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Antibodies That Distinguish between the Serine-158 Phospho- and Dephospho-Form of Spinach Leaf Sucrose-Phosphate Synthase[1]

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Serum antibodies were raised against a synthetic peptide corresponding to the amino acid sequence surrounding the major inactivating phosphorylation site (serine-158) of spinach (*Spinacia oleracea*) leaf sucrose-phosphate synthase (SPS). The anti-peptide antibodies precipitated highly activated SPS preferentially to ATP-inactivated SPS and interacted only weakly with the sodium dodecyl sulfate-denatured enzyme bound to a membrane. The antibodies blocked phosphorylation but not dephosphorylation of SPS. Highly activated SPS was not entirely dephosphorylated and ATP-inactivated SPS was not completely phosphorylated on serine-158, as indicated by the sensitivities of immunopurified serine-158 phospho- and dephospho-SPS to inhibition by inorganic phosphate. The anti-peptide antibodies can be used to detect changes in the phosphorylation state of serine-158, and they are useful to purify and characterize distinct kinetic forms of SPS.

SPS is a key enzyme in the pathway of Suc biosynthesis in higher plants (Stitt et al., 1987). In spinach (*Spinacia oleracea*) leaves, SPS appears to be regulated by multisite phosphorylation (Huber and Huber, 1992), and Ser^{158} has recently been identified as the major inactivating phosphorylation site (McMichael et al., 1993). Phosphorylation of SPS does not affect the apparent maximum activity (V_{max}) of SPS but enhances the sensitivity of the enzyme to inhibition by Pi and decreases the affinity for the substrate Fru-6-P and the activator Glc-6-P (limiting assay conditions) (Huber and Huber, 1990; Siegl and Stitt, 1990). Therefore, phosphorylation of SPS can be monitored as a change in the activation state, which is defined as the ratio of the apparent SPS activities under V_{max} and limiting conditions. However, the end points of this phosphorylation-dependent scale of activation and thus the correlation between activity changes and phosphorylation have not yet been defined.

The most widely used procedure for correlating changes in the activity of a phosphoenzyme with phosphorylation of a particular site is labeling with [^{32}P]Pi and peptide mapping. Although this procedure is sensitive and extremely useful, several problems are associated with it, such as incomplete isotopic equilibration between cellular phosphate pools and [^{32}P]Pi or between ATP and individual phosphorylation sites (Sefton, 1991), protein phosphatase activity, and partial cleavage of the phosphoprotein before phosphopeptide mapping (Boyle et al., 1991). Immunochemical methods may provide an attractive alternative for detecting changes in the state of phosphorylation of a protein. For example, it has been shown that antibodies to phosphorylatable, short synthetic peptides recognize the corresponding epitopes in proteins and select for either the phosphorylated or dephosphorylated form of these proteins (Czernik et al., 1991). In the present study we used this approach and produced anti-peptide antibodies that cross-reacted preferentially with highly activated SPS. The objectives were: (a) to determine whether these antibodies immunoprecipitate SPS in an activation state-dependent manner; (b) to separate phosphoserine-158-SPS from the corresponding dephosphoenzyme form; and (c) to correlate the phosphorylation state of Ser^{158} with the sensitivity of SPS to inhibition by Pi.

MATERIALS AND METHODS

Partial Purification of Enzymes, Experimental Treatments

Spinach (*Spinacia oleracea* cv Hybrid 424) was grown in soil in growth chambers as described previously (Huber et al., 1989). The following procedures were carried out essentially as described by Weiner et al. (1993): (a) preparation of desalted crude extracts; (b) partial purification of SPS, SPS kinase, and SPS phosphatase via fractionation by PEG 8000 and anion-exchange chromatography on a Mono-Q column using a Pharmacia/LKB fast protein liquid chromatography system; (c) assay of SPS with limiting substrates plus Pi (3 mM Fru-6-P, 12 mM Glc-6-P, 10 mM UDP-Glc, and 10 mM Pi) or with saturating substrates (V_{max} assay: 10 mM Fru-6-P, 40 mM Glc-6-P, and 10 mM UDP-Glc; the ratio of the two activities multiplied by 100 is termed the activation state [in percent]); (d) SPS kinase assay as the ATP-dependent inactivation of SPS (phosphorylation of SPS); and (e) SPS phosphatase-dependent activation (dephosphorylation) of SPS. ATP was determined as described by Lowry and Passoneau (1972).

