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Phosphorylation of Serine-15 of Maize Leaf Sucrose Synthase[1]

Occurrence in Vivo and Possible Regulatory Significance

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Experiments were conducted to determine whether sucrose synthase (SuSy) was phosphorylated in the elongation zone of maize (*Zea mays* L.) leaves. The approximately 90-kD subunit of SuSy was ^{32}P-labeled on seryl residue(s) when excised shoots were fed [^{32}P]orthophosphate. Both isoforms of SuSy (the SS1 and SS2 proteins) were phosphorylated in vivo, and tryptic peptide-mapping analysis suggested a single, similar phosphorylation site in both proteins. A combination of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and automated Edman sequencing analysis unequivocally identified the phosphorylation site in the maize SS2 protein as serine-15. This site was phosphorylated in vitro by endogenous protein kinase(s) in a strictly Ca^{2+}-dependent manner. A synthetic peptide, based on the phosphorylation site sequence, was used to identify and partially purify an endogenous Ca^{2+}-dependent protein kinase(s) from the maize leaf elongation zone and expanding spinach leaves. Phosphorylation of SuSy in vitro selectively activates the cleavage reaction by increasing the apparent affinity of the enzyme for sucrose and UDP, suggesting that phosphorylation may be of regulatory significance. Conservation of the phosphorylation site, and the sequences surrounding it, among plant species suggests that phosphorylation of SuSy may be widespread, if not universal, in plants.

SuSy (EC 2.4.1.13) catalyzes a readily reversible reaction but is thought to play a primary role in Suc breakdown in vivo (Chourey and Nelson, 1979; Cobb and Hannah, 1988; Geigenberger and Stitt, 1993; Geigenberger et al., 1993). Consequently, SuSy activity is highest in heterotrophic "sink" tissues that are importing Suc and actively growing or accumulating starch (Chourey, 1981; Ho, 1988; Sung et al., 1989, 1994). In young maize (*Zea mays* L.) leaves SuSy activity is highest in the elongation zone, which is contained within the leaf sheath (Kalt-Torres and Huber, 1987). There are two major isoforms of SuSy in higher plants: the SS1 and SS2 proteins, which are encoded in maize by the *Sh1* and *Sus1* genes, respectively (Echt and Chourey, 1985). In the maize leaf elongation zone SuSy activity consists primarily of the SS2 protein (Nguyen-Quoc et al., 1990).

Regulation of SuSy activity is thought to involve primarily control of the steady-state level of enzyme protein. In addition to cell-specific expression and developmental control, the expression of SuSy genes is known to be regulated by tissue carbohydrate status (Koch et al., 1992). In contrast, mechanisms for the regulation of the enzymatic activity of SuSy are not well understood. The enzymatic activity of the SuSy protein may be controlled in vivo by free hexose sugars (Doehlert, 1987), which could produce end-product inhibition of the cleavage reaction. The possibility of the control of SuSy by protein phosphorylation was recently introduced by the observation that the SuSy protein was ^{32}P-labeled when cultured maize suspension cells were provided with [γ-^{32}P]ATP (Shaw et al., 1994) or when excised root tips were provided with [^{32}P]Pi (Koch et al., 1995). However, in neither study was incorporation of ^{32}P into a phosphoamino acid demonstrated. Conse-

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Abbreviations: FPLC, fast protein liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; RP-HPLC, reverse-phase HPLC; SuSy, Suc synthase; TLE, thin-layer electrophoresis; TOF-MS, time-of-flight MS.

quently, the objectives of the present study were to: (a) determine whether SuSy was phosphorylated in vivo in the elongation zone of maize leaves and, if so, (b) identify the specific residue(s) phosphorylated and (c) identify the possible regulatory significance. We show that both isoforms of SuSy are phosphorylated in vivo on a single seryl residue and that the phosphorylation site is Ser-15 of the maize SS2 protein (*Sus1* product). In vitro phosphorylation of SuSy appears to be of regulatory significance, because the kinetic properties of the enzyme are affected. These results are noteworthy because they identify a previously unrecognized mechanism that may function in the regulation of a key enzyme of carbohydrate utilization in plants.

MATERIALS AND METHODS

[γ-^{32}P]ATP (3000 Ci mmol^{-1}) and [^{32}P]Pi (carrier-free) were obtained from New England Nuclear[3]; microcystin-London Resin White was obtained from Calbiochem. All other chemicals were obtained from Sigma. Synthetic peptides SP1 through SP4 were synthesized as amides on a Synergy 432A peptide synthesizer (Perkin-Elmer, Applied Biosystems Division) by the solid-phase peptide synthesis process using traditional 9-fluorenylmethyloxycarbonyl chemistry.

Maize (*Zea mays* L.) and spinach (*Spinacea oleracea* L. cv Bloomsdale) plants were grown in a soil mixture in a greenhouse and fertilized twice weekly with a modified Hoagland solution. Maize genotypes included wild type (cv Pioneer 3864) and two mutant genotypes, lacking either the *Sh1*-encoded SS1 protein (Chourey and Nelson, 1976) or the *Sus1*-encoded SS2 protein (Chourey et al., 1988).

Enzyme Extraction and SuSy Assays

Tissue that was obtained from either mature leaves or the elongation zone, as indicated, was frozen in liquid nitrogen and stored at −80°C prior to assay. Frozen tissue was homogenized in extraction buffer (1 g fresh weight/4 mL) containing 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, and 0.1% (v/v) Triton X-100 (Sigma). The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 10,000*g* for 1 min. Clarified extracts were desalted by centrifugal filtration on 4-mL columns of Sephadex G-25 (Fisher Scientific/Sigma) into a buffer containing 50 mM Mops-NaOH (pH 7.5), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM DTT. SuSy activity was assayed immediately after desalting or after the extracts were supplemented with 0.5 μM microcystin-London Resin White (control) or 1 mM Mg·ATP plus 0.5 μM microcystin-London Resin White and preincubated at 25°C for 15 min.

SuSy was assayed in the cleavage direction using a fixed-time assay followed by the enzymatic determination of UDP-Glc. The reaction mixture for the SuSy assay contained 50 mM Mops-NaOH (pH 7.5), 1.8 mM $MgCl_2$, 2.5 mM DTT, 2 to 50 mM Suc, 0.05 to 0.5 mM UDP, and 30 μL of desalted leaf extract in a total volume of 80 μL. Reactions initiated by the addition of extract were run at 25°C for 3 to 10 min and were terminated by immersion of tubes in a 95°C water bath for 2 min. Blanks were routinely run with the same reaction mixture but were heat inactivated without prior incubation at 25°C. It was verified that product formation was linear with respect to time and amount of extract added to the assay. The UDP-Glc formed was determined using UDP-Glc dehydrogenase as described by Zrenner et al. (1995).

SuSy was assayed in the synthetic direction using a fixed-time assay followed by the colorimetric determination of Suc. Reaction mixtures contained 50 mM Mops-NaOH (pH 7.5), 1 to 10 mM UDP-Glc, 2 to 10 mM Fru, 10 μL of extract, and other additions as indicated, in a total volume of 75 μL. After 3 to 10 min at 25°C, reactions were terminated by the addition of 70 μL of 30% (w/v) KOH and immersion of tubes in a boiling water bath for 10 min. After the samples were cooled, 1 mL of 0.15% (w/v) anthrone in H_2SO_4 was added, the tubes were incubated at 40°C for 20 min, and the A_{620} was recorded.

