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Protein Phosphorylation as a Mechanism for Osmotic-Stress Activation of Sucrose-Phosphate Synthase in Spinach Leaves[1]

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Experiments were performed to investigate the mechanism of sucrose-phosphate synthase (SPS) activation by osmotic stress in darkened spinach (*Spinacia oleracea* L.) leaves. The activation was stable through immunopurification and was not the result of an increased SPS protein level. The previously described Ca^{2+}-independent peak III kinase, obtained by ion-exchange chromatography, is confirmed to be the predominant enzyme catalyzing phosphorylation and inactivation of dephosphoserine-158-SPS. A new, Ca^{2+}-dependent SPS-protein kinase activity (peak IV kinase) was also resolved and shown to phosphorylate and activate phosphoserine-158-SPS in vitro. The peak IV kinase also phosphorylated a synthetic peptide (SP29) based on the amino acid sequence surrounding serine-424, which also contains the motif described for the serine-158 regulatory phosphorylation site; i.e. basic residues at P-3 and P-6 and a hydrophobic residue at P-5. Peak IV kinase had a native molecular weight of approximately 150,000 as shown by gel filtration. The SP29 peptide was not phosphorylated by the inactivating peak III kinase. Osmotically stressed leaves showed increased peak IV kinase activity with the SP29 peptide as a substrate. Tryptic ^{32}P-phosphopeptide analysis of SPS from excised spinach leaves fed [^{32}P]inorganic P showed increased phosphorylation of the tryptic peptide containing serine-424. Therefore, at least part of the osmotic stress activation of SPS in dark leaves results from phosphorylation of serine-424 catalyzed by a Ca^{2+}-dependent, 150-kD protein kinase.

The metabolic response of higher plants to osmotic stress is extremely complex (Bray, 1993). Among the most important components of this response is thought to be the reduction of cellular water potential by osmolyte synthesis, which facilitates increased water uptake from the environment (Zrenner and Stitt, 1991; Schwall et al., 1995). Carbon partitioning toward Suc is increased in numerous higher plant species in response to osmotic stress (Quick et al., 1989, 1992; Foyer et al., 1992; Huber and Huber, 1996). SPS (EC 2.4.1.14) has been implicated as a key metabolic control point in Suc synthesis (Barber, 1985; Stitt et al., 1988; Lunn and ap Rees, 1990). In a number of higher plant species, including spinach (*Spinacia oleracea* L.), increased partitioning of carbon toward Suc in response to osmotic stress has been largely attributed to an increase in the activation state of SPS (Quick et al., 1989; 1992; Zrenner and Stitt, 1991; Reimholz et al., 1994). The SPS enzyme is under complex regulation involving fine control by allosteric effectors and coarse control by protein phosphorylation (for review, see Huber and Huber [1996]). Site-specific seryl phosphorylation of spinach SPS on Ser-158 is the mechanism underlying light/dark modulation (McMichael et al., 1993), but the basis for activation during osmotic stress is not known.

The regulatory phosphorylation motif targeted by SPSk contains the consensus motif B-Hy-X-B-X_2-Ser, where Ser represents the phosphate acceptor, X is any amino acid, Hy is a hydrophobic residue, B is a basic residue, and the subscript number is the number of residues (McMichael et al., 1995b). The motif appears only at two places in the SPS sequence, around Ser-158 (the regulatory site involved in light/dark modulation) and Ser-424. It is not known whether Ser-424 is phosphorylated, and if so, if it has any regulatory function.

Previous work with spinach and other higher plant species under water deficit has shown increased Suc synthesis and SPS activation states associated with decreased starch synthesis (Quick et al., 1989, 1992; Zrenner and Stitt, 1991). In these reports the osmotic stress activation of SPS was detected only with selective assays (i.e. limiting substrate concentrations plus the inhibitor Pi) that distinguish between different kinetic forms of SPS (Quick et al., 1989, 1992; Zrenner and Stitt, 1991). In addition to increases in the activation state of SPS, Huber et al. (1993) observed changes in the ^{32}P-labeling patterns of tryptic ^{32}P-phosphopeptides derived from SPS immunopurified from osmotically stressed spinach leaves fed [^{32}P]Pi. Total ^{32}P-labeling of SPS was increased slightly, as was labeling of the regulatory phosphopeptide containing Ser-158. Thus, dephosphorylation of the regulatory site (Ser-158) cannot explain the activation. However, the osmotically stressed leaves also contained prominent ^{32}P-labeling of a peptide with high mobility during TLE, which was only weakly labeled in nonstressed controls. The high mobility during TLE suggests that the tryptic peptide is highly

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Abbreviations: MAb, monoclonal antibody; SPS, Suc-P synthase; SPSk, SPS protein kinase; SP2 and SP29, synthetic peptides based on the spinach SPS sequence surrounding Ser-158 and Ser-424, respectively; TLE, thin-layer electrophoresis.

charged (positive) and relatively small. Considering all of the possible tryptic peptides that can be derived from SPS, there is only one peptide that meets the criteria and also contains a Ser residue: GVS-424-CHGR. However, there is no experimental evidence that Ser-424 in SPS is phosphorylated in stressed or unstressed leaves.

