS	Low-activity SPS assayed with		High-activity SPS assayed with	
	Zero P_i	2.5 mM P_i	Zero P_i	2.5 mM P_i
None	80.9 ± 0.5	8.8 ± 0.8	72.3 ± 3.8	40.6 ± 1.7
60 mU·ml^{-1} PP2A	78.2 ± 1.9	28.4 ± 0.4	65.1 ± 9.6	34.9 ± 0.6
60 mU·ml^{-1} PP2A + 5 μM okadaic acid	87.8 ± 0.8	10.6 ± 0.9	72.3 ± 3.1	38.3 ± 1.6
65 mU·ml^{-1} PP1	83.3 ± 6.2	16.2 ± 2.2	–	–
65 mU·ml^{-1} PP1 + 5 μM okadaic acid	83.3 ± 4.7	10.8 ± 0.7	–	–

that the low-activity form of SPS is the phosphorylated form and indicate that spinach leaf SPS can be dephosphorylated by mammalian PP2A and to a much lesser extent by mammalian PP1.

3.2. *Evidence that PP2A is the major SPS phosphatase in spinach leaves*

In order to determine which of the PPs in spinach leaves are responsible for dephosphorylating SPS, extracts were prepared from leaves that had been darkened before harvesting and were then fractionated from 0 to 55% ammonium sulfate and desalted. SPS remained in its low-activity form provided that the temperature was kept at 0°C. Subsequent incubation at 30°C led to a slight increase in maximal SPS activity and a 4–6-fold increase in activity in the presence of 2.5 mM P_i (Fig. 1). After 30–40 min, the activity in the presence of P_i had increased to about 60% of that in the absence of P_i. This is approaching the activation state observed when SPS is assayed in extracts from leaves that have been illuminated. Activation of SPS could be largely prevented by addition of 1 μM okadaic acid or microcystin-LR to the extract (Fig. 1) demonstrating that at least some of the activation of SPS was due to dephosphorylation by endogenous PP1 and/or PP2A. Incubation with 50 mM NaF (a general phosphatase inhibitor) in the absence of divalent cations had the same effect as okadaic acid or microcystin-LR which suggested that no other serine/threonine-specific phosphatases were involved in the conversion. Since none of these phosphatase inhibitors could completely prevent the activation of SPS the possibility exists that the unblocked activation was due to some alternative mechanism (which may or may not be physiologically relevant). This may be proteolysis, which mimics the effects of dephosphorylation on several mammalian enzymes (e.g. [21]). However, inclusion of trypsin inhibitor and leupeptin had no effect.

PP1 accounted for 73% and PP2A for 27% of the phosphorylase phosphatase activity in the ammonium sulphate fraction (data not shown). In order to determine the relative contributions of PP1 and PP2A to the activation of SPS, the desalted ammonium sulfate fraction was supplemented with inhibitor-2, a specific inhibitor of PP1. The activation of SPS was unaffected by inclusion of inhibitor-2 at 0.5 μM (Fig. 2). Control experiments in which the extract was supplemented with ^{32}P-labelled phosphorylase kinase, established that 0.5 μM inhibitor-2 inhibited by about 75% the initial rate of dephosphorylation of the β-subunit of phosphorylase kinase (data not shown). Since PP1 is the only enzyme which dephosphorylates the β-subunit specifically [13], this experiment indicated that most, or all, of the PP1 in the fraction was being inhibited. In contrast, dephosphorylation of the α-subunit, which is predominantly a substrate for PP2A, was hardly affected (data not shown). Thus 0.5 μM inhibitor-2, which was sufficient to inactivate PP1 in the extract, had no effect on SPS; demonstrating that, in spinach leaves, PP2A is solely responsible for dephosphorylation of SPS.

The ammonium sulphate fraction contained about 1 mU·ml^{-1} of the Mg^{2+}-dependent, okadaic acid insensitive protein phosphatase, PP2C. However, inclusion of Mg^{2+} up to 20 mM had no effect in the rate of activation of SPS in the presence or absence of 5 μM okadaic acid or microcystin (data not shown). We conclude that PP2C made no contribution to the dephosphorylation of SPS.

