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Spinach Leaf Sucrose-Phosphate Synthase and Nitrate Reductase Are Phosphorylated/Inactivated by Multiple Protein Kinases in Vitro[1]

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The regulation of sucrose-phosphate synthase (SPS) and nitrate reductase (NR) activities from mature spinach (*Spinacia oleracea* L.) leaves share many similarities in vivo and in vitro. Both enzymes are light/dark modulated by processes that involve, at least in part, reversible protein phosphorylation. Experiments using desalted crude extracts show that the ATP-dependent inactivation of spinach SPS and NR is sensitive to inhibition by glucose-6-phosphate. Also, a synthetic peptide homolog of the spinach SPS phosphorylation site inhibits the ATP-dependent inactivation of both enzymes with a similar concentration dependence. We have addressed the possibility that SPS and NR are regulated by the same protein kinase by partially purifying the protein kinases involved. Three unique kinase activities can be separated by anion-exchange and size-exclusion chromatography. Each peak of activity has a different substrate specificity. By gel filtration, they have apparent molecular masses of approximately 45, 60, and 150 kD. Additionally, the activities of the two smaller kinases are dependent on micromolar concentrations of Ca^{2+}, whereas the 150-kD kinase is not. Finally, the 150-kD kinase has a subunit molecular mass of about 65 kD as determined by renaturing the kinase activity in situ following sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Nitrogen and carbon assimilation both respond to light/dark signals that act to keep these processes generally coordinated with photosynthesis. This coordination is accomplished by regulation of several key enzymatic steps in each pathway. SPS is one of the regulated enzymes in Suc synthesis (Huber et al., 1986; Stitt et al., 1987; Worrell et al., 1991), and NR is an important control point in nitrogen assimilation (Beevers and Hageman, 1969). Recent investigations have revealed that reversible phosphorylation of Ser residues is one of the mechanisms by which both enzymes are regulated (Huber et al., 1989a, 1992a; Kaiser and Spill, 1991). Upon transition of leaves from dark to light, both enzymes show a marked decrease in the phosphorylation state of specific Ser residues, whereas phosphate incorporation into these sites is increased on transition to the dark (Huber et al., 1989a, 1992a). The major regulatory phosphorylation site in spinach (*Spinacia oleracea* L.) leaf SPS has been identified as Ser^{158} (McMichael et al., 1993). These changes in phosphorylation correlate inversely with the activation state of the enzymes, which is defined for SPS as the ratio of activity measured under conditions of limiting substrates (plus the inhibitor Pi) to V_{max} activity (Huber et al., 1989a) and for NR as the ratio of the activities measured in the presence and absence of 5 mM Mg^{2+} (Huber et al., 1992a). In spinach leaves, diurnal changes in activation state of both enzymes also follow a similar pattern; activation state is low in the morning before the lights come on, increases rapidly when illumination begins, and then slowly decreases throughout the day nearly reaching dark levels by the end of the photoperiod (Huber et al., 1992b). Also, the rapid dephosphorylation and concomitant activation that occurs upon illumination for both SPS and NR can be mimicked in darkness by feeding Man to excised leaves (Huber et al., 1992b; Weiner et al., 1992).

One important difference between the inactivation of SPS and NR is that in the NR system two protein components are required to inactivate the enzyme (Spill and Kaiser, 1994; Bachmann et al., 1995; MacKintosh et al., 1995). The first component is the protein kinase that phosphorylates NR, and the second component is a protein that causes inactivation of phospho-NR in the presence of divalent cations. We have designated this protein IP (Bachmann et al., 1995). On the other hand, phosphorylation of SPS by its protein kinase is sufficient to inactivate the enzyme. Nevertheless, the strikingly similar responses of SPS and NR to environmental signals raises the possibility that the two enzymes might be phosphorylated by the same protein kinase. This hypothesis was supported by experiments with desalted crude extracts that showed that inactivation of both enzymes was inhibited by Glc-6-P, a known inhibitor of SPS-PK activity in partially purified preparations (Huber and Huber, 1990; Weiner et al., 1992), and by two synthetic peptides derived from the major regulatory phosphorylation site in spinach SPS (McMichael