Phosphorylation of SuSy in Vivo and in Vitro

Shoots of approximately 3-week-old seedlings were cut at the soil line 2 to 3 h after the start of the photoperiod, recut under water, and placed in tubes containing 1 mL of degassed water, 10 μM Pi, and 0.5 mCi of [^{32}P]Pi (carrier-free). The shoots were kept in the light (150 μmol m^{-2} s^{-1}) for 2 h and thereafter were in darkness (25°C constant temperature). At the times indicated, the leaf elongation zone (approximately 5 cm of the enclosed leaf base; approximately 0.5 g fresh weight) was harvested directly into liquid nitrogen. The tissue was extracted, and the SuSy protein was purified by immunoprecipitation with monoclonal antibodies (Chourey et al., 1991) followed by SDS-PAGE. The amount of SuSy protein was estimated based on the specific activity of 15 units mg^{-1} enzyme protein or based on the observation that the elongation zone of maize leaves contains approximately 0.2 mg SuSy protein g^{-1} fresh weight (Nguyen-Quoc et al., 1990). For some experiments radioactivity that was associated with the approximately 90-kD subunit of SuSy was determined by liquid scintillation counting, and in other experiments the SuSy monomer was transferred to a membrane prior to phosphoamino acid analysis or peptide mapping.

Phosphorylation of SuSy in vitro was studied using desalted extracts prepared from plants that had been in the dark for 20 h prior to harvest. The extracts, prepared as described above, were incubated with 1 mM [γ-^{32}P]ATP (500 cpm pmol^{-1}) and 1 μM microcystin-London Resin White in the presence of 0.1 mM Ca^{2+} or 0.1 mM Ca^{2+} plus 0.5 mM EGTA. After the extracts were incubated at 25°C for 5 or 10 min, reactions were terminated by addition of excess EDTA, and the approximately 90-kD subunit of SuSy was purified by immunoprecipitation and SDS-PAGE.

[3] 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.

Phosphoamino Acid Analysis

The approximately 90-kD subunit of SuSy was transferred electrophoretically to Immobilon-P (Millipore). Partial acid hydrolysis was performed on the membrane in 5.7 N HCl for 1 h at 110°C (Kamps and Sefton, 1989), and the released phosphoamino acids were resolved and analyzed by two-dimensional TLE and autoradiography (King et al., 1983).

Peptide Mapping

The SuSy protein, radiolabeled in vivo or in vitro, was immunopurified, electrophoresed, and transferred to Immobilon-P membrane. The membrane strip containing the SuSy polypeptide was excised, and the protein was digested at 37°C with sequencing-grade trypsin as previously described (King et al., 1983). After digestion was complete, the solution was taken to dryness, and the peptides were resolved by two-dimensional TLE/TLC. Autoradiography was performed using X-Omat AR film (Kodak) and intensifying screens at −80°C.

Partial Purification and Assay of SuSy-Kinase

The elongation zone of maize leaves (5 g) was frozen in liquid nitrogen, powdered, and homogenized in 25 mL of extraction buffer using a mortar and pestle. The extraction and all subsequent steps were conducted at 4°C. The homogenate was filtered through Miracloth and fractionated with PEG-8000. Proteins precipitating between 5 and 20% (w/v) PEG were collected by centrifugation at 38,000*g* for 15 min and resuspended in 3 mL of buffer A (50 mM Mops-NaOH [pH 7.5], 10 mM $MgCl_2$, and 2 mM DTT). After the sample was clarified by centrifugation, it was applied onto a 1-mL ResourceQ column (Pharmacia). Following washing with buffer A, bound proteins were eluted with a 35-mL linear gradient of 0 to 500 mM NaCl in buffer A at a flow rate of 1 mL min^{-1}. Peak-activity fractions were pooled and loaded onto a Fractogel TSK HW55(S) column (Merck, Darmstadt, Germany; 1.6 × 60 cm) equilibrated with buffer A containing 100 mM NaCl. The column was developed at a flow rate of 1 mL min^{-1} with the same buffer, and 2-mL fractions were collected and assayed for SuSy activity, as described above, and for peptide kinase activity using the synthetic peptides specified.

Typical peptide phosphorylation assays consisted of a 4-μL kinase fraction in 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM [γ-^{32}]ATP (500 cpm $pmol^{-1}$), and 0.1 mg/mL peptide in a total volume of 40 μL. After 10 min at room temperature, 20-μL aliquots were spotted onto Whatman P81 phosphocellulose paper squares (2 × 2 cm) and washed in 75 mM H_3PO_4 to remove unincorporated ATP. After four washes of 5 min each, radioactivity bound to the paper was measured by liquid scintillation counting. For the Ca dependence of SuSy-kinase, the assay mixtures contained 5 mM EGTA and various concentrations of $CaCl_2$. Free [Ca^{2+}] was calculated using a computer program (BASIC) based on a program (FORTRAN) reported by Perrin and Sayce (1967).

MS

All MALDI mass spectra were obtained on a Voyager Elite (Perspective Biosystems, Farmingham, MA) time-of-flight mass spectrometer, used in the linear mode, equipped with a nitrogen laser (337 nm, 3-ns pulse). The accelerating voltage in the ion source was 26 kV. Data were acquired with a transient recorder with 2-ns resolution. The matrix used in this work was α-cyano-4-hydroxycinnamic acid dissolved in water:acetonitrile (1:1, v/v) to give a saturated solution at room temperature. To prepare the sample, 1 μL of the peptide solution was added to 1 μL of the matrix solution and applied to a stainless steel sample plate. The mixture was then allowed to air dry before being introduced into the mass spectrometer. Each spectrum was produced by accumulating data from 100 to 256 laser shots. Time-to-mass conversion was achieved by external or internal calibration using bradykinin ($[M+H]^+$ at *m/z* 1061.2) and insulin ($[M+H]^+$ at *m/z* 5734.6).

The computer program MSU MassMap (Liao et al., 1994) was used to calculate the average masses of possible peptide and possible phosphopeptide fragments from the protein and the *m/z* value of the mass spectral peak for the corresponding $[M+H]^+$ ion.

Edman Degradation

SuSy was ^{32}P-labeled in vivo, and the approximately 90-kD subunit was digested with chymotrypsin as described above. The resultant peptide mixture was applied to an Ultremex 5 C_{18} preparative RP-HPLC column (10 × 250 mm; Phenomenex, Torrance, CA) and eluted using a linear gradient of 0 to 70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 2.5 mL min^{-1}. Fractions (0.5 mL) were collected and checked for radioactivity by Cerenkov counting; a single radioactive peak was obtained (Fig. 6). The radioactive phosphopeptide fraction was further fractionated using a microbore RP-HPLC system (Michrom BioResources, Ringwood, NJ) with a 9 to 86% (v/v) gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid and a flow rate of 50 μL min^{-1} on a Reliasil C_{18} column (5 μm, 300 Å, 1 × 150 mm, Michrom BioResources). Fourteen fractions, which gave strong A_{214} peaks, were collected manually. The HPLC fraction containing a pure peptide corresponding to *m/z* 1706 was subjected to N-terminal sequencing by automated Edman degradation using a 494A protein sequencer (Applied Biosystems).