Recently, the possibility of multiple SPS genes and gene products in potato (Müller-Röber et al., 1992) and citrus (Komatsu et al., 1996) have been reported. However, Sakamoto et al. (1995) reported the map-based cloning of a single SPS gene in rice source organs, and Worrell et al. (1991) reported that SPS enzyme activity was conferred by a single gene product in maize. To date, no evidence has been presented to our knowledge for the occurrence of spinach-leaf SPS isoforms that differ in molecular weight from the native enzyme under conditions of abiotic stress (for review, see Guy et al. [1992]). However, the presence of multiple genes encoding SPS in some species raises the possibility that different forms may be expressed in response to stress. Thus, osmotic stress activation of SPS could arise by covalent modification of existing SPS protein or by enhanced expression of an isoform(s) with different kinetic and/or regulatory properties.

To our knowledge, osmotically stressed SPS has not been purified beyond the crude extract to ascertain whether the osmotic stress activation is persistent. For example, it is known that SPS can form an association with other proteins such as its inactivating kinase (Huber and Huber, 1996) and with Suc-P phosphatase (Salerno et al., 1996). In particular, the association in the latter case is thought to stimulate SPS activity and it is not known whether such associations are affected by stress. Thus, there are a number of fundamental questions concerning the nature and molecular mechanism of osmotic-stress activation of SPS. The overall objective of the present work was to determine whether osmotic-stress activation of spinach SPS is the result of covalent modification, and if so, whether protein phosphorylation could be the mechanism. To this end, in vivo and in vitro experiments were performed with purified spinach-SPS, SPSk, SP2, and SP29 (see Table I).

MATERIALS AND METHODS

Growth and Harvest of Plant Material

Spinach (*Spinacia oleracea* L. cvs Bloomsdale and Tyee) was grown under standard greenhouse conditions as previously described (for review, see Huber et al. [1989]). Highly activated (dephospho-Ser-158) SPS was obtained by feeding 50 mM D-Man to excised leaves as described by Huber and Huber (1992). Highly inactivated (phospho-Ser-158) SPS was obtained from dark-adapted spinach leaves. Leaves were harvested directly into liquid nitrogen after placing plants into a dark growth chamber for 1 h. Osmotic-stress-activated SPS was obtained by the administration of 0.4 M sorbitol solutions to the cut petioles of excised spinach leaves for 4 h in the dark. Leaves were harvested directly into liquid nitrogen at the end of the osmotic stress period and stored at −80°C.

Extraction of Plant Material

Frozen leaf tissue was ground in a chilled mortar with extraction buffer (2–4 mL g^{-1} fresh weight) containing 50 mM Mops-NaOH, pH 7.5, 10 mM $MgCl_2$, 0.2 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 1 mM ϵ-amino-*n*-caproic acid, 1 mM benzamidine, and 0.1% (v/v) Triton X-100. The homogenates were passed through four layers of Miracloth (Calbiochem).[2] In experiments in which crude extracts were used, plant material was ground as described above, except that the extraction was carried out with 2 mL of buffer g^{-1} fresh weight. An aliquot of the filtered homogenate was centrifuged at 14,000 rpm for 2 min and immediately desalted on a Sephadex G-25 spin column (4-mL column bed volume) equilibrated with 50 mM Mops-NaOH, pH 7.5, 10 mM $MgCl_2$, and 2.5 mM DTT (Weiner et al., 1992; McMichael et al., 1995a). When extracts were being prepared for purification, extraction was with 4 mL g^{-1} fresh weight, as described below.

Synthetic Peptides

The sequences of synthetic peptides SP2 and SP29 were based on the spinach SPS native sequence surrounding Ser-158 and Ser-424, respectively (see Table I). Peptide SP2 was a kind gift from Dr. Jan Kochansky (U.S. Department of Agriculture-Agricultural Research Service, Insect Neurobiology and Hormone Laboratory, Beltsville, MD). SP29 was synthesized as its amide on a peptide synthesizer

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

Table I. *Amino acid sequences and consensus motif of synthetic peptides SP2 and SP29 used in the present study*

The actual or putative target Ser residues are shown in bold italics. Amino acids are designated using the standard single-letter code, and their positions are numbered relative to the actual or putative phosphorylatable Ser, which is set at position 0. Presently known positive-recognition elements, identified for SP2, are underlined and the consensus motif is given. B, Basic; Hy, hydrophobic; X, any amino acid.

Synthetic Peptide	Amino Acid Sequence												
			−6		−4		−2		0		+2		+4
	residue position												
SP2	G	R	R	J	R	R	I	S	***S***	V	E	J	J
SP29	–	A	R	M	R	R	G	V	***S***	C	H	G	R
Consensus motif	–	–	B	Hy	X	B	X	X	***S***	X	X	–	–

(Synergy 432A, Perkin-Elmer) according to the manufacturer's instructions and stored at −20°C.