Unless otherwise mentioned, the following buffer was used during purification and enzyme assays (Mops buffer):

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Abbreviations: CNBr, cyanogen bromide; SPS, sucrose-phosphate synthase; TBS, Tris-buffered saline.

50 mM Mops/NaOH, 10 mM $MgCl_2$, 2 mM benzamidine, 2 mM ε-amino-*n*-caproic acid, 0.5 mM PMSF, 5 mg/L leupeptin, and 2.5 mM DTT, pH 7.5. Enzyme preparations were supplemented with 0.5% (w/v) BSA and desalted prior to assay. Addition of BSA and protease inhibitors was essential to prevent proteolytic breakdown of the 120-kD subunit of SPS (Salvucci et al., 1990; Bruneau et al., 1991). Proteolysis resulted in the appearance of additional polypeptides in SDS-PAGE-fractionated immunoprecipitates (not shown).

Production of Peptide-Specific Antisera

A synthetic peptide (CGRMRRISSVEMMDN, amino acids 151–164 of spinach leaf SPS [McMichael et al., 1993] with an additional amino-terminal Cys) was coupled to a purified protein derivative of tuberculin (Statens Seruminstitute, Copenhagen, Denmark) with *N*-maleimido-6-aminocaproylester of 1-hydroxy-2-nitro-4-benzenesulfonic acid (Bachem, Heidelberg, Germany). The reactions were carried out as described by Aldwin and Nitecki (1987). The peptide conjugate (80–120 μg) was emulsified in incomplete Freund's adjuvant and injected intradermally into rabbits (New Zealand Whites) that had been rendered tuberculin sensitive with 6×10^5 viable units of bacillus of Calmete and Guerin vaccine (Behringwerke AG, Marburg, Germany) (Vyakarnam et al., 1981). Every 6 weeks boosts were given with about 50 μg of peptide conjugate. Blood was taken from the ear artery every 2 weeks after each injection with peptide conjugate, and antibody production was monitored by ELISA using ovalbumin-peptide conjugate or by immunoprecipitation of SPS (see below). For affinity purification, the peptide was linked to aminohexyl-Sepharose (Pharmacia) as recommended by the manufacturer, using the same cross-linking reagents as mentioned above. After antiserum was loaded on this column, peptide-specific antibodies could not be not eluted by standard methods (Harlow and Lane, 1988) without using additional denaturating conditions (see "Results and Discussion").

Antibodies to SPS total protein were generated as follows. SPS was immunoprecipitated from Mono-Q-purified enzyme preparations with anti-peptide antibodies (see below). Purified immunocomplexes (coupled to protein A beads) containing about 5 μg of SPS (assuming 150 units mg^{-1} SPS protein [Walker and Huber, 1989]) were emulsified with complete Freund's adjuvant to produce rabbit antibodies using the same immunization schedule as described above for anti-peptide antibodies. For the present study all antibodies were used as whole sera.

ELISA and Western Blotting

ELISA plates (Maxi-Sorb; Inter-Med, Nunc, Wiesbaden, Germany) were coated with 300 ng of ovalbumin-peptide conjugate (prepared as described above) per well, blocked with TBS (25 mM Tris-HCl, 135 mM NaCl, 3 mM KCl, pH 8.0) containing 3% (w/v) BSA, and incubated with primary antibodies in TBS containing 1% BSA. Goat anti-rabbit IgG-coupled peroxidase (Bio-Rad) was used as a secondary antibody. Peroxidase activity was quantified using *o*-phenylinediamine (0.6 mg/mL) and H_2O_2 (0.012%, v/v) in 200 μL of 50 mM citrate buffer, pH 5.0. The reaction was stopped with 200 μL of 1 N H_2SO_4 and A_{492} was measured.

Western blots were carried out as follows. After electrophoresis in 7.5% SDS polyacrylamide gels (Laemmli, 1970) the proteins were transferred onto Immobilon-P (Millipore) or nitrocellulose membranes (Bio-Rad) (Towbin et al., 1979). The blots were blocked in TBS containing 0.2% (v/v) Triton X-100, 0.1% (v/v) Tween-20, and 3% (w/v) skimmed milk and incubated with primary antibody for 1 to 2 h (dilutions of antisera are given in "Results and Discussion"). Goat anti-rabbit IgG-coupled alkaline phosphatase (Bio-Rad) was used at a 1:3000 dilution for 30 to 45 min to detect the primary antibody. Alkaline phosphatase activity was measured using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.

