Regulation of a Plant SNF1-Related Protein Kinase by Glucose-6-Phosphate[1]

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One of the major protein kinases (PK_{III}) that phosphorylates serine-158 of spinach sucrose-phosphate synthase (SPS), which is responsible for light/dark modulation of activity, is known to be a member of the SNF1-related family of protein kinases. In the present study, we have developed a fluorescence-based continuous assay for measurement of PK_{III} activity. Using the continuous assay, along with the fixed-time-point ^{32}P-incorporation assay, we demonstrate that PK_{III} activity is inhibited by glucose-6-phosphate (Glc-6-P). Relative inhibition by Glc-6-P was increased by decreasing pH from 8.5 to 5.5 and by reducing the concentration of Mg^{2+} in the assay from 10 to 2 mM. Under likely physiological conditions (pH 7.0 and 2 mM Mg^{2