Partial Purification of SPS and Kinase Activities by Fast-Protein Liquid Chromatography Fractionation

SPS and SPSk activities were partially purified from extracts of illuminated control, Man-treated, and osmotically stressed leaves (usually 10–20 g fresh weight) by PEG fractionation and chromatography on Resource-Q resins (Pharmacia) following the procedures of McMichael et al. (1993). Crude extracts were prepared as described above and fractionated by the gradual addition of a 50% (w/v) solution of PEG-8000 (Sigma) to a concentration of 3% (w/v). After the sample was stirred for 10 min at 4°C, it was centrifuged at 38,000*g* for 10 min. The supernatant was decanted and PEG was added to a final concentration of 25% (w/v). After 15 min of stirring at 4°C, precipitated proteins were collected by centrifugation at 38,000*g* for 10 min and resolubilized in one-tenth the original volume of buffer A (50 mM Mops-NaOH, pH 7.5, 10 mM $MgCl_2$, and 2.5 mM DTT). The solution was again centrifuged at 38,000*g* for 10 min, and the clarified supernatant was applied onto a 2-mL Resource-Q column. The column was washed with 10 bed volumes of buffer A. Bound proteins were eluted with a 45-mL linear gradient from 0 to 500 mM NaCl in buffer A. One-milliliter fractions were collected and assayed for SPS and SPSk activities as outlined below.

Assay for Kinase Activity

Using Synthetic Peptide Phosphorylation

Typically, a 40-μL reaction mixture contained 0.1 mg mL^{-1} of synthetic peptide (SP2: $GRRJRRISSVEJJDKK_{NH2}$ or SP29: $ARMRRGVSCHGRFMK_{NH2}$), 4 μL of Resource-Q purified kinase fraction, and 4 μL of [γ-^{32}P]ATP (150 cpm $pmol^{-1}$) in buffer A. Reactions were started by the addition of ATP. Following a 10-min incubation at ambient temperature, a 20-μL aliquot of the reaction mixture was spotted onto a 2- × 2-cm square piece of P81 phosphocellulose paper. The papers were washed three times in excess 75 mM H_3PO_4 (5 min per wash). ^{32}P incorporation into the peptide was determined by liquid scintillation counting of the washed paper squares.

Using SPS-Holoenzyme Inactivation

Typically, 45-μL assays contained 20 milliunits (nmol min^{-1}) of kinase/phosphatase-free SPS (prepared as described below), 15 μL of kinase fraction, 1 mM ATP or buffer, and additives as indicated in the figure legends, in the final buffer concentration of 50 mM Mops-NaOH, pH 7.5, 10 mM $MgCl_2$, and 2.5 mM DTT. The final mixture was adjusted to contain 2 nM okadaic acid. The samples were incubated for 30 min at 25°C and then assayed for SPS activity under selective (limiting) and nonselective (V_{max}) conditions (Huber et al., 1989). Apparent SPSk activity was expressed as the ATP-dependent modification (activation or inactivation) in SPS activity.

Assay for SPS Activity

Crude extracts, Resource-Q, and antibody-purified SPS fractions obtained from control and osmotically stressed leaves were assayed for SPS activity for 10 min using selective and nonselective conditions (Huber et al., 1989). Background samples were killed with equivalent volumes of 30% (w/v) KOH at assay initiation. Anthrone reagent was used for color development, which was measured at 620 nm.

Preparation of Kinase/Phosphatase/Protease-Free SPS

A MAb-based method was routinely used to isolate enzymatically active SPS for use in phosphorylation and activation/inactivation experiments. SPS from crude extracts and Resource-Q fractions were immunopurified using the spinach-leaf SPS-specific MAbs described by Walker and Huber (1989), with Immunoprecipitin (Gibco-BRL) as the precipitating agent. The final Immunoprecipitin-MAb-SPS complex obtained was collected by centrifugation at 5000*g* for 3 min at 4°C. The immune complex was washed five times with buffer A containing 200 mM NaCl, 0.1% (v/v) Triton X-100, and 25 mM NaF, and finally with buffer A containing 25 mM NaF. The bound SPS was free of interconverting enzymes and retained an activation state similar to that of the free enzyme in solution.

Size Determination of Kinase Activities with Fractogel TSK HW55 (S)

Fractions of kinase activity (typically 2–3 fractions) from Resource-Q separations were pooled and precipitated by the dropwise addition of saturated NH_4SO_4 to 50% (w/v) saturation at 4°C. Precipitated proteins were collected by centrifugation at 38,000*g* for 10 min at 4°C, and resolubilized in a minimal volume of buffer A. A Fractogel TSK (HW55 S) column (1.6 × 60 cm; Merck) was equilibrated with buffer A containing 100 mM NaCl. Kinase samples were applied to the column and separation was performed by ascending chromatography with the same buffer (flow rate, 1 mL min^{-1}; 2-mL fractions). Kinase activity was assayed with synthetic peptides using the procedures outlined above.

In Vitro SPS-Phosphorylation Experiments

Immunoprecipitated SPS was prepared as described above and phosphorylated using Resource-Q purified SPSk activities and [γ-^{32}P]ATP (radioisotopic specific activity, 150 cpm $pmol^{-1}$) using the procedures described for the assay of kinase activity. Phosphorylation of the enzyme was determined by separation on SDS-PAGE gels (Laemmli, 1970). Gels were stained with Coomassie blue and exposed against Kodak X-Omat film at −80°C. SPS bands were excised and ^{32}P incorporation was determined by liquid scintillation counting.