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Abbreviations: IC_{50}, 50% inhibition constant; IP, inhibitor protein; Nle, norleucine; NR, NADH:nitrate reductase; NR-PK, NR-protein kinase; SPS, Suc-phosphate synthase; SPS-PK, SPS-protein kinase.

et al., 1993). We addressed the question of whether SPS and NR are regulated by the same protein kinase or by similarly regulated but distinct protein kinases by partially purifying the kinase(s) based on their ability to inactivate SPS and/or NR in an ATP-dependent manner. The results indicate that NR and SPS are phosphorylated by multiple kinases in vitro.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea* L. cv Bloomsdale) was grown in soil in growth chambers or in the greenhouse as previously described (Huber et al., 1989a). Activated (dephospho) SPS and NR were obtained from leaves harvested in the light (400 and 800 μmol m^{-2} s^{-1} PPFD for growth chamber and greenhouse plants, respectively). Leaves were harvested directly into liquid nitrogen approximately 1 h after the beginning of the 12-h photoperiod.

Partial Purification of SPS-PK and NR-PK

Frozen leaf tissue (approximately 20 g fresh weight) was ground in a chilled mortar with extraction buffer (2 mL/g) containing 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, and 0.1% (v/v) Triton X-100. The homogenates were filtered through two layers of cheesecloth and one layer of Miracloth[2] (Calbiochem). In experiments in which crude extracts were used, an aliquot of this filtrate was desalted on a Sephadex G-25 spin column equilibrated with buffer A (50 mM Mops-NaOH [pH 7.5], 10 mM $MgCl_2$, 2.5 mM DTT; Weiner et al., 1992) and used directly. Otherwise, the filtrate was fractionated by the addition of a 50% (w/v) solution of PEG-8000 (Sigma) to a final concentration of 5% (w/v). After the sample was incubated for 20 min with stirring at 4°C, the supernatant fluid was recovered after centrifugation at 38,000*g* for 15 min. The PEG concentration in the supernatant fraction was increased to 12% (w/v) with an additional aliquot of the 50% (w/v) stock solution. The protein that precipitated during the 20-min incubation at 4°C was collected by centrifugation at 38,000*g* for 15 min and resuspended in buffer A (0.5 mL/g tissue used).

The resuspended pellet was clarified at 38,000*g* for 15 min and loaded onto two 1-mL Resource Q columns (Pharmacia) connected in series and equilibrated with buffer A. After the sample was washed with buffer A to remove unbound material, bound proteins were eluted at a flow rate of 4 mL/min by a 35-mL linear gradient from 0 to 500 mM NaCl in buffer A. Fractions of 1 mL were collected and assayed for SPS-PK and NR-PK activities as described below. Appropriate fractions were pooled (as indicated, usually two to three fractions) and loaded onto a Fractogel TSK HW55 (S) column (Merck; 1.6 × 60 cm) equilibrated with buffer A containing 100 mM NaCl. The column was developed at a flow rate of 1 mL/min with the same buffer, and 2-mL fractions were collected and assayed for SPS-PK and NR-PK activities. The kinase-containing fractions were pooled and dialyzed against buffer A in a microdialysis apparatus (Pierce) for 1 h. These fractions were used immediately for inhibitor studies. At this stage of purification the kinase fractions were stable for several days at 4°C, but activity was greatly reduced or lost upon freeze (−20°C)/thawing.

Assay of SPS-PK

Typical 45-μL assays contained 20 milliunits (nmol/min) of kinase/phosphatase-free dephospho-SPS (see below), 15 μL of kinase fraction, 1 mM ATP or buffer, and additives as indicated in the text, in a final buffer concentration of 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 2.5 mM DTT. Inclusion of protein phosphatase inhibitors was not necessary (see below). The samples were incubated at 25°C for 20 min and then assayed for SPS activity under limiting conditions, as previously described (Huber et al., 1989a). In this system, apparent SPS-PK activity was expressed as the ATP-dependent decrease in SPS activity. In all cases, control reactions preincubated with or without ATP were assayed under V_{max} conditions to ensure that SPS activity remained constant. In routine column assays, the carryover of NaCl from the chromatography steps into the assay (80 mM for Resource Q and 33 mM for Fractogel TSK) produced some inhibition of SPS kinase activity (data not shown) but was not corrected for. No additional Ca^{2+} was added to the reactions; however, there appears to be sufficient Ca^{2+} in the buffers to activate the Ca^{2+}-dependent kinases.