RESULTS

Suc Synthase Is Phosphorylated in Situ on Seryl Residues

Preliminary studies were conducted to determine whether SuSy was phosphorylated in situ in the elongation zone of maize leaves. Shoots were excised at the soil line early in the photoperiod and provided with [^{32}P]Pi via the transpiration stream. The excised shoots were kept in the light for the first 2 h and thereafter remained in the dark. The approximately 90-kD SuSy subunit was readily labeled with ^{32}P and reached a maximum level of incorporation within about 2 h (Fig. 1). Incorporation of radiolabel re-

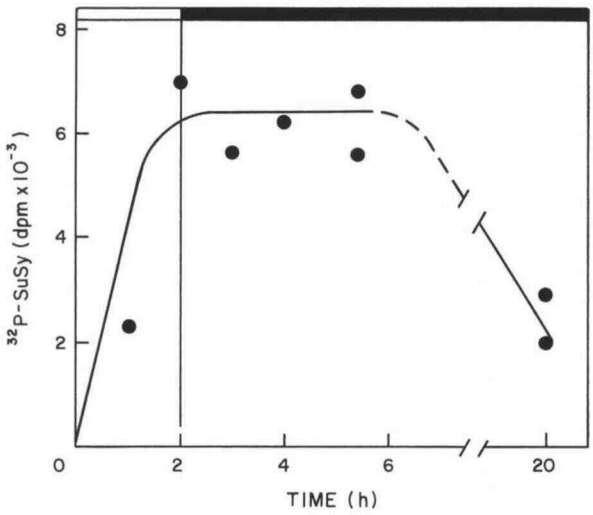

Figure 1. Time course of ^{32}P labeling of SuSy in the elongation zone of maize leaves. Each point represents the ^{32}P labeling of approximately 50 μg of SuSy protein. Each sample was from a different plant, and results from two separate experiments were combined. See "Materials and Methods" for details.

mained relatively constant when shoots were darkened for at least 4 h, but ^{32}P-labeling was sharply reduced after 20 h of darkness. There was no appreciable decrease in SuSy protein after the extended dark treatment (data not shown). The results suggested that SuSy was phosphorylated in situ in a reaction that was not light-dependent per se, but radiolabeling was apparently reduced after extended periods of darkness.

Source leaf tissue from the ^{32}P-labeled shoots used in the experiment presented in Figure 1 was also harvested and analyzed for labeling of SuSy. Mature maize leaves contain a low activity of SuSy (Kalt-Torres and Huber, 1987; Nguyen-Quoc et al., 1990), which is probably confined to the vascular tissues (Geigenberger et al., 1993; Nolte and Koch, 1993). The SuSy in source leaves was also ^{32}P-labeled (data not shown).

Phosphoamino acid analysis of the elongation zone of shoots harvested in the light or dark revealed that label in SuSy was exclusively associated with P-Ser (Fig. 2). Thus, for the first time, to our knowledge, incorporation of ^{32}P into the approximately 90-kD subunit of SuSy was demonstrated to involve a traditional phosphorylation event, and this eliminates the possibility of other explanations for ^{32}P incorporation such as adenylation or the formation of a stable phosphoenzyme intermediate.

SuSy Is Phosphorylated in Vitro by a Ca-Dependent Protein Kinase

Incubation of a desalted extract prepared from the elongation zone of maize plants subjected to 20 h of darkness with [γ-^{32}P]ATP and 0.1 mM $CaCl_2$ resulted in the time-dependent labeling of the approximately 90-kD subunit of SuSy in vitro (Fig. 3). When the incubation mixture contained 0.5 mM EGTA, phosphorylation of SuSy was essen-

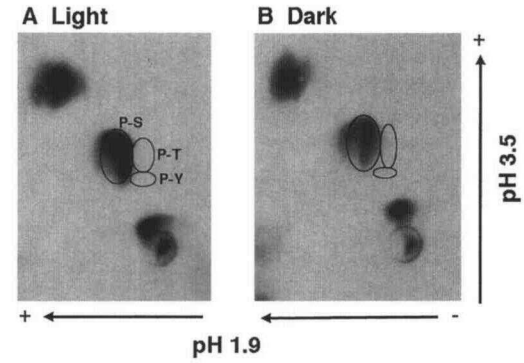

Figure 2. Phosphorylation of SuSy in situ exclusively on seryl residues in the elongation zone of light- and dark-adapted maize shoots. Maize shoots were labeled with [^{32}P]Pi as described in the legend of Figure 1. After immunoprecipitation and SDS-PAGE, the ^{32}P-labeled subunit of SuSy was transferred electrophoretically to Immobilon-P and hydrolyzed in 5.7 N HCl at 110°C for 1 h. Phosphoamino acids were resolved by TLE at pH 1.9 in the first dimension and pH 3.5 in the second dimension. P-S, phosphoserine; P-t, phosphothreonine; P-Y, phosphotyrosine.

tially eliminated. These results indicate that SuSy is phosphorylated in vitro by a Ca^{2+}-dependent protein kinase.

Identification of the Phosphorylation Site

Peptide-mapping analysis of ^{32}P-labeled SuSy was performed to obtain an estimate of the number of potential phosphorylation sites. SuSy was ^{32}P-labeled either in vivo (Fig. 4A) or in vitro (Fig. 4B) prior to digestion with trypsin and resolution of ^{32}P-peptides by two-dimensional TLE/TLC. In both cases there was a single major ^{32}P-phosphopeptide, suggesting that there is a single phosphorylation site and that the same site can be phosphorylated either in vivo or in vitro.

The ^{32}P-labeled tryptic peptide derived from the major phosphorylation site of SuSy was relatively mobile in both the electrophoretic and chromatographic dimensions, suggesting that the phosphopeptide was highly charged and probably contained several hydrophobic residues. In addition to containing at least one seryl residue, the peptide may possess a basic residue at the P-3 position (relative to the seryl residue at position 0) and a hydrophobic residue

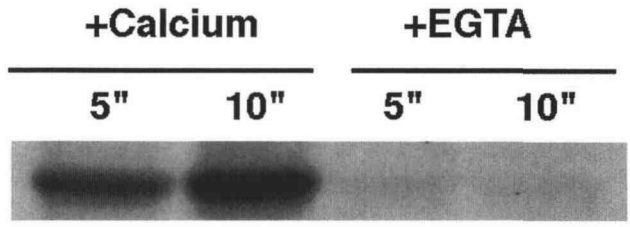

Figure 3. Phosphorylation of SuSy in desalted crude leaf extracts is Ca^{2+}-dependent. The elongation zone was harvested from plants after 20 h of darkness. A crude extract was prepared and desalted, and an aliquot (containing approximately 20 μg of SuSy protein) was incubated with 1 mM [γ-^{32}P]ATP in the presence of 0.1 mM Ca^{2+} or 0.5 mM EGTA. At 5 and 10 min, reactions were terminated by the addition of excess EDTA, and the approximately 90-kD subunit of SuSy was purified by immunoprecipitation and SDS-PAGE.