In Vivo SPS-Phosphorylation Experiments

Spinach leaves (typically 7–12 g fresh weight) were fed 0.75 mCi of [^{32}P]Pi in 50 μM carrier potassium phosphate in

the dark as described by Huber and Huber (1990; 1992). Following the initial [^{32}P]Pi uptake leaves were transferred to 0.4 M sorbitol for 4 h (osmotic-stress treatment). Control leaves remained in water for the same duration. Leaves were frozen in liquid nitrogen at the end of the experimental period and stored at −80°C.

Phosphopeptide Mapping by TLE/TLC Analysis

^{32}P-labeled SPS from osmotically stressed and control leaves was immunoprecipitated and resolved by SDS-PAGE as described previously (Huber and Huber, 1990). Following electrotransfer to PVDF membrane, the strips containing the 120-kD SPS subunit were excised and incubated with 0.5% (w/v) PVP-40 in 100 mM acetic acid for 1 h at 37°C. The membranes were washed five times with water and the SPS was digested with trypsin (sequencing grade and L-[tosylamido-2-phenyl] ethyl chloromethyl ketone-treated, Worthington Biochemicals, Freehold, NJ) in 50 mM NH_4HCO_3 and 10 mM β-mercaptoethanol. Trypsin was added at time zero and again after 5 h, and digestion was carried out overnight at 37°C. The digestion solution was removed, the PVDF strips were rinsed with water, and the two solutions were combined and lyophilized. The ^{32}P-phosphopeptides were then dissolved in 400 μL of TLE buffer (pyridine:acetic acid:water, 1:10:89, v/v, pH 3.5) and again lyophilized. Finally, samples were dissolved in a minimal volume of TLE buffer, applied to cellulose TLC plates (13255, Kodak), and resolved by electrophoresis at 1000 V for 1 to 1.5 h, followed by chromatography in the second dimension (pyridine:butanol:acetic acid: water, 15:10:3:12, v/v) for 10 cm or until the solvent front was 2 cm from the top of the plate. Autoradiography of the plates was performed for appropriate lengths of time with Kodak X-Omat film and intensifying screens at −80°C. For radioactivity determinations, spots were scraped into vials containing scintillation cocktail (Scintiverse, Fisher Scientific) and counted for radioactivity.

RESULTS AND DISCUSSION

The Osmotic-Stress Activation of SPS Persists during Immunopurification

As discussed in the introduction, in previously reported osmotic-stress studies with spinach SPS, the possibility that the observed increases in the activation state of SPS may have been caused by unidentified cofactor(s) was not considered. SPS from Man-treated, dark-treated control, and dark-treated osmotically stressed spinach leaves was purified to electrophoretic homogeneity by immunoprecipitation using the MAb protocol described in "Materials and Methods." Immobilized SPS, bound to the MAb and Immunoprecipitin, was enzymatically active, and the activation state of the immobilized enzyme was essentially the same as that of the soluble enzyme in the original extract. Thus, the osmotic-stress activation of SPS was maintained during this one-step purification (Fig. 1A), which is consistent with the hypothesis that posttranslational modification of SPS occurs with osmotic-stress treatment.

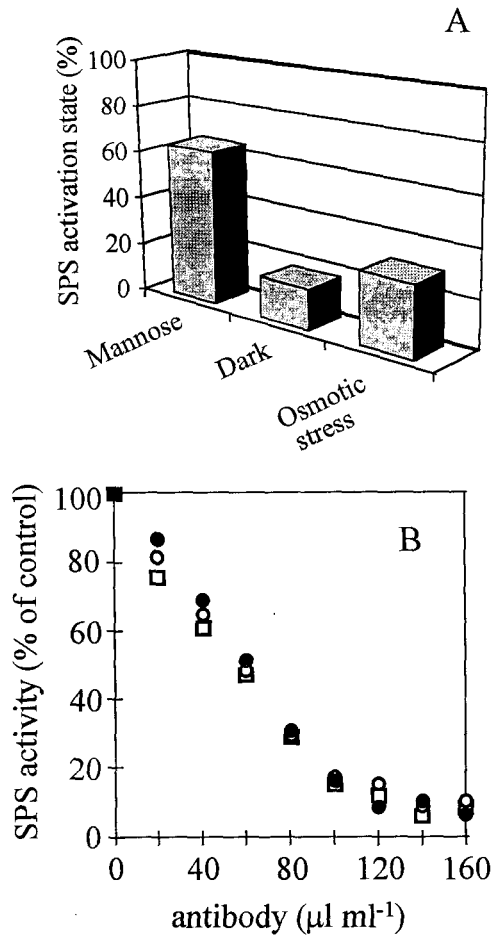

Figure 1. Osmotic-stress activation of SPS in the dark involves covalent modification of existing SPS protein. A, Osmotic-stress activation of SPS persists during immunopurification of the enzyme with monoclonal antibodies. B, Immunotitration of SPS from crude spinach extracts prepared from Man-treated (□), dark-treated (○), and osmotically stressed (●) spinach leaves.