Assay of NR-PK

Our assay of NR-PK activity is based on the Mg^{2+} sensitivity induced by the interaction of phospho-NR with IP. Typical NR-PK assays contained 10 to 12 milliunits (nmol/min) of kinase-free dephospho-NR (see below), 10 μL of kinase fraction, 10 μL of partially purified IP, 0.75 μM microcystin-LR, 0.75 mM ATP and other additions as indicated in the text in a final volume of 80 μL and buffer concentration of 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 2.5 mM DTT. Following incubation at 25°C for 15 min, the reaction mixture was assayed for NADH:NR activity in the presence of 5 mM Mg^{2+} as described previously (Huber et al., 1992a). In this system, apparent NR-PK activity was expressed as the ATP-dependent decrease in NR activity when IP was present. In all cases, control reactions preincubated with or without ATP were assayed in the absence of Mg^{2+} to ensure that NR activity remained constant. Under these conditions, the carryover of NaCl from the chromatography steps (35 mM for Resource Q and 14 mM for Fractogel TSK) had little effect on NR kinase activity (data not shown). No additional Ca^{2+} was added to the reactions; however, there appears to be sufficient Ca^{2+} in the buffers to activate the Ca^{2+}-dependent kinases.

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Preparation of Kinase/Phosphatase-Free Dephospho-SPS

A resolubilized, clarified 5 to 12% PEG fraction derived from 25 g of illuminated tissue (Weiner et al., 1992) was loaded onto a 1.6- × 10-cm column of 5′-AMP-agarose (Sigma) equilibrated in buffer A. The column was washed with buffer A until no more protein eluted. SPS was not retained on this column but SPS-PK activity was bound. The SPS-containing fractions from the flowthrough were pooled and loaded onto a 1-mL Resource Q column, which was eluted at a flow rate of 1 mL/min with a 35-mL linear gradient from 0 to 500 mM NaCl in buffer A. Fractions of 1 mL were collected and assayed for SPS activity. After desalting on Sephadex G-25 spin columns equilibrated with buffer A, the SPS was used as substrate to assay for SPS-PK activity. Following inactivation of this fraction by SPS-kinase, the ATP could be removed from the reaction mixture without any subsequent increase in activation state, indicating that no phosphatases were present in the SPS preparation (data not shown). Also, adding protein phosphatase inhibitors such as microcystin-LR and okadaic acid to the reaction mixture had no effect on SPS inactivation and thus were not used in assays utilizing SPS prepared by this method.

Preparation of Kinase-Free/IP-Free Dephospho-NR

NR was partially purified from a crude extract by batch absorption to Blue Sepharose as described by Campbell and Smarelli (1978). Briefly, Blue Sepharose (made according to the method of Bohme et al., 1972) was added to the extract at 1 g resin/10 g tissue and incubated for 45 min with stirring at 4°C. The resin was then collected on a fritted glass filter and washed with 40 mL/g resin of extraction buffer and 40 mL/g resin of buffer A. The washed resin was resuspended in buffer A and poured into a glass column. The resin was washed with 2 volumes of 0.4 M NaCl in buffer A and 2 volumes of 0.9 M NaCl in buffer A. Kinase-free NR was eluted from the resin with 4 volumes of buffer A containing 100 mM NaCl and 0.2 mM NADH. NR-containing fractions were pooled and desalted on an Econo-Pac 10DG column (Bio-Rad) equilibrated with buffer A.

In Situ Renaturation and Assay of the 150-kD Kinase following SDS-PAGE

This procedure was identical with the modification of Kameshita and Fujisawa (1989) described by Wang and Chollet (1993) except that 0.4 mg/mL of the 26-kD recombinant truncated SPS protein, corresponding to residues 50 to 243 of the spinach protein (Klein and Salvucci, 1993), was included in the gel during polymerization and a 10% polyacrylamide separating gel was used. Phosphorylation was detected by autoradiography at −70°C using Kodak X-Omat AR film with an intensifying screen.