Immunoprecipitation and Phosphopeptide Mapping

To immunoprecipitate SPS, partially purified or crude enzyme preparations were incubated on ice with anti-peptide or anti-SPS total protein antisera in Mops buffer containing 0.5% BSA (dilutions of sera, SPS activities, and volumes are given in figure legends). After 1 h, protein A-Sepharose beads were added and the resulting suspension was incubated on a multi-axle rotating mixer at 4°C for 30 to 45 min. The immune complexes bound to the beads were pelleted and washed twice in TBS containing 0.1% (v/v) Triton X-100 and three times in TBS, followed by a final wash in 10 mM Tris-HCl, pH 7.5. For analysis of the immunoprecipitated proteins the beads were boiled in sample buffer and centrifuged, and the supernatant was analyzed by SDS-PAGE on 7.5% gels (Laemmli, 1970). For activity measurements the beads were resuspended in 50 μL of Mops buffer containing 0.5% BSA and preincubated at 25°C for 5 min prior to measuring SPS activity. The assay was started by adding limiting or saturating substrates (see above), followed by an incubation for 5 min with regular mixing (30-s intervals). Reactions were stopped by pelleting the beads, and the formation of Suc and Suc-6-P in the supernatant was measured as described by Weiner et al. (1993).

Phosphopeptide mapping was carried out in the following way. The SPS protein was radiolabeled in vivo (see "Results and Discussion"), immunopurified, electrophoresed, and transferred to nitrocellulose. The membrane piece containing the SPS subunit was excised and the protein was cleaved with CNBr (Luo et al., 1991). Phosphopeptides were analyzed by a two-dimensional separation as described by Boyle et al. (1991). Samples were centrifuged for 10 min at 20,000*g* to pellet insoluble material, and supernatants were spotted in 0.5-μL steps onto thin-layer cellulose plates (Merck, Darmstadt, Germany). Separation in the first dimension was carried out by electrophoresis in 7.8% acetic acid, 2.2% formic acid (pH 1.9) at 10°C and at 1000 V for 30 min with a 2117 Multiphor II electrophoresis unit (Pharmacia). Chromatography in the second dimen-

sion was carried out with *n*-butanol:pyridine:acetic acid: H_2O (75:50:15:60) for 8 h at 25°C. Cellulose plates were dried and exposed for 3 d at −75°C.

RESULTS AND DISCUSSION

Specificity of Anti-Peptide Antibodies

We raised serum antibodies against a synthetic peptide corresponding to the amino acid sequence surrounding the major inactivating phosphorylation site (Ser^{158}) of SPS from spinach leaves (the amino acid sequence is given in "Materials and Methods"). These antibodies immunoprecipitated spinach leaf SPS from partially purified (Fig. 1A) and crude enzyme preparations (not shown). However, the SDS-denatured 120-kD subunit of SPS immobilized in a Immobilon-P membrane was only weakly bound to the antibodies as indicated by the weak staining during immunodecoration (Fig. 1B). In the presence of competing peptide (the synthetic peptide used for immunization) the antibodies failed to immunoprecipitate native SPS or detect denatured and immobilized SPS. Also, SPS was not immunoprecipitated in the presence of 0.05% SDS (not shown), possibly because of a drastically reduced immunoreactivity of the region around Ser^{158} after denaturation with SDS. In an attempt to affinity purify the antibodies with Sepharose-immobilized synthetic peptide we failed to recover the anti-peptide antibodies from the affinity matrix without applying harsh or denaturating conditions (solutions containing more than 1% SDS, or pH below 2.0), probably because of their strong interaction with the synthetic peptide. Taken together the results suggest that the anti-peptide serum contains antibodies that bind strongly to SPS in its native form, specifically interacting with the sequence surrounding Ser^{158}. For comparison, we produced serum antibodies against SPS total protein. Unlike the anti-peptide antibodies, these antibodies immunoprecipitated SPS in the presence or absence of SDS and produced a strong signal during immunodecoration (Fig. 1).