The possibility that different isoforms of SPS may be expressed as a result of the osmotic-stress treatments was also addressed with the use of anti-spinach-SPS MAbs. SDS-PAGE analysis of antibody purified SPS from osmotically stressed and control leaves showed no differences in subunit size. In all cases, only a single subunit of approximately 120 kD was observed (data not shown). The volume of antibody required for complete precipitation of enzyme activity can be used as a relative measure of the amount of enzyme protein in the extract (e.g. see Betts and Mayer [1977]). Using desalted crude extracts from Man-treated, dark-treated control, and dark-treated osmotically stressed leaves, the activity of SPS remaining in the supernatant was assessed after the addition of increasing amounts of MAb plus Immunoprecipitin. The immunoprecipitation profiles were identical for the three treatments, suggesting that the amount of SPS enzyme protein was not increased by the Man or osmotic-stress treatments (Fig. 1B).

In summary, SPS from osmotically stressed leaves has a higher activation state compared with controls, and the increase is stable with purification, with no requirement for

any other factor(s) in vitro. It is important that the activation does not appear to involve an increase in SPS enzyme protein. Collectively, these results support the hypothesis that the osmotic-stress activation of spinach SPS is due to covalent modification of the enzyme.

Protein Kinase Activities from Osmotically Stressed Leaves Targeting Spinach SPS and SP2

Resource-Q fractionation was used to resolve protein kinase activities from osmotically stressed spinach leaves that phosphorylate the SP2 synthetic peptide (GRRJR-$RISS_{158}$-VEJJDKK$_{NH2}$; see Table I) and also inactivate dephospho-Ser-158-SPS (obtained from Man-fed leaves). The SP2 peptide kinase assays revealed three major regions of kinase activity (designated peaks I, IV, and III, listed in order of elution) that recognized the regulatory motif (Fig. 2A). The motif is present in only two regions of the SPS sequence, around Ser-158 and Ser-424. The predominant ATP-dependent inactivation of SPS (by phosphorylation of Ser-158) was carried out by the kinase activity that eluted in the region of peak III (McMichael et al., 1993). The inability of peak I to inactivate SPS (Fig. 2A) is in marked contrast to an earlier report from McMichael et al. (1995a) and cannot be readily explained at the present time. In

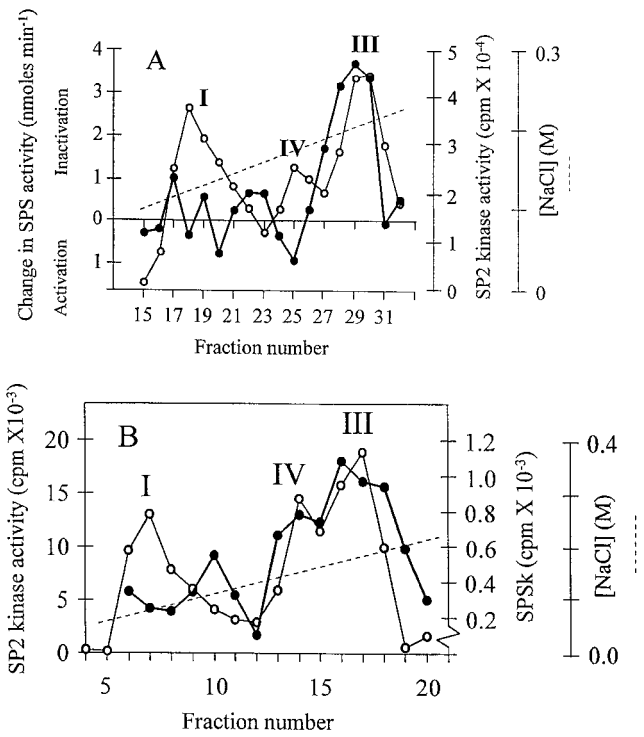

Figure 2. Resolution of protein kinases by Resource-Q ion-exchange chromatography that phosphorylates SPS holoenzyme and SPS-derived peptides. A, Comparison of spinach leaf SP2-kinase activities with ATP-dependent SPS inactivation. B, In vitro phosphorylation of dephospho-Ser-158-SPS with protein kinases resolved on Resource-Q. Incorporation of radioactivity into dephospho-Ser-158-SPS (–●–) was determined by liquid scintillation counting of excised bands. For direct comparison, SP2 kinase activity (–○–) determined with the filter-binding assay is shown.

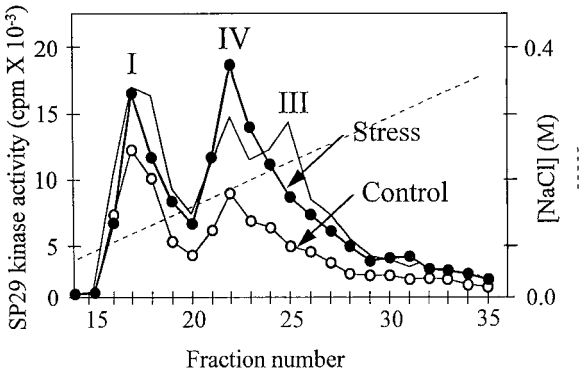

Figure 3. Osmotic-stress activation of a protein kinase that phosphorylates Ser-424 in a synthetic SPS peptide kinase. Proteins from osmotically stressed (–●–) and control (–○–) leaves are shown. For reference, the SP2 kinase activity is also shown (no data points).

other experiments using soluble SPS rather than the immobilized enzyme as in Figure 2A, we have also been unable to demonstrate phosphorylation of SPS catalyzed by the peak I kinase (data not shown). Even though the peak I kinase can phosphorylate the SP2 peptide it cannot phosphorylate Ser-158 in the holoenzyme.