Assay for Synthetic Peptide Phosphorylation

A 50-μL reaction mixture contained 0.1 mg/mL synthetic peptide (SP1: GRMRRISSVEMMDNWANTFK$_{NH2}$), 5 μL of kinase fraction, and 100 μM [γ-^{32}P]ATP (500 cpm/pmol) in buffer A with and without 0.2 mM EGTA. Reactions were initiated by the addition of ATP. Following a 10-min incubation at ambient temperature, 20 μL of the reaction was spotted on a 2 × 2-cm square of P81 phosphocellulose paper and immediately washed in 75 mM H_3PO_4. The filters were then washed five times with 500 mL of 75 mM H_3PO_4 for 5 min/wash. ^{32}P incorporation was determined by liquid scintillation counting.

Synthetic Peptides

Peptide SP1 was purchased from Multiple Peptide Systems (San Diego, CA), and peptide SP2 was a generous gift of Dr. Jan Kochansky (U.S. Department of Agriculture-Agricultural Research Service, Insect Neurobiology and Hormone Laboratory, Beltsville MD). Both peptides were routinely used without further purification.

RESULTS AND DISCUSSION

The strikingly similar in vivo response of SPS and NR activity to light/dark transitions and other signals raised the possibility that the inactivation of these two enzymes utilized the same protein kinase. This possibility was strengthened by in vitro experiments with crude extracts that showed that the ATP-dependent inactivation of SPS and NR was inhibited similarly by several compounds. The first compound tested was Glc-6-P, since it has been reported previously as an inhibitor of both SPS inactivation (Weiner et al., 1992) and SPS phosphorylation (Huber and Huber, 1990) in partially purified preparations. Glc-6-P inhibited SPS inactivation in desalted crude extracts as well, with an IC_{50} of approximately 12 mM (Fig. 1A, ■). The ATP-dependent inactivation of NR was also inhibited by Glc-6-P (Fig. 1A, □), and it appears to be more sensitive than SPS inactivation, having an IC_{50} of approximately 2 mM. The other inhibitor tested was a synthetic peptide homolog of the major regulatory phosphorylation site in spinach SPS (McMichael et al., 1993), which we have designated SP2 (GRNleRRISSVENleNleDKK). This peptide inhibited the ATP-dependent inactivation of SPS and NR in desalted crude extracts (Fig. 1B). Both activities were inhibited similarly with IC_{50} values in the 30- to 40-μg/mL range. Although it was expected that the peptide would have an effect on SPS inactivation, we saw the effect on NR inactivation as an indication that either SPS and NR are phosphorylated by the same protein kinase or their phosphorylation sites are very similar.

To determine whether SPS-PK and NR-PK are distinct proteins, we attempted to fractionate the enzymes activities. The protein that precipitates between 5 and 12% (w/v) PEG was separated on a Resource Q column and the resulting fractions were assayed for the ATP-dependent inactivation of SPS and IP/ATP-dependent inactivation of NR. This analysis (Fig. 2) revealed three distinct kinase activities, which have been designated peaks I, II, and III. Peak I activity recognized both SPS and NR, whereas peak II inactivated only NR and peak III inactivated only SPS. These substrate specificities are also borne out by compe-