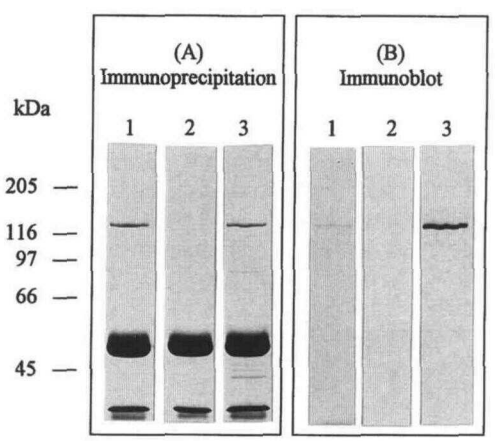

Figure 1. Immunoprecipitation and western blot analysis using different antisera. Mono-Q-purified SPS was immunoprecipitated (0.03 μmol min^{-1} on a V_{max} basis in 120 μL) and analyzed by SDS-PAGE and Coomassie blue staining (A) or subjected to SDS-PAGE (12 μL containing 0.003 μmol min^{-1} SPS), protein blotting, and immunodecoration (B). Immunoprecipitation and immunodecoration were performed with anti-peptide serum in the absence (lanes 1) or presence (lanes 2) of 10 μM of competing peptide and with anti-SPS total protein antiserum (lanes 3). The final dilutions of antisera were 1:50 during immunoprecipitation or 1:1000 during immunodecoration. The polypeptide above 116 kD is the subunit of SPS. The heavily stained polypeptides represent the IgG heavy (about 50 kD) and light (about 25 kD) chains. The minor stained polypeptides below 97 and 45 kD in lanes 3 are also serum proteins.

In addition to spinach leaf SPS, the peptide-specific antibodies immunoprecipitated two polypeptides with about 120 and 130 kD from crude enzyme extracts of potato tubers (H. Weiner, unpublished data). The presence of identical epitopes in these antigens, which are likely to represent SPS subunits, suggests phosphorylation sites with high sequence homology in these proteins. The ability of the anti-peptide antibodies to identify this particular phosphorylation site sequence was confirmed in an attempt to immunoprecipitate SPS from maize leaves. Maize and spinach leaf SPS have a lower degree of sequence homology around the phosphate acceptor of their major inactivating phosphorylation sites (McMichael et al., 1993), and, therefore, as could be expected, the anti-peptide antibodies failed to immunoprecipitate SPS from maize leaves (not shown).

Activation State-Dependent Interaction of SPS with Anti-Peptide Antibodies

Preliminary experiments using partially purified enzymes and anti-peptide serum indicated an increased recovery of SPS in immunoprecipitates after in vitro dephosphorylation and activation of SPS by spinach leaf endogenous SPS phosphatase before immunoprecipitation. Also, the immunosignal of SPS on western blots was slightly improved after this dephosphorylation (although the signal was still very weak; not shown). The interaction of the antibodies with the major phosphorylation site was obviously enhanced after dephosphorylation of this site. Thus, it was of interest to analyze the efficiency of immunoprecipitation further. SPS was highly phosphorylated or dephosphorylated and subjected to immunoprecipitation with increasing amounts of anti-peptide serum. The SPS activities remaining in the supernatant after the immunocomplexes were collected were measured to determine the efficiency of immunoprecipitation (Fig. 2). With phospho-SPS about 10 times more antiserum was necessary to precipitate 50% of the SPS activity than with dephospho-SPS, suggesting that the peptide-specific antibodies interacted preferentially with the phosphorylation site sequence in its dephosphorylated form. Given the size of the synthetic peptide used as an antigen one might expect that the anti-peptide serum contains an antibody fraction interacting with phosphorylation-independent epitopes, which are present in both SPS forms. However, it is also possible that all peptide antibodies are directed to phosphorylation-sensitive epitopes, which may or may not include Ser^{158} and which greatly reduce, but not completely abolish, their immunoreactivity upon phosphorylation of Ser^{158}.