It is essential that phosphorylation of a protein by transfer of ^{32}P from [γ-^{32}P]ATP is demonstrated before it can be concluded that the protein may serve as a substrate for the kinase in vivo. In particular, since the peptide kinase activity termed peak IV has not been detected previously in kinase preparations from spinach, it was necessary to determine whether peak IV targeted the SPS enzyme. To this end, purified (dephosphorylated) SPS was used as a substrate for the peptide kinase activities detected with the SP2 synthetic peptide. Phosphorylation of the SPS enzyme was greatest in the region of peak III kinase activity, but significant phosphorylation of the holoenzyme was also catalyzed by the peak IV kinase (Fig. 2B).

Osmotic-Stress Activation of a Protein Kinase That Phosphorylates Ser-424 in a Synthetic SPS Peptide

As discussed in the introduction, several lines of evidence suggest that Ser-424 may be specifically phosphorylated in SPS under osmotic-stress conditions. To test this hypothesis, SP29 was prepared. The SP29 and SP2 synthetic peptides were tested as substrates for the kinase activities isolated from control and osmotically stressed spinach leaves (Fig. 3). The SP29 synthetic peptide was recognized by two major kinase activities isolated from spinach leaf tissue; it was readily phosphorylated by peaks I and IV (Fig. 3). Importantly, peak IV kinase activity with SP29 as a substrate was significantly increased as a result of osmotic-stress treatment of spinach leaves (Fig. 3). Although the two synthetic peptides tested as substrates for the kinase activities from Resource-Q separations contain the same motif (B-Hy-X-B-X_2-Ser; see the introduction), peak III kinase activity (the predominant inactivating kinase) did not phosphorylate the SP29 peptide (Fig. 3). It is feasible that, in vivo, the increase in peak IV activity as a result of osmotic stress leads to increased phosphorylation

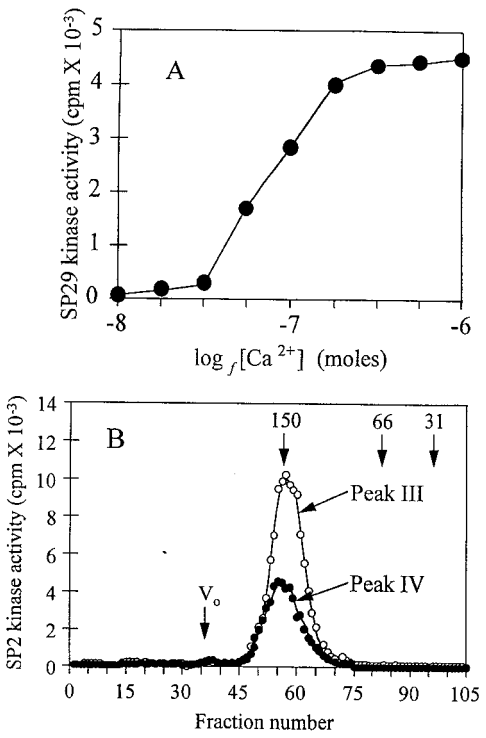

Figure 4. Characterization of the partially purified peak IV protein kinase. A, Effect of free [Ca^{2+}] on peak IV kinase activity. B, Size-exclusion chromatography of peak III and IV peptide kinase activities. Partially purified kinase III (–○–) and kinase IV (–●–) activities are shown. The void volume and elution of standard proteins are indicated by vertical arrows: alcohol dehydrogenase (150 kD), BSA (66 kD), carbonic anhydrase (31 kD), and Cyt *c* (12.4 kD).

of Ser-424, which could explain, at least in part, the increases in SPS phosphorylation and activation state reported by Huber et al. (1993).

Only recently have reports of induction of protein kinase activities as a result of environmental stress emerged (for review, see Hardie [1994]). Holappa and Walker-Simmons (1995) reported increased accumulation of transcript for an ABA-responsive protein kinase (PKABA1) in wheat with osmotic, cold, and salt stress. Speculation on the function of stress-related protein kinases in various other higher plant systems is also emerging (e.g. see Mizoguchi et al. [1996]). However, at present, there is relatively little information on the physiological roles of stress-induced kinases in plants (Battey et al., 1992; Pestenácz and Erdei, 1996). The peak IV protein kinase activity represents the first reported SPS protein kinase that shows modification in activity with environmental stress.

Properties of Peak IV Protein Kinase

The Ca^{2+} dependence of the peak IV kinase was investigated in experiments utilizing the SP29 synthetic peptide and EGTA/Ca^{2+} buffer systems. In contrast to the previously reported major SPS (inactivating) protein kinase activity (SPSk III), which is Ca^{2+} independent (McMichael et al., 1995a), the new kinase activity was found to be strictly Ca^{2+} dependent (Fig. 4A). The peak IV kinase is likely to have a high-affinity Ca^{2+}-binding domain that renders the kinase sensitive to submicromolar concentrations of Ca^{2+}. It is not presently known whether it has any further similarities to other reported Ca^{2+}-dependent protein kinases.