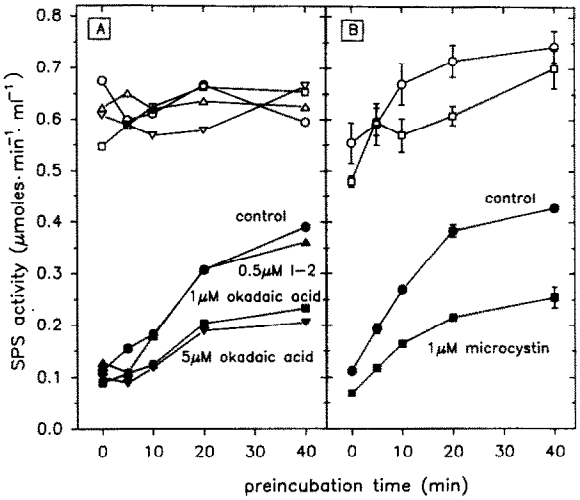

Fig. 1. Activation of SPS in an ammonium sulphate fraction prepared from spinach leaves. Spinach plants were preilluminated for 15 h, darkened for 30 min and an extract prepared containing SPS in the low-activity form. The extract was incubated at 30°C with or without additions, and aliquots were removed at different times and assayed for SPS in the presence (●, ■, ▼, ▲) or absence (○, □, ▽, △) of P_i. (A) Extract incubated without additions (control, ●,○) or with 1 μM (■, □) or 5 μM okadaic acid (▼, ▽), or with 0.5 μM inhibitor-2 (I-2, ▲, △). (B) Extract incubated without additions (control, ●,○), or with 1 μM microcystin-LR (■, □) (s_e for $n = 3$).

3.3. *Okadaic acid or microcystin-LR prevent activation (dephosphorylation) of SPS in vivo in spinach leaf discs*

Spinach leaf discs were incubated for 12 h in the dark in the presence of various concentrations of either okadaic acid or microcystin-LR and were then illuminated for 15 min. Subsequent assays revealed that in the absence of toxin, SPS had been converted during illumination to the expected high-activity (dephosphorylated) form. In the discs that had been incubated with toxin before illumination, this activation of SPS was prevented, with half-maximal effects occurring at 0.24 μM okadaic acid (Fig. 2A) or 0.84 μM microcystin-LR (Fig. 2B). Thus both okadaic acid and microcystin-LR can enter leaf discs and prevent activation (dephosphorylation) of SPS in the manner expected from the in vitro experiments.

3.4. *Okadaic acid and microcystin-LR prevent the stimulation of photosynthetic sucrose synthesis*

If SPS plays a pivotal role in regulation of sucrose biosynthesis [1], then inhibition of SPS dephosphorylation in vivo by okadaic acid or microcystin-LR (Fig. 2) should be reflected in changes in the overall rate of this pathway. Leaf discs were therefore incubated for 12 h in the dark in the presence of various concentrations of okadaic acid (Fig. 3A) or microcystin-LR (Fig. 3B) and illuminated for 25 min in the presence of ^{14}C-labelled CO_2. The discs were then analyzed for labelling of sucrose and other metabolites. Fig. 3 shows that okadaic acid or microcystin-LR did indeed cause a dose-dependent decrease in incorporation of ^{14}C into sucrose. The toxins acted at similar concentrations to those which prevented activation of SPS in vivo (Fig. 2).

Sucrose is synthesized in the cytosol, and starch is synthesized in the chloroplast, and it is well established that more photosynthate is diverted to starch when sucrose synthesis is specifically inhibited [1,5]. In agreement, both toxins led to a small stimulation of starch synthesis (Fig. 3). The synthesis of organic acids and amino acids was essentially unaffected (Fig. 3).

To provide evidence that inactivation of SPS was responsible for the decreased flux to sucrose, we measured the concentration of its substrates. These should remain high or increase, even though the flux to sucrose decreases. After supplying 1 μM okadaic acid, there was an increase of Fru6P (from 67.3 ± 2.4 to 88.1 ± 3.3 nmol·mg Chl^{-1}) and UDPGlc (from 54.5 ± 2.9 to 68.5 ± 4.6 nmol·mg Chl^{-1}). After adding microcystin, UDPGlc increased slightly (from 59.0 ± 1.7 to 76.8 ± 4.4 nmol·mg Chl^{-1}), but Fru6P decreased slightly (from 87.0 ± 2.2 to 76.4 ± 5.9 nmol·mg Chl^{-1}), (s_e for n = 4).

Both toxins also decreased the rate of photosynthesis (Fig. 3). A similar inhibition was observed within 1 h when microcystin was supplied to spinach leaves via the transpiration stream (data not shown). The decreased rate of photosynthesis could be a consequence of the lower rate of sucrose synthesis [1,5], but it is also possible that these toxins are exerting further effects on cytosolic metabolism. A direct effect on chloroplast metabolism via inhibition of plastid protein phosphatases is unlikely, because subcellular fractionation of various plant tissues has shown that only trace amounts of PP1 and PP2A are associated with chloroplasts, and these enzymes are therefore unlikely to be directly involved in regulation of chloroplast metabolism [14].