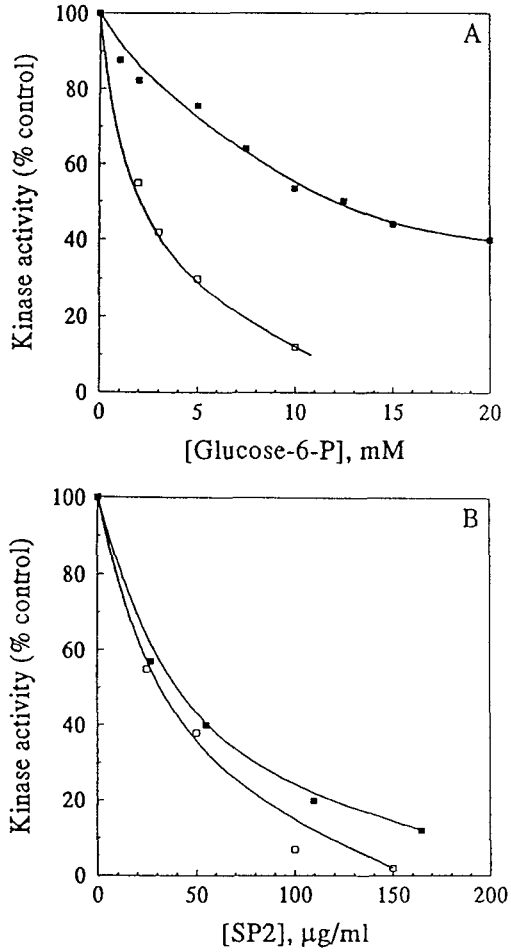

Figure 1. Inhibition of ATP-dependent inactivation of SPS and NR in desalted crude extracts. Extracts were incubated with or without 1 mM ATP and varying concentrations of potential inhibitor and then assayed for SPS or NR activity as described in "Materials and Methods" to determine the extent of ATP-dependent inactivation (apparent phosphorylation). The inhibition of the inactivation of SPS (■) and NR (□) caused by Glc-6-P (A) or a synthetic peptide homolog (SP2) of the major regulatory phosphorylation site in spinach SPS (B) are plotted.

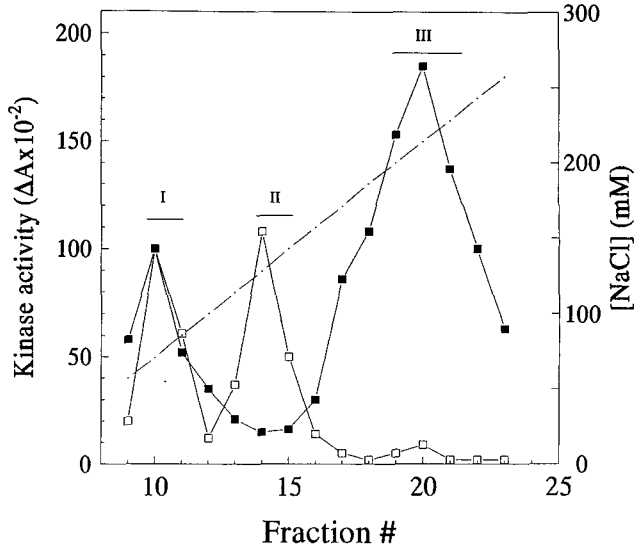

Figure 2. Anion-exchange chromatography of the 5 to 12% PEG fraction on Resource Q. See "Materials and Methods" for details. ■, ATP-dependent inactivation of SPS; □, ATP-dependent inactivation of NR; —·—, NaCl gradient. The horizontal bars indicate the fractions that were pooled for further purification.

tition experiments between the native protein substrates and a recombinant 26-kD fragment of spinach SPS (Klein and Salvucci, 1993), which contains the phosphorylation site (Table I). When peak I was assayed, NR and the 26-kD protein competed but not when peak II was assayed. Peak III did not recognize NR, but, as expected, the 26-kD protein competed with SPS. It is interesting to note that, whereas the synthetic peptide SP2 was capable of inhibiting almost all of the NR-PK activity in crude extracts (see Fig. 1B), the isolated peak II enzyme showed no affinity for SPS (Fig. 2) or the 26-kD protein (Table I). Also, SP2 was not phosphorylated by peak II (data not shown). Most likely the phosphorylation sites in SPS and NR have some similarities allowing the small peptide SP2 to bind to the peak II enzyme without being phosphorylated. The larger, more complex structure of SPS and the 26-kD protein might shield the phosphorylation site from the peak II kinase.