The native size of the Ca^{2+}-dependent peak IV activity was determined by size-exclusion chromatography on Fractogel TSK HW55. The peak III and IV protein kinases had similar apparent molecular masses (approximately 150 kD; Fig. 4B). Since protease inhibitors were routinely included, and the two kinases have similar molecular masses, proteolytic degradation, which is known to occur with some protein kinases (Roberts and Harmon, 1992), is unlikely to account for the occurrence of the peak IV activity in the present kinase preparations.

ATP-Dependent Activation of Phospho-Ser-158-SPS by Peak IV Kinase in Vitro

Preliminary experiments with phospho-Ser-158-SPS (from dark-treated leaves) and a broad kinase preparation (i.e. protein precipitated between 5 and 20% PEG) from osmotically stressed leaves indicated that an ATP-dependent SPS activation was detectable in vitro with selective assays (data not presented). We wanted to determine whether a distinct protein kinase activity could account for the ATP-dependent activation of SPS. As was shown in Figure 2, A and B, the peak IV kinase activity phosphorylated dephospho-Ser-158-SPS, but did not cause inactivation. Further experiments were performed to determine whether the peak IV kinase from osmotically stressed spinach leaves catalyzed the ATP-dependent activation of phospho-Ser-158-SPS. As shown in Figure 5, there was an ATP-dependent increase in SPS activity (selective assay) that was coincident with the Ca^{2+}-dependent peak IV kinase activity (Fig. 5).

Evidence for in Vivo Phosphorylation of Ser-424 in Spinach-Leaf SPS

As shown in Figure 3, the SP29 peptide is a good substrate for the osmotic-stress-activated peak IV kinase (Fig.

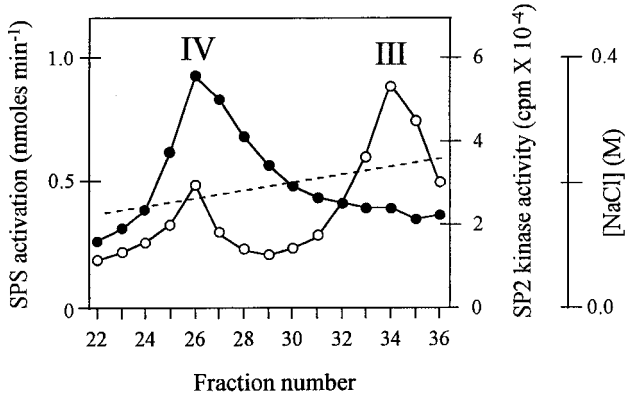

Figure 5. ATP-dependent activation of SPS activity by the peak IV kinase. The ATP-dependent increase in SPS activity (–●–), determined with the selective assay, and SP2 synthetic-peptide (–○–) phosphorylation from filter-binding assays are presented.

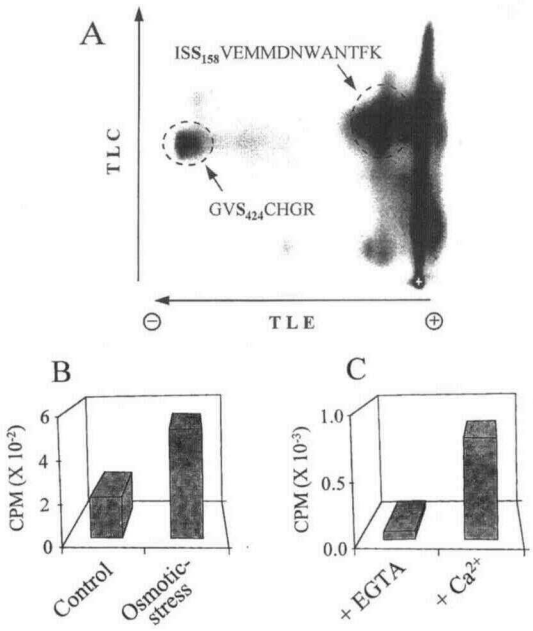

Figure 6. Phosphorylation of SPS-Ser-424 in vivo and in vitro. A, Evidence for phosphorylation of SPS-Ser-424 in vivo. Two-dimensional TLC/TLE analysis of ^{32}P-labeled tryptic phosphopeptides derived from SPS in relation to ^{32}P-phospho-SP29. The position of the single spot obtained from the tryptic ^{32}P-SP29 synthetic peptide is indicated by the dashed circle. B, Increased in vivo phosphorylation of SPS-Ser-424 with osmotic stress. C, Ca^{2+}dependence of SPS-Ser-424 phosphorylation in vitro.

3). It is not known, however, whether Ser-424 is phosphorylated in vivo, and if so, whether there is an increase in the level of phosphorylation with osmotic stress. Excised spinach leaves were fed [^{32}P]Pi and SPS immunopurified using anti-spinach SPS monoclonal antibodies. Tryptic digests were prepared from SDS-PAGE-purified SPS isolated from control and osmotically stressed spinach leaves. In accordance with the reports of Huber et al. (1993), the level of total ^{32}P incorporation (from [^{32}P]Pi) into SPS was 20 to 40% higher in the osmotic-stress treatments relative to the controls (data not presented). However, it was necessary to determine the level of ^{32}P incorporation into specific phosphopeptides.