Further evidence that the anti-peptide antibodies bound strongly and predominantly to the Ser^{158}-dephosphory-

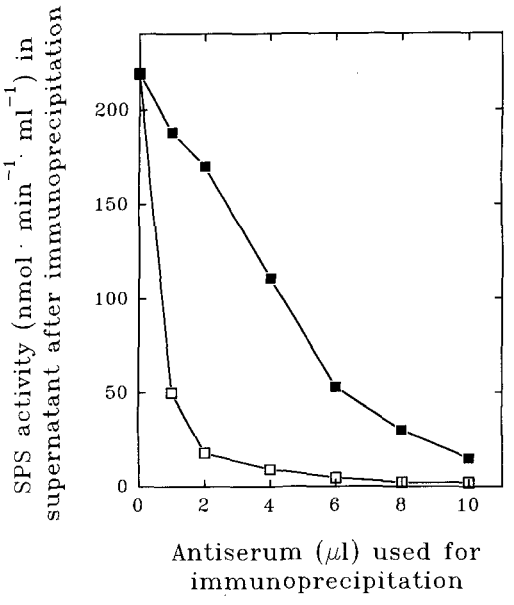

Figure 2. Preferential immunoprecipitation of dephospho-SPS. Mono-Q SPS (0.06 μmol min^{-1} on a V_{max} basis in 250 μL) was highly dephosphorylated to 74% activation state (□) or phosphorylated to 24% activation state (■) and immunoprecipitated using anti-peptide serum (for details see "Materials and Methods"). After SPS-immunocomplexes were collected the SPS activity (V_{max}) remaining in the supernatant (i.e. not immunoprecipitated) was measured. Results of one experiment are shown. A second independent experiment gave very similar results.

lated form of SPS is provided in Figure 3. Highly activated SPS and SPS kinase were incubated with ATP to phosphorylate and inactivate SPS in the presence of anti-peptide antibodies. The antibodies blocked SPS phosphorylation (Fig. 3A), possibly by preventing access of SPS kinase or ATP to the phosphorylation site. In control experiments without SPS and SPS kinase, the ATP concentration of the incubation mixture was not reduced after precipitation of the anti-peptide antibodies. Thus, it is unlikely that the anti-peptide antibodies bound ATP in a nonspecific manner, making ATP less available for the kinase reaction. Unlike SPS phosphorylation, activation of phospho-SPS by SPS phosphatase was not affected (Fig. 3B), which may indicate a less efficient binding of anti-peptide antibodies to phospho-SPS.

Analysis of the Phosphate Inhibition of SPS prior to and after Immunoprecipitation

It has been proposed that SPS exists in several distinct forms in vivo, with at least one form being phosphorylated and strongly inhibited by Pi and another form being dephosphorylated and less sensitive to Pi inhibition (Huber and Huber, 1990; Siegl and Stitt, 1990). Because the anti-peptide serum contained antibodies that distinguish between these enzyme forms (Fig. 2), we attempted to separate phosphoserine-158-SPS from the corresponding dephosphoenzyme with these antibodies. We wanted to examine the sensitivity of distinct SPS forms to inhibition by Pi to correlate phosphorylation of Ser^{158} with Pi inhibition of the SPS activity and to estimate the phosphorylation state of Ser^{158} after in vitro phosphorylation and dephosphorylation of SPS.

We examined the Pi sensitivity of SPS in the supernatant after partial immunoprecipitation of highly activated or ATP-inactivated SPS (Fig. 4). Partial immunoprecipitation denotes that a limited amount of antiserum was used to ensure predominant binding of the anti-peptide antibodies to dephospho-SPS and thereby to reduce their interaction with the phosphoenzyme. As expected, we observed a pronounced difference between the Pi sensitivities of highly phosphorylated and dephosphorylated SPS in supernatants in control experiments using preimmune serum, which failed to immunoprecipitate SPS. However, this difference in the Pi sensitivity was abolished if anti-peptide serum instead of preimmune serum was used, probably because the SPS form that was less sensitive to Pi inhibition was removed from the solution by the anti-peptide antibodies. After 70% of SPS V_{max} activity from ATP-inactivated SPS was precipitated instead of 20% (as in