At this point we were still unable to say whether or not SPS and NR were regulated by the same protein kinase because peak I inactivated both enzymes, whereas peaks II and III recognized only one or the other. Since both peaks II and III co-elute with their substrate proteins (data not shown) and it has been shown that SPS and the co-eluting protein kinase (i.e. peak III) have an affinity for one another (Huber and Huber, 1990), we were concerned that the presence of multiple peaks of kinase activity could be due to some type of protein-protein interaction. This possibility was addressed by pooling each activity peak separately and analyzing them on a Fractogel TSK HW55 (S) size-exclusion column (Fig. 3). On this column, peaks I, II, and

Table I. *Substrate specificity of peaks I, II, and III*

Kinase fractions from Figure 2 were incubated with NR or SPS in the presence or absence of 40 μg/mL 26-kD recombinant SPS protein (Klein and Salvucci, 1993). Inactivation, assayed as described in "Materials and Methods," is reported as the percentage difference in substrate enzyme (SPS or NR) activity after incubation with kinase, with or without 1 mM ATP. N.D., Not determined.

Kinase	Addition	Inactivation of Substrate	
		SPS	NR
		%	
Peak I	None	22.6	20.4
	+26 kD	N.D.	6.1
Peak II	None	0.7	25.0
	+26 kD	N.D.	24.8
Peak III	None	31.2	−1.1
	+26 kD	13.2	N.D.

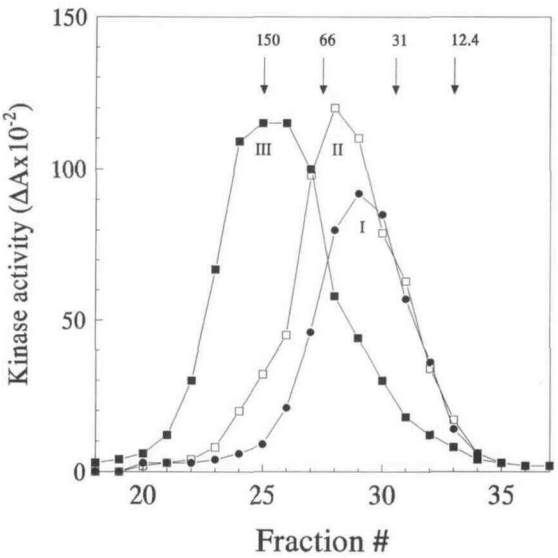

Figure 3. Size-exclusion chromatography of peak I, II, and III protein kinase activities on Fractogel TSK HW55 (S). See "Materials and Methods" and Figure 2 for details. ■, ATP-dependent inactivation of SPS by peak III; □, ATP-dependent inactivation of NR by peak II; ●, ATP-dependent inactivation of NR by peak I. Note that each profile represents a separate run on the column. Elution of protein molecular mass standards are indicated by arrows: alcohol dehydrogenase (150 kD), BSA (66 kD), carbonic anhydrase (31 kD), and Cyt *c* (12.4 kD).

III had apparent molecular masses of 45, 60, and 150 kD, respectively. This column also separated peaks II and III from their protein substrates, and subsequent analysis of the kinases on a Resource Q column showed that they both eluted at the original salt concentrations (data not shown). Therefore, interactions between the kinases and their protein substrates were not responsible for their co-elution on this anion-exchange column. This adds strength to the hypothesis that each peak of activity represents a different protein kinase with different substrate specificities. Another possibility is that the 45-kD kinase could be the catalytic subunit of peak III that was released during purification, giving rise to an activity with a broad substrate specificity. Further information about the subunit structure of peak III was obtained using in situ renaturation and assay of this protein kinase after SDS-PAGE. This technique has been used to identify the catalytic subunits of many protein kinases (for a review of this technique, see Hutchcroft et al., 1991). With our preparations it was necessary to include the 26-kD recombinant SPS truncated protein in the gel matrix as a substrate for the SPS-PK to detect activity in the approximately 65-kD band (Fig. 4A). These results strongly suggest that peak I was not the free catalytic subunit from peak III. If no protein was added or a similarly sized nonsubstrate protein, in this case carbonic anhydrase (31 kD), was added instead of the 26-kD protein, then no activity was detected (Fig. 4B).