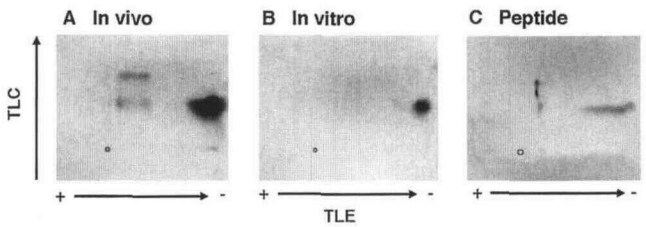

Figure 4. Tryptic peptide map of ^{32}P-labeled SuSy and synthetic peptide SP1. SuSy was labeled in vivo with [^{32}P]Pi (as in Fig. 1) or in vitro with [γ-^{32}P]ATP (as in Fig. 3). The synthetic peptide SP1 was labeled in vitro with [γ-^{32}P]ATP and partially purified SuSy-kinase. The ^{32}P-SuSy protein (approximately 25 μg of protein) and the ^{32}P-SP1 peptide (approximately 20 μg) were digested with trypsin, and the released peptides were resolved by TLE in the first dimension and TLC in the second dimension. In each panel the origin is in the lower left corner and is marked with a small box. A, SuSy labeled in vivo with [^{32}P]Pi; B, SuSy labeled in vitro with [γ-^{32}P]ATP; C, synthetic peptide SP1 labeled in vitro with [γ-^{32}P]ATP and partially purified SuSy-kinase.

at P-5 by analogy to the substrate specificity of the Ca^{2+}-dependent protein kinase that phosphorylates and inactivates NADH-nitrate reductase (Bachmann et al., 1996). Inspection of the deduced sequence of the maize *Sus1* gene product for tryptic peptides indicated that there were only a few seryl residues contained within tryptic sequences that met the previously mentioned possible criteria, namely Ser-11, Ser-15, and Ser-385. Synthetic peptides were made to portions of the polypeptide corresponding to residues 6 to 21 (designated synthetic peptide SP1, encompassing the first two possibilities) and residues 378 to 393 (designated SP2, encompassing the third possibility):

SP1: GDRVLSRLHSVRERIGK

SP2: GIVRKWISRFEVWPYLKK

The sequence of the synthetic peptides corresponds to that of the native protein, with the exception of the C-terminal Lys residue(s) that was added to facilitate the P81 phosphocellulose filter-binding assay for peptide phosphorylation.

The synthetic peptides were tested as possible substrates for endogenous protein kinases in extracts of the maize leaf elongation zone, which were resolved by anion-exchange chromatography on FPLC-ResourceQ. As shown in Figure 5, the synthetic peptide based on the sequence surrounding Ser-385 (SP2) was not phosphorylated, whereas the synthetic peptide based on the Ser-11 and Ser-15 sequences (SP1) was a good substrate for an endogenous protein kinase(s). Because the SP1 peptide contains two seryl residues, two separate peptides (SP3 and SP4) were made; each contained only a single seryl residue, by replacement with Ala residues (underlined) as shown below:

11 15

Native sequence: GDRVLSRLHSVRERIG

SP3: GDRVLSRLHAVKK

SP4: VLARLHSVRERIKK

Figure 5 shows that only SP4 was a substrate for the protein kinase(s), suggesting that the phosphorylated residue in native SuSy is Ser-15 and not Ser-11. It is important to note that the tryptic peptide map derived from ^{32}P-labeled SP1 had a mobility identical with the phosphopeptide derived from the SuSy holoenzyme phosphorylated in vivo or in vitro (Fig. 4). Collectively, these results strongly suggest that Ser-15 is the phosphorylated residue in the approximately 90-kD subunit of SuSy.

MALDI-TOF-MS was used to provide direct confirmation of the phosphorylation site. In these experiments SuSy was ^{32}P-labeled in vivo, immunopurified, and digested with either trypsin or chymotrypsin. The peptide mixtures were subjected to RP-HPLC, and fractions were collected and analyzed for radioactivity by Cerenkov counting. Only a single peak of radioactivity was resolved from a tryptic digest (data not shown). When the ^{32}P-labeled tryptic peptide fraction was subjected to MALDI-TOF-MS analysis, a peak at *m/z* 691.8 was observed (data not shown), which is consistent with the expected $[M+H]^+$ ion (*m/z* 691.7) of the tryptic phosphopeptide containing Ser-15, LHS(P)VR. No other phosphopeptide with a similar mass can be derived from the deduced sequence of the enzyme. Therefore, these results also suggest that Ser-15 is the phosphorylated residue. However, small peptides are detected in a region of the spectrum that is complicated by a high background of matrix-related peaks. For this reason, confirmatory MALDI-MS analysis after dephosphorylation was not possible, and digestion with chymotrypsin was pursued for an additional confirmation of the phospho-Ser-15 assignment.

As shown in Figure 6, digestion of ^{32}P-labeled SuSy with

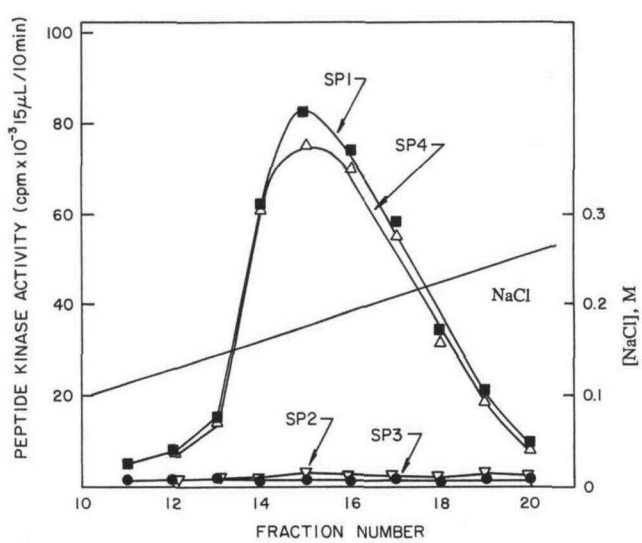

Figure 5. Phosphorylation of synthetic peptides based on the sequence surrounding Ser-15 in the SS2 form of SuSy. An extract was prepared from the elongation zone of maize leaves and concentrated by fractionation with 5 to 20% (w/w) PEG. The protein mixture was applied to an FPLC-ResourceQ column, and the bound proteins were eluted with a linear gradient of NaCl. Fractions were assayed for SuSy activity, and peptide kinase activity was as described in "Materials and Methods." SP1, GDRVLSRLHSVRERIGK; SP2, GIVRKWISRFEVWPYLKK; SP3, GDRVLSRLHAVKK; SP4, VLARLHSVRERIKK.