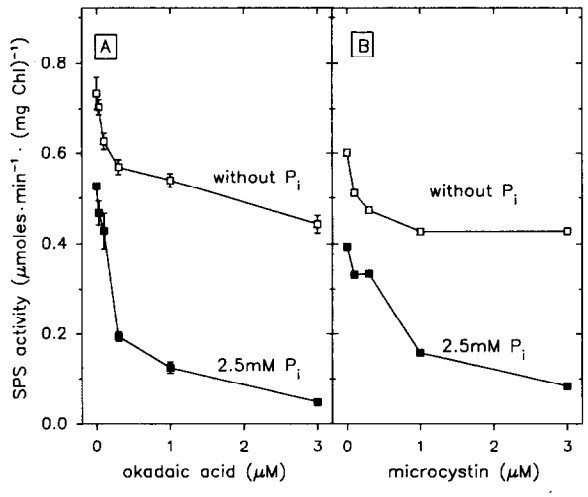

Fig. 2. Inactivation of SPS in vivo. Spinach leaf discs (0.2 g FW) were incubated overnight on various concentrations of (A) okadaic acid (s_e for n = 3) and (B) microcystin-LR (volume of solution 0.5 and 1 ml, respectively) and were then illuminated in saturating CO_2 for 15 min before preparing extracts and assaying for SPS in the absence (□) and presence of 2.5 mM P_i (■).

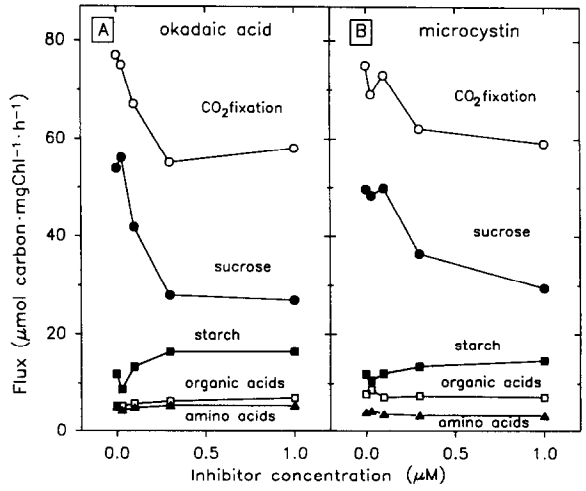

Fig. 3. Inhibition of sucrose synthesis by (A) okadaic acid and (B) microcystin-LR. Spinach leaf discs were pretreated with toxin as in Fig. 2 and were then illuminated for 25 min in a leaf disc O_2 electrode in the presence of ^{14}C-labelled CO_2 before extracting them and analyzing for label in starch (■, insolubles) and sucrose (●, neutral solubles), amino acids (▲, basic solubles) and organic acids (□, acidic solubles) (s_e for n = 3).

4. CONCLUDING REMARKS

Two potent and specific inhibitors of PP1 and PP2A, okadaic acid and microcystin-LR, have enabled us to study the regulatory properties of SPS at both the molecular and the cellular level. The in vivo experiments demonstrate directly that SPS is regulated by protein phosphorylation, and changes in protein phosphorylation have a profound effect on the distribution of carbon fixed during photosynthesis.

SPS is the third plant enzyme identified as a substrate for PP2A. The others are PEP carboxylase in the CAM plant *Bryophyllum fedtschenkoi* [22] and quinate dehydrogenase in carrot cells [14].

Okadaic acid is proving an extremely useful probe for identification and analysis of physiological processes in mammalian tissues that are regulated by reversible phosphorylation of proteins. Microcystin, despite having a completely different chemical structure [17], inhibits PP1 and PP2A in a very similar way to okadaic acid, and indeed the binding of these two toxins to the protein phosphatases is mutually exclusive [17]. The results presented in this paper are the first demonstration that microcystin is able to enter intact plant leaves and change the phosphorylation state of proteins in a similar manner to okadaic acid. The role of protein phosphorylation as a regulatory mechanism in plants is only just starting to emerge. Clearly there is enormous potential for these two toxins employed in this study to be used as probes to investigate (the role of PP1 and/or PP2A in) the miriad of plant responses to environmental and physiological stimuli.

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