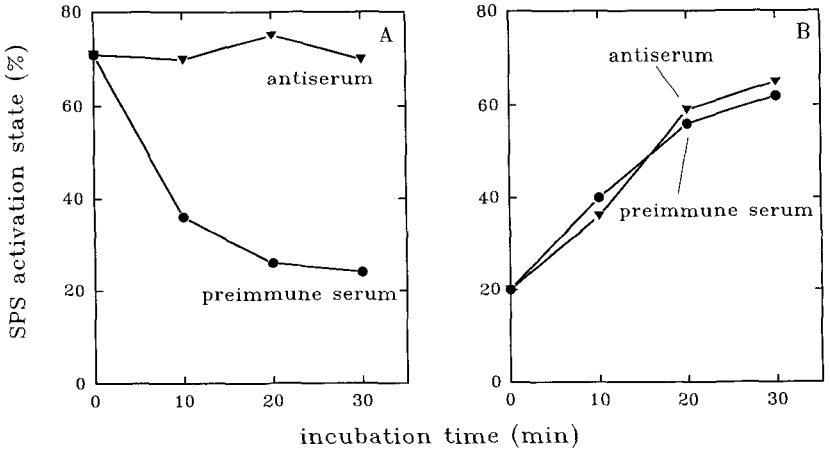

Figure 3. Anti-peptide antibodies block ATP-dependent inactivation (phosphorylation) but not activation (dephosphorylation) of SPS. A, Highly activated Mono-Q SPS (0.09 μmol min^{-1} on a V_{max} basis in 300 μL) containing the co-purifying SPS kinase was supplemented with 2 μL of anti-peptide serum or preimmune serum (i.e. serum collected before immunization of the rabbits) and incubated for 15 min prior to addition of 10 μM ATP. B, Phosphorylated SPS preparations (same V_{max} and volume as in A) were preincubated with sera as in A and supplemented with 15 μL of partially purified SPS phosphatase. SPS activation was not observed without addition of SPS phosphatase. Activation and ATP-dependent inactivation of SPS were identical with or without preimmune serum. Data are mean values of two independent experiments.

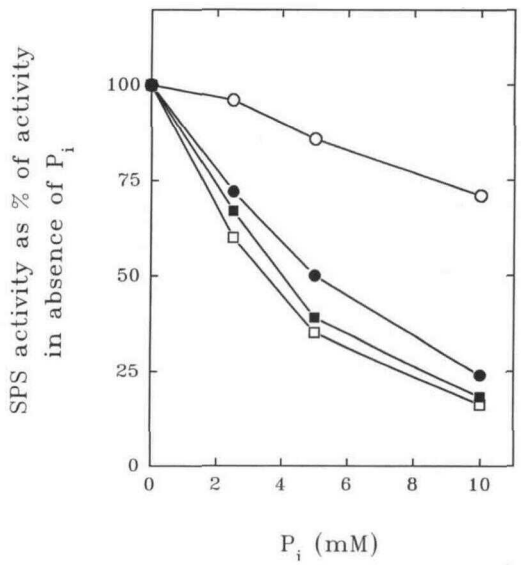

Figure 4. Phosphate inhibition of SPS. SPS was phosphorylated or dephosphorylated as described in Figure 2. The sensitivities of highly phosphorylated (●, ■) and dephosphorylated SPS (○, □) toward Pi inhibition were determined with limiting substrates in supernatants after partial immunoprecipitation with 2 μL of preimmune serum (○, ●) or antiserum (□, ■). SPS protein was not immunoprecipitated with preimmune serum. The presence of serum or anti-peptide serum had no effect on either the limiting or V_{max} activities of SPS. The results of one experiment are shown and were confirmed in a second independent experiment.

Fig. 4), the inhibition of SPS activity in the supernatant was not enhanced further. Therefore, we conclude that dephospho-SPS was completely removed by the anti-peptide antibodies and that the SPS activities in the supernatants were entirely due to the corresponding phosphoenzyme, which is strongly but not completely inhibited by 10 mM Pi. The SPS activities in these supernatants were more strongly inhibited by Pi than the activity of ATP-inactivated SPS of the control experiment (Fig. 4). This suggests that ATP-inactivated SPS still contains some dephosphoserine-158.