More evidence that peak I and peak III are different proteins is that peak I and peak II are Ca^{2+}-dependent protein kinases (Bachmann et al., 1995), whereas Ca^{2+} has little effect on peak III activity. These results are exemplified by Figure 5. Here, fractions from a Resource Q separation of the 5 to 12% PEG precipitate were assayed for their ability to phosphorylate the SP1 synthetic peptide substrate, derived from the major phosphorylation site of spinach SPS, in the presence and absence of 0.2 mM EGTA. The activity without EGTA matched the SPS inactivation profile shown in Figure 2, but addition of a low concentration of the Ca^{2+} chelator virtually eliminated peak I activity while leaving peak III activity essentially unaffected. It is clear from these results that the three kinase activity peaks are different protein kinases.

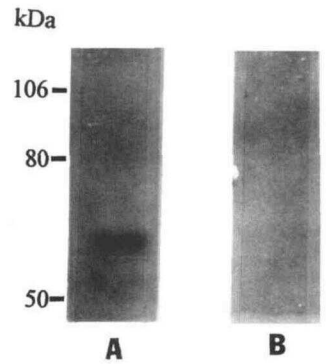

Figure 4. Estimation of SPS-PK (peak III) subunit molecular mass by in situ renaturation and assay of SPS-PK activity following SDS-PAGE. Aliquots of SPS-PK were separated on 10% SDS-PAGE gels containing either 0.4 mg/mL 26-kD recombinant SPS (A) or 0.4 mg/mL carbonic anhydrase (B). The gels were then subjected to the in situ procedure as described in "Materials and Methods." Indicated on the left of the autoradiographs is the position of the prestained protein standards.

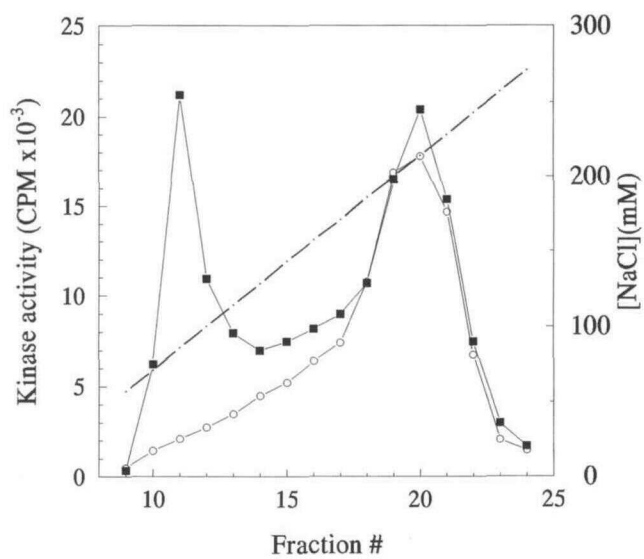

Figure 5. The effect of EGTA on the phosphorylation of the synthetic peptide SP1 by Resource Q column fractions. See "Materials and Methods" for details. Phosphorylation of SP1 in the absence (■) and presence (○) of 0.2 mM EGTA. The salt gradient is indicated by the dashed line.

Determination of which kinases were acting in vivo cannot be made at this time. Peak I could have been a protein kinase with broad substrate specificity that had been generated as an artifact of purification and was not involved in the regulation of SPS or NR. On the other hand, peak I could have been the kinase that acted in vivo while peaks II and III were artifactual. The more exciting possibility is that these three kinase activities allow SPS and NR to be regulated independently via the peak II and peak III kinases, while allowing coordination of their regulation under specific circumstances through the peak I kinase. This hypothesis is an obvious next step that we are pursuing. There are also other avenues of further study indicated by this work. The mechanisms by which these kinases are controlled, thereby regulating SPS and NR activity, are of great interest and have been examined in greater detail by Bachmann et al. (1995). Finally, the ability of the synthetic peptide SP2 to inhibit NR inactivation will help direct current investigations to map the phosphorylation site(s) in NR. Examination of the deduced primary structure of spinach NR (Prosser and Lazarus, 1990) for sites that are similar but not identical with the major SPS phosphorylation site has revealed several candidates that we are in the process of examining.

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