Tryptic digestion of SPS yields a complex mixture of peptides (McMichael et al., 1993). If Ser-424 was phosphorylated, the tryptic ^{32}P-phosphopeptide would be a small, highly charged peptide that would be predicted to migrate rapidly toward the cathode. Following isolation of [^{32}P]SPS from osmotically stressed leaves, tryptic peptides were resolved by TLE/TLC analysis (Fig. 6A). The standard was trypsin-digested ^{32}P-phospho-SP29, which gave a single spot by TLE/TLC analysis that corresponded in both electrophoretic mobility and R_F value to the fast-moving spot resolved from digests of osmotically stressed spinach leaf SPS (Fig. 6A). Levels of radioactivity recovered from this spot were determined by liquid scintillation counting and compared for incorporation of radioactivity from osmotically stressed and control leaves. The level of radioactivity incorporated into the tryptic peptide containing Ser-424 increased almost 3-fold in the osmotic-stress treatment relative to control leaves (Fig. 6B).

Ca^{2+} Dependence of SPS-Ser-424 Phosphorylation in Vitro

Phosphorylation of the SP29 synthetic peptide by the peak IV kinase showed a dependence on submicromolar concentrations of free Ca^{2+} (Fig. 4A). It was of interest to determine whether the phosphorylation of Ser-424 on the SPS holoenzyme might also show a strict Ca^{2+} dependence. SPS was phosphorylated in vitro using a Ca^{2+}/EGTA buffer system. Following tryptic digestion, peptides were resolved by one-dimensional TLE (1000 V for 1 h). The phosphorylation patterns obtained from the separation suggest that phosphorylation of Ser-424 was completely Ca^{2+} dependent (Fig. 6C).

CONCLUSIONS

Our results suggest that with osmotic stress there is an increase in the phosphorylation of Ser-424 in spinach leaf SPS. Identification of this site is based on identical movement of the high-mobility peptide and the tryptic ^{32}P-phosphopeptide derived from SP29 (Fig. 6). Given that no other portion of the SPS sequence could provide a tryptic peptide with the same characteristics, we conclude that Ser-424 is phosphorylated to a greater extent in osmotically stressed leaves relative to unstressed controls. A 150-kD, Ca^{2+}-dependent protein kinase was identified that readily phosphorylated a synthetic peptide containing Ser-424 and catalyzed the ATP-dependent activation of phospho-Ser-158-SPS. We postulate that phosphorylation of Ser-424 may partially antagonize the inhibitory effect of phosphorylation of Ser-158 (in the selective assay), and that this could be the basis for the osmotic-stress activation of SPS.

The putative osmotic-stress phosphorylation site is conserved among a number of higher plant species (Table II). It is attractive to speculate that osmotic-stress activation of SPS by protein phosphorylation may be a widespread phenomenon among higher plants. In sugar beet leaves, osmotic stress caused a doubling of the SPS activation state, whereas SPS transcript levels remained constant (concomitant with a 3-fold increase in Suc and a 90% decrease in starch content; Harn and Daie, 1992). We speculate that the phosphorylation of Ser-414 in sugar beet SPS may be responsible for the activation observed. In spinach the putative osmotic-stress phosphorylation site shares the B-Hy-X-B-X_2-Ser motif (see the introduction and Table II) of the major regulatory phosphorylation site involved in light/dark modulation (i.e. Ser-158). Therefore, it is not immediately obvious why the alternate site would not be phosphorylated even in control leaves. The sequence surrounding Ser-424 in spinach may contain negative elements that prevent phosphorylation by the peak III kinase. Conceivably, the conserved Cys residue at P+1 and/or the conserved Arg residue at P+4 could be responsible. Studies are under way to characterize further the substrate-specificity of peak III and IV kinases with respect to both positive and negative recognition elements. The results

Table II. *Conservation of the putative osmotic-stress SPS phosphorylation site and surrounding residues in various higher plant species*

The putative target Ser residues are shown in bold italics. Amino acids are designated using the standard single-letter code, and their positions are numbered relative to the putative phosphorylatable Ser, which is set at position 0. Conserved residues are indicated. B, Basic; X, any amino acid.

Species	Residues	Amino Acid Sequence											Reference
		−6		−4		−2		0		+2		+4	
		residue position											
Spinach	418–428	R	M	R	R	G	V	***S***	C	H	G	R	Klein et al. (1993)
Sugar beet	408–418	R	M	K	R	G	V	***S***	C	Y	G	R	Hesse et al. (1995)
Potato	409–419	R	I	K	R	N	V	***S***	C	Y	G	R	Sonnewald and Basner (1993)
Rice	418–428	R	A	R	R	G	V	***S***	C	H	G	R	Valdez-Alarcón et al. (1996)
Maize	418–428	R	A	R	R	G	V	***S***	C	H	G	R	Worrell et al. (1991)
Citrus	410–420	R	I	K	R	N	V	***S***	C	Y	G	K	Komatsu et al. (1996)
Conserved motif		R	X	B	R	X	V	*S*	C	X	G	R	

obtained could yield insights about other stress responses in plants.

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