In contrast to the SPS activity in the supernatants, we observed only 5% inhibition of the SPS activity by 10 mM Pi in resuspended partial immunoprecipitates of highly activated SPS (not shown; for these particular experiments the amounts of antiserum were chosen to immunoprecipitate only 20% of SPS V_{max} activity to minimize interaction with phosphoserine-158-SPS). The SPS activity in these immunoprecipitates was clearly less sensitive to Pi inhibition than highly activated SPS (Fig. 4). Overall, and without further quantification, the results reinforce the notion that SPS was not completely phosphorylated or dephosphorylated on Ser^{158} after its activity was manipulated in vitro. Incomplete dephosphorylation of phosphoserine-158 prior to "back phosphorylation" with [γ-^{32}P]ATP may at least partly explain why the stoichiometry of ^{32}P incorporation per SPS subunit determined in vitro was below unity (about 0.5 mol of phosphate per mol of SPS subunit; Huber and Huber, 1992). Currently, we are examining activation of SPS to explain the mechanism that prevents SPS from reaching activation states close to 100%.

Composition of Different SPS Forms in Immunoprecipitates

Next, we examined the activation state-dependent pattern of the Coomassie blue-stained 120-kD polypeptide of SPS in immunoprecipitates. We subjected partially purified SPS preparations containing SPS with different phosphorylation states but identical activities on a V_{max} basis to a sequential immunoprecipitation, a precipitation of SPS in two steps using two different antibodies (Fig. 5). After a partial immunoprecipitation step with anti-peptide antibodies, the amounts of SPS protein recovered in the precipitates paralleled increasing activation states of SPS. To monitor the SPS protein that remained in the supernatant after this step, we performed a second immunoprecipitation using antiserum to SPS total protein. As expected, we obtained decreasing amounts of SPS protein in the second precipitates that balanced the amounts of SPS protein recovered in the first step. Hence, when appropriate amounts of anti-peptide antibodies are used to allow predominant binding of dephospho-SPS during the first immunoprecipitation, this sequential precipitation of SPS can be used to detect changes in the phosphorylation state of Ser^{158}.

Because the anti-peptide antibodies did not seem to be entirely specific for Ser^{158}-SPS but also appeared to interact with the corresponding phosphoserine enzyme (see Fig. 2), we assumed that the immunoprecipitates obtained after the first step of sequential immunoprecipitation also contained a fraction of this phospho-SPS form. The first precipitates presumably contained much more dephospho- than phospho-SPS when highly activated SPS was used. However, this assumption may not have held when ATP-inactivated SPS was used, which contained a high ratio of phosphoserine-158 to Ser^{158}. This would have favored immunoprecipitation of phosphoserine-158-SPS, which was further examined.

Highly activated Mono-Q SPS, containing the co-purifying regulatory kinase activity, was labeled with [γ-^{32}P]ATP to phosphorylate and inactivate SPS and subjected to sequential immunoprecipitation. The SPS recovered in the first immunoprecipitate contained 20% of the total SPS activity (V_{max}) and 10% of the total SPS label prior to

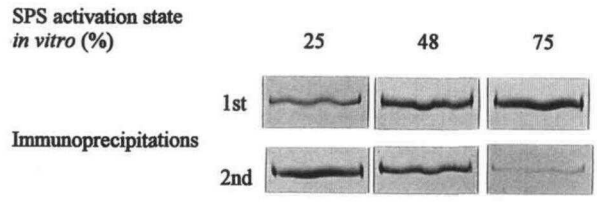

Figure 5. Sequential immunoprecipitation. Mono-Q-purified preparations with equal V_{max} (0.06 μmol min^{-1} in 250 μL) but different activation states of SPS (obtained after phosphorylation and dephosphorylation as described in Fig. 2) were subjected to immunoprecipitation using 2 μL of anti-peptide serum. SPS protein that remained in the supernatant after this step was completely removed by a second immunoprecipitation step with a different antiserum, directed against SPS total protein. Immunoprecipitates were fractionated by SDS-PAGE and stained to compare the amount of immunoprecipitated 120-kD polypeptide of SPS after each step.