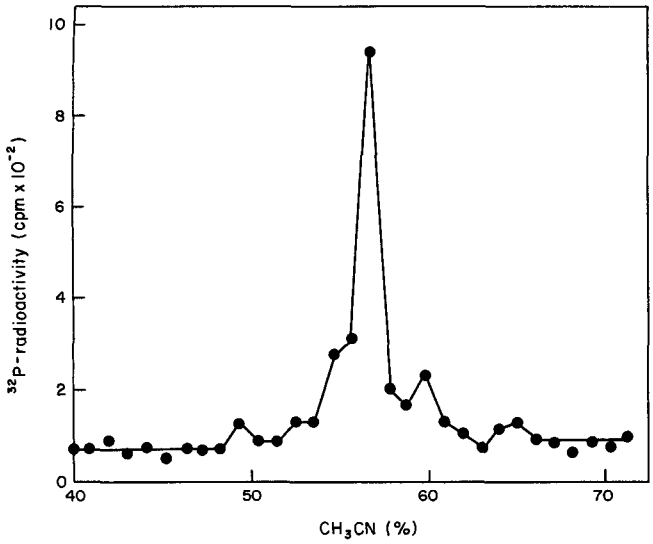

Figure 6. Resolution of a single major ^{32}P-phosphopeptide fraction by RP-HPLC of a chymotryptic digest of SuSy labeled in vivo. Maize shoots were labeled in the light for 2 h with [^{32}P]Pi, and the ^{32}P-labeled SuSy (approximately 25 μg of protein) was prepared as described in the legend of Figure 4, except that the digestion was performed with chymotrypsin, and the peptides were fractionated by reverse-phase chromatography.

chymotrypsin yielded a single peak of radioactivity, which eluted at about 56% (v/v) CH_3CN during RP-HPLC. An initial MALDI-TOF-MS analysis of the major radioactive fraction indicated that it contained several peptides (Fig. 7A). After treatment of the sample with alkaline phosphatase, the peak at *m/z* 1706.2 disappeared and a new peak at *m/z* 1625.9 appeared (Fig. 7B), i.e. a decrease of approximately 80 mass units, which would be expected for the removal of a single phosphate group (Liao et al., 1994). Based on the deduced amino acid sequence of the *Sus1* gene product and the possible chymotryptic fragments, these results are consistent only with the peptide sequence: phospho-SRLHSVRERIGDSL (corresponding to residues S11–L24). Because this sequence contains three seryl residues, Ser-11, Ser-15, or Ser-23 could be the residue phosphorylated. To solve this unequivocally, the crude phosphopeptide fraction resolved by RP-HPLC (as in Fig. 6) was further fractionated by a microbore RP-HPLC system.

By analyzing the collected fractions by MALDI-MS, we found one fraction (which contained the radioactivity) to be a pure peptide with a corresponding $[M+H]^+$ ion at *m/z* 1706 (Fig. 7C). This purified chymotrypic ^{32}P-phosphopeptide was subjected to automated Edman sequence analysis. As shown in Table I, analysis through the first six cycles produced the sequence S-R-L-H-X-V, which was consistent with the result obtained by MALDI-TOF-MS analysis. Given the sequence, a Ser signal would have been expected in the fifth cycle; instead, a small phenylthiohydantoin-dehydroalanine signal was observed, consistent with the occurrence of phospho-Ser at this position. Collectively, the results obtained by MALDI-TOF-MS analysis of tryptic and chymotryptic phosphopeptides as well as by direct Edman

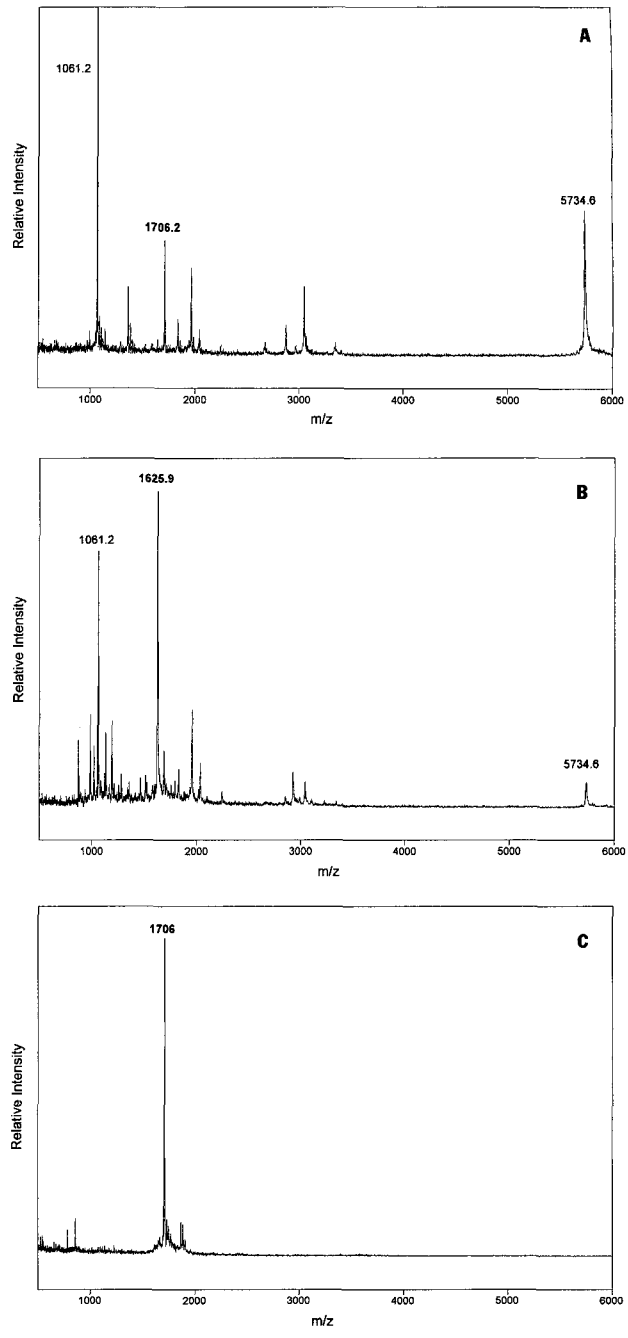

Figure 7. Mass spectra of the fraction containing the major chymotryptic ^{32}P-phosphopeptide before and after phosphatase treatment. SuSy was ^{32}P-labeled in vivo, the immunopurified protein was digested with chymotrypsin, and the major ^{32}P-labeled chymotryptic phosphopeptide fraction was resolved by RP-HPLC (as in Fig. 6). This fraction, which contained a mixture of peptides, was analyzed by MALDI-TOF-MS before and after incubation with alkaline phosphatase at 37°C for 30 min. A, Crude chymotryptic fraction before phosphatase treatment; B, crude chymotryptic fraction after phosphatase treatment; C, purified chymotryptic fraction (i.e. after sequential preparative RP-HPLC and microbore RP-HPLC), before phosphatase treatment, used for Edman degradation.

Table I. *Edman sequence analysis of the purified chymotryptic ^{32}P-phosphopeptide obtained from SuSy labeled in vivo*

The ^{32}P-peptide was purified by microbore RP-HPLC prior to microsequence analysis (see Fig. 7 for details). The first six cycles are shown, and the phenylthiohydantoin derivative obtained at each cycle is indicated.

Cycle	Residue	pmol Recovered
1	S	57.3
2	R	136.3
3	L	135.4
4	H	61.8
5	S[a]	7.4[a]
6	V	60.9

[a] Recovery of a phenylthiohydantoin-Ser peak was extremely low in cycle 5, but a phenylthiohydantoin-dehydroalanine peak was observed instead.

sequencing indicate that Ser-15 is the phosphorylated residue in maize SuSy.

If identification of the phosphorylation site as Ser-15 (in the SS2 protein) is correct, then both isozymes of SuSy should be phosphorylated and their peptide maps should be indistinguishable because the predicted ^{32}P-tryptic peptides from the two isozymes are almost identical (see Fig. 9 for their deduced N-terminal sequences). The phosphorylation of the two isozymes was tested in mutants that are deficient in each of the isozymes: *sh1Sus1* is deficient in the SS1 protein and expresses only SS2 (Chourey and Nelson, 1976), whereas *Sh1sus1* is deficient in the SS2 protein and expresses only SS1 (Chourey et al., 1988). Figure 8 shows that SuSy was phosphorylated in both mutants and that their tryptic peptide maps were indistinguishable from that of the wild type, which primarily expresses the SS2 protein in the leaf elongation zone (Nguyen-Quoc et al., 1990).

The phosphorylation site near the N terminus was also conserved among plant species. As shown in Figure 9, a homologous seryl residue was contained in the deduced sequences of SuSy from a wide variety of monocot and dicot species. Amino acid residues surrounding the target Ser were highly conserved, so that possible recognition elements (e.g. basic and hydrophobic residues N-terminal to the phosphorylated Ser) would also be present. These results suggest that phosphorylation of plant SuSy may be widespread.

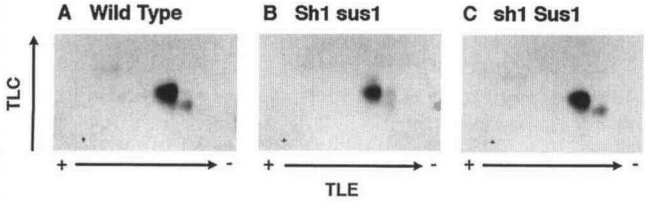

Figure 8. Two-dimensional phosphopeptide mapping of the phosphorylation site in the two isoforms of SuSy. SuSy was ^{32}P-labeled in vivo in excised shoots of maize genotypes as described in Figure 4. A, Wild-type maize, which primarily expresses the SS2 protein in the elongation zone; B, *Sh1sus1* mutant, which expresses only the SS1 protein; C, *sh1Sus1* mutant, which expresses only the SS2 protein.

Species	Protein	Residues	Deduced Sequence
Maize	SS2	8-17	R V L S R L H S V R
	SS1	3-12	A K L T R L H S L R
Rice	SS2	8-17	R V L S R L H S V R
	SS1	3-12	A K L A R L H S L R
Barley	SS2	8-17	R A L S R V H S V R
Carrot	--	4-13	P V L T R V H S L R
Arab	SS2	6-15	R M I T R V H S Q R
	--	14-23	G F L N K F L S F W
Potato	--	4-13	R V L T R V H S L R
Mung	SS1	4-13	D R L T R V H S L R
Broad	--	4-13	E R L T R V H S L R

Figure 9. Conservation of the phosphorylation site in the SuSy proteins from various plant species. Arab, *Arabidopsis thaliana L.*; Broad, broad bean (*Vicia faba* L.); Mung, mung bean (*Vigna radiata* L.).

Characterization of SuSy-Kinase

SuSy-kinase was partially purified from the elongation zone of maize leaves using phosphorylation of the synthetic peptide SP1 as the assay. During chromatography of leaf proteins on anion-exchange (FPLC-ResourceQ) a single broad peak of activity was observed (Fig. 5) that was completely inhibited by 0.5 mM EGTA (data not shown). When peak activity fractions from anion exchange were pooled and subjected to size-exclusion chromatography on a Fractogel TSK Hw55(S) column, a single peak of peptide kinase activity was detected with an estimated M_r of 65,000 (data not shown). It is important that the peptide kinase activity was strictly dependent on the presence of free Ca^{2+}, since a low concentration of EGTA strongly inhibited activity after gel filtration (data not shown). The Ca^{2+} dependence of the partially purified maize SP1-kinase was investigated in more detail using Ca^{2+}-EGTA buffers. As shown in Figure 10, kinase activity was dramatically stimulated by submicromolar concentrations of Ca^{2+}.

Initial experiments were conducted to determine whether rapidly growing leaves of a dicot species contained endogenous protein kinase(s) capable of phosphorylating peptide SP1. FPLC-ResourceQ fractionation of an extract prepared from spinach sink leaves resolved a major and minor peak of SP1-kinase activity (data not shown). Activity of both peaks was strictly dependent on free Ca^{2+} because the addition of 0.5 mM EGTA completely inhibited activity. Collectively, these results suggest that SuSy may be phosphorylated by a Ca^{2+}-dependent protein kinase in both monocot and dicot species. Furthermore, the dependence of activity on submicromolar concentrations of Ca^{2+} suggests that physiologically relevant changes in cytosolic [Ca^{2+}] may control the phosphorylation of SuSy by regulating the activity of the requisite kinase(s).

Effect of in Vitro Phosphorylation on SuSy Activity

Experiments were conducted to determine whether phosphorylation affected the activity of SuSy in either the cleavage or synthetic direction. In the initial studies the elongation zone was harvested from plants that had been in the dark for approximately 20 h, because SuSy appeared to be at least partially dephosphorylated in situ after extended darkness (Fig. 1) and extracts from dark-adapted tissue yielded SuSy that could be phosphorylated in vitro (Fig. 3). The basic protocol was to assay SuSy activity in desalted extracts (a) immediately, (b) after a 15-min preincubation at 25°C with 0.5 μM microcystin-London Resin White, or (c) after phosphorylation in vitro (15 min at 25°C in the presence of 1 mM ATP plus 0.5 μM microcystin-London Resin White). In the initial experiments assays of SuSy activity were conducted at pH 7.5 with saturating substrate concentrations to give an estimate of maximum activity. None of the preincubation conditions affected the maximum SuSy activity assayed in either the synthetic or cleavage direction (data not shown). However, when SuSy was assayed in the cleavage direction with rate-limiting substrate concentrations, activity was enhanced after phosphorylation in vitro. Consequently, kinetic analysis was conducted to determine whether the affinity for either one or both substrates was affected. As shown in Table II, preincubation of extracts with ATP reduced the K_m(UDP) approximately 5-fold and the K_m(Suc) about 2.5-fold; as expected, calculated V_{max} activities were unaffected. The synthetic activity of SuSy was also assayed in vitro with rate-limiting substrate concentrations (2 mM Fru plus 2 mM UDP-Glc), but no changes in activity as a result of preincubation (with or without ATP) were detected (data not shown). These results suggest that phosphorylation of SuSy in vitro activates the cleavage reaction without affecting the synthetic activity.

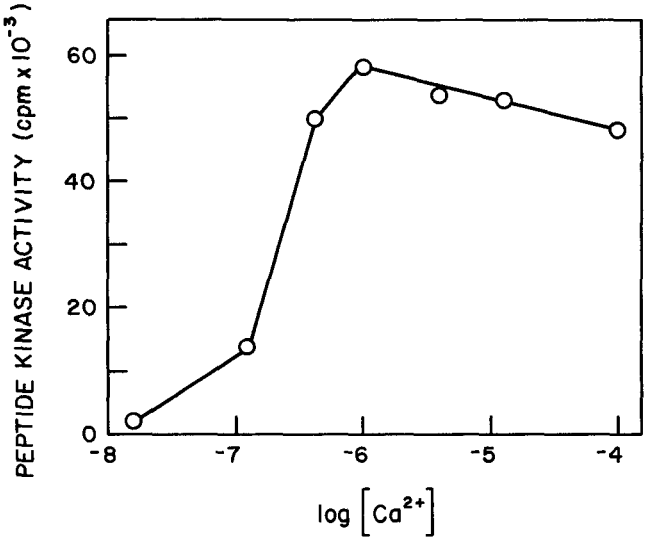

Figure 10. Ca^{2+} dependence of partially purified maize leaf SP1-kinase. The enzyme was partially purified from the elongation zone of maize leaves by sequential PEG fractionation, FPLC-ResourceQ chromatography, and gel filtration. For details, see "Materials and Methods."

Table II. *Phosphorylation of SuSy in vitro activates the cleavage reaction by increasing apparent affinities for substrates*

Parameter	Preincubation Conditions[a]	
	−ATP	+ATP
K_m(UDP) (mM)[b]	0.35 ± 0.03	0.07 ± 0.07
K_m(Suc) (mM)[c]	32.0 ± 5.1	12.3 ± 2.3
V_{max}[d]	24.7 ± 3.4	22.9 ± 1.9

[a] The leaf elongation zone was harvested from maize plants after 20 h of darkness. Extracts were prepared, desalted, and supplemented with 0.5 μM microcystin-London Resin White ± 1 mM ATP. Preincubation was at 25°C for 15 min. Assays were then performed in 50 mM Mops (pH 7.5), 1.8 mM $MgCl_2$, 2.5 mM DTT, and substrates as indicated. [b] Measured with 5 mM Suc. [c] Measured with 1 mM UDP. [d] μmol Suc cleaved g^{-1} fresh weight h^{-1}.

After preincubation at 25°C with ATP, extracts could be desalted to remove unreacted ATP, and the increased activity of SuSy (assayed in the cleavage reaction with rate-limiting substrates) was still apparent relative to control extracts preincubated without ATP (data not shown). In addition, SuSy activity in the cleavage reaction was linear with reaction times up to 15 min when assayed both before and after phosphorylation in vitro (data not shown).

DISCUSSION

The results obtained in the present study confirm the earlier reports that SuSy is ^{32}P-labeled in vivo (Shaw et al., 1994; Koch et al., 1995) and extend that information in several important ways. First, we have established that SuSy is, in fact, phosphorylated (as opposed to other modifications that could also result in ^{32}P incorporation) exclusively on seryl residues. Second, the requisite protein kinase(s) involved in phosphorylation of SuSy in vivo is probably a Ca^{2+}-dependent protein kinase in both the monocot and dicot species. Third, the major in vivo phosphorylation site was unequivocally identified as Ser-15 in the maize SS2 protein, and it was demonstrated that the phosphorylation site was conserved in both SuSy isozymes (SS1 and SS2 proteins, products of the *Sh1* and *Sus1* genes, respectively). Fourth, preliminary results suggest that phosphorylation of SuSy may be of regulatory significance, because the cleavage reaction was specifically activated after phosphorylation of the enzyme in vitro. Because the phosphorylation site appears to be conserved among isozymes and plant species (Fig. 9), our results establish the potential for control of Suc degradation by phosphorylation in a wide range of tissues and genotypes.

An endogenous protein kinase(s) that can phosphorylate a synthetic peptide based on the sequence surrounding Ser-15 has been partially purified. It is not clear how the kinase targets this specific seryl residue. It is likely that a basic residue at the P-3 position is not sufficient because peptides based on sequences surrounding Ser-11 and Ser-385, which also contain a basic residue at P-3, were not substrates for the protein kinase (Fig. 5). In addition, these latter seryl residues were not phosphorylated in the native SuSy molecule. The lack of phosphorylation at these sites in the native protein could reflect steric hindrance, but this cannot explain the lack

of phosphorylation of the corresponding peptide substrates. We postulate that efficient phosphorylation of the target seryl residue at position 15 requires a basic residue at P-3 and a hydrophobic residue at P-5, by analogy with the Ca^{2+}-dependent protein kinase that phosphorylates nitrate reductase (Bachmann et al., 1996). These requirements could explain the lack of phosphorylation of peptide SP3 (which includes Ser-11) because the hydrophobic residue is replaced by Gly. It is not clear at present why peptide SP2 (which includes Ser-385) is not phosphorylated in vitro but one possibility is that the Trp residue at P-2 is a strong, negative determinant. Studies with peptide analogs are currently under way to identify both positive and negative recognition elements for SuSy-kinase.

To our knowledge, this is the first report suggesting that phosphorylation activates SuSy. In previous studies direct effects of adenylates on SuSy activity were examined. For example, Tsai (1974) reported that nucleotides in general inhibited the cleavage activity of SuSy in developing maize endosperm. However, those experiments were performed with partially purified enzyme, which may have been devoid of the requisite kinase(s), and preincubation with ATP was not performed. Moreover, the direct effect of ATP was minimal (about 5% inhibition at 1 mM ATP in the assay) and the cleavage activity of SuSy was not measured with the rate-limiting substrate concentrations, which we have found to be essential in detecting a kinetic effect of phosphorylation.

The data reported here show that phosphorylation of SuSy in vitro clearly affects the apparent affinity for substrates when activity is assayed in the direction of Suc cleavage, although no alteration in the synthetic direction was detected. Similar unidirectional effects were noted in various physiological and genetic investigations reported previously. For example, Pontis et al. (1981) noted that the oxidized form of SuSy catalyzed Suc synthesis almost exclusively, whereas the reduced form preferentially catalyzed the cleavage reaction. Partial digestion of SuSy with trypsin generates a truncated form of the enzyme that can synthesize, but not catabolize, Suc (Pontis and Wolosiuk, 1972). Various proteins from wheat inhibit the cleavage reaction without affecting the synthetic reaction (Pontis and Salerno, 1982), and several glycosides selectively inhibit the cleavage reaction (Wolosiuk and Pontis, 1974). Furthermore, from genetic studies, Chourey and Nelson (1979) reported that Suc cleavage activity was elevated 30% in a heterozygote containing two mutant *sh1* alleles, whereas activity in the synthetic direction was equal to the average of the two mutants. Hence, results from a number of independent physiological, genetic, and biochemical investigations point to differential effects on either the synthetic or cleavage reactions of SuSy. It is intriguing that phosphorylation may also have differential effects on the synthesis and cleavage reactions of SuSy, and this aspect is being studied further.

Another important aspect is to establish whether the phosphorylation status and kinetic properties of SuSy change in vivo and, if so, in response to which stimuli/factors. Thus far our studies have been confined to the elongation zone of maize leaves harvested from plants after 20 h of darkness because earlier experiments indicated that SuSy was at least partially dephosphorylated in situ after extended dark adaptation (Fig. 1). It will be important in the future to study the enzyme in leaf extracts that have been prepared under a variety of conditions. In addition, although the requisite protein kinase(s) has been partially purified and characterized with respect to Ca^{2+} dependence (Fig. 10), nothing is known about the possible regulation of the kinase. It will be important to identify the requisite protein phosphatase(s) necessary to dephosphorylate/inactivate the enzyme. Last, in view of the recent observation that SuSy can be associated with the plasma membrane in cotton fibers (Amor et al., 1995) and in maize (S.J. Carlson and P.S. Chourey, unpublished data), it will be important to determine whether the membrane interaction is modified in any way by phosphorylation of the enzyme.

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LITERATURE CITED

Amor Y, Haigler CH, Johnson S, Wainscott M, Delmer DP (1995) A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. Proc Natl Acad Sci USA **92:** 9353–9357

Bachmann M, Shiraishi N, Campbell WH, Yoo B-C, Harmon AC, Huber SC (1996) Identification of the major regulatory phosphorylation site as Ser-543 in spinach leaf nitrate reductase and its phosphorylation by a Ca^{2+}-dependent protein kinase in vitro. Plant Cell **8:** 505–517

Chourey PS (1981) Genetic control of sucrose synthase in maize endosperm. Mol Gen Genet **184:** 372–376

Chourey PS, DeRobertis GA, Still PE (1988) Altered tissue specificity of the revertant *shrunken* allele upon *dissociation (Ds)* excision is associated with loss of expression and molecular rearrangement of the corresponding non-allelic isozyme locus in maize. Mol Gen Genet **214:** 300–306

Chourey PS, Nelson OE (1976) The enzymatic deficiency conditioned by the *shrunken-1* mutation in maize. Biochem Genet **14:** 1041–1055

Chourey PS, Nelson OE (1979) Interallelic complementation at the *sh* locus in maize at the enzyme level. Genetics **91:** 317–325

Chourey PS, Taliercio EW, Kane EJ (1991) Tissue-specific expression and anaerobically induced posttranscriptional modulation of sucrose synthase genes in *Sorghum bicolor* M. Plant Physiol **96:** 485–490

Cobb BG, Hannah LC (1988) Shrunken-1 encoded sucrose synthase is not required for sucrose synthesis in the maize endosperm. Plant Physiol **88:** 1219–1221

Doehlert DC (1987) Substrate inhibition of maize endosperm sucrose synthase by fructose and its interaction with glucose inhibition. Plant Sci **52:** 153–157

Echt CS, Chourey PS (1985) A comparison of two sucrose synthetase isozymes from normal and *shrunken-1* maize. Plant Physiol **79:** 530–536

Geigenberger P, Langenberger S, Wilke I, Heineke D, Heldt HW, Stitt M (1993) Sucrose is metabolized by sucrose synthase and glycolysis within the phloem of *Ricinus communis* L. seedlings. Planta **190:** 446–452

Geigenberger P, Stitt M (1993) Sucrose synthase catalyzes a readily reversible reaction in vivo in developing potato tubers and other plant tissues. Planta **189:** 329–339

Ho LC (1988) Metabolism and compartmentation of imported sugar in sink organs in relation to sink strength. Annu Rev Plant Physiol **39:** 355–378

Kalt-Torres W, Huber SC (1987) Diurnal changes in maize leaf photosynthesis. III. Leaf elongation rate in relation to carbohydrates and activities of sucrose metabolizing enzymes in elongating leaf tissue. Plant Physiol **83:** 289–293

Kamps MD, Sefton BM (1989) Acid and base hydrolysis of phosphoproteins bound to Immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. Anal Biochem **176:** 22–27

King MM, Fitzgerald TJ, Carlson GM (1983) Characterization of initial autophosphorylation events in rabbit skeletal muscle phosphorylase kinase. J Biol Chem **258:** 9925–9930

Koch KE, Nolte KD, Duke ER, McCarty DR, Avigne WT (1992) Sugar levels modulate differential expression of maize sucrose synthase genes. Plant Cell **4:** 59–69

Koch KE, Xu J, Duke ER, McCarty DR, Yuan C-X, Tan B-C, Avigne WT (1995) Sucrose provides a long distance signal for coarse control of genes affecting its metabolism. *In* HG Pontis, G Salerno, E Echeverria, eds, Sucrose Metabolism, Biochemistry, Physiology and Molecular Biology, Vol 14. American Society of Plant Physiologists, Rockville, MD, pp 266–277

Liao P-C, Leykam J, Andrews PC, Gage DA, Allison J (1994) An approach to locate phosphorylation sites in a phosphoprotein: mass mapping by combining specific enzymatic degradation with matrix-assisted laser desorption/ionization mass spectrometry. Anal Biochem **219:** 9–20

Nguyen-Quoc B, Krivitzky M, Huber SC, Lecharny A (1990) Sucrose synthase in developing maize leaves. Regulation of activity by protein level during the import to export transition. Plant Physiol **94:** 516–523

Nolte KD, Koch KE (1993) Companion-cell specific localization of sucrose synthase in zones of phloem loading and unloading. Plant Physiol **101:** 899–905

Perrin DD, Sayce IG (1967) Computer calculations of equilibrium concentrations in mixtures of metal ions and complexing species. Talanta **14:** 833–842

Pontis HG, Babio JR, Salerno G (1981) Reversible unidirectional inhibition of sucrose synthase activity by disulfides. Proc Natl Acad Sci USA **78:** 6667–6669

Pontis HG, Salerno GL (1982) Inhibition of sucrose synthetase cleavage activity by protein factors. FEBS Lett **141:** 120–123

Pontis HG, Wolosiuk RA (1972) Studies on sucrose synthetase. I. Effect of trypsin on the cleavage activity. FEBS Lett **28:** 86–88

Shaw JR, Ferl RJ, Baier J, St Clair D, Carson C, McCarty DR, LC Hannah (1994) Structural features of the maize *Sus1* gene and protein. Plant Physiol **106:** 1659–1665

Sung SS, Sheih WJ, Geiger DR, Black CC Jr (1994) Growth, sucrose synthase, and invertase activities of developing *Phaseolus vulgaris* L. fruits. Plant Cell Environ **17:** 419–426

Sung SS, Xu WP, Black CC Jr (1989) Identification of actively filling sucrose sinks. Plant Physiol **89:** 1117–1121

Tsai C-Y (1974) Sucrose-UDP glucosyltransferase of *Zea mays* endosperm. Phytochemistry **13:** 885–891

Wolosiuk RA, Pontis HG (1974) The role of sucrose and sucrose synthase in carbohydrate plant metabolism. Mol Cell Biochem **4:** 115–123

Zrenner R, Salanoubat M, Willmitzer L, Sonnewald U (1995) Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). Plant J **7:** 97–107