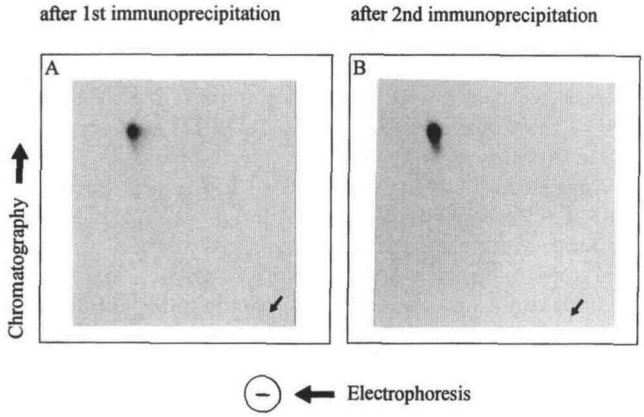

Figure 6. Autoradiogram of SPS-phosphopeptides in sequential immunoprecipitates. Mono-Q-purified fractions containing highly activated SPS and SPS kinase were incubated with 10 μM [γ-^{32}P]ATP (800 Cerenkov cpm/pmol ATP) to phosphorylate and thereby inactivate SPS to a 24% activation state. SPS was subjected to sequential immunoprecipitation as described in Figure 5. The first (A) and the second (B) immunoprecipitates were fractionated by SDS-PAGE and transferred to nitrocellulose. The 120-kD SPS proteins were excised and cleaved with CNBr. Phosphopeptides (400 or 800 Cerenkov cpm for A and B, respectively) were analyzed by two-dimensional cellulose thin-layer electrophoresis/TLC and autoradiography (the origins are denoted by arrows in the lower right corners). The results of one experiment are shown, which were confirmed in two additional independent experiments.

immunoprecipitation. This suggests that one-half of the precipitated SPS was phosphorylated on Ser^{158} if one assumes that SPS was labeled exclusively at this site. However, since Mono-Q SPS can be phosphorylated on more than one site by the co-purifying kinase activity (Huber and Huber, 1992; McMichael et al., 1993), this 10% label may also have been due to ^{32}P at a site other than Ser^{158}. Therefore, we produced two-dimensional phosphopeptide maps of SPS to compare the incorporation of label into Ser^{158} after the first (10% of total SPS label) and second (90% of total SPS label) immunoprecipitations (Fig. 6). SPS phosphopeptides from both immunoprecipitates mapped very similarly. In both maps the label appeared as one major spot. These spots co-migrated after equal amounts of SPS label from both immunoprecipitates were mixed prior to CNBr cleavage and mapping (not shown), which suggests that the two phosphopeptides are identical. SPS was obviously not labeled on more than one site in the present study, in the course of which in vitro instead of in vivo dephosphorylated SPS (McMichael et al., 1993) was used before "back-phosphorylation." It is very likely that no phosphorylation site other than Ser^{158} was dephosphorylated in the present study and, as could therefore be expected, no other site was labeled with [γ-^{32}P]ATP.

CONCLUDING REMARKS

As described in the introduction, phosphorylation of SPS is a critical factor for the biosynthesis of Suc in higher plants. In this study we established the correlation between the degree of phosphorylation of Ser^{158} and the activation state of SPS using standard assay conditions: fully phosphorylated SPS does not appear to be totally inactive and fully dephosphorylated SPS does not appear to be 100% active. Our results strongly support the original proposal of Stitt and co-workers (1988), based on a kinetics study, that SPS exists in at least two interconvertible forms that differ in substrate and effector affinities. These enzyme forms have not yet been separated by conventional procedures. Clearly, a more precise analysis of SPS function will require direct examination of distinct SPS forms. Affinity purification of different SPS forms with anti-peptide antibodies as defined in the present study would greatly facilitate this.

Conventional procedures to monitor phosphorylation of a particular site in a protein are often limited by their reliance on using tedious procedures such as labeling with ^{32}P and peptide mapping. In this study we used an alternative and more flexible method (Czernik et al., 1991) using anti-peptide antibodies directed against a phosphorylation site sequence of SPS. These antibodies bound preferentially to Ser^{158} dephospho-SPS, which provides the functional basis to detect changes in the phosphorylation state of this site. In general, synthetic peptides as antigens give rise to antibodies that cross-react well with the denatured form of the corresponding protein and may or may not recognize the native protein (Harlow and Lane, 1988). The anti-peptide antibodies described here interact preferentially with the native protein, presumably because the synthetic peptide used for immunization approximates epitopes of the native protein. This might be fortuitous or a more widespread feature of synthetic peptides corresponding to a certain group of phosphorylation site sequences and warrants further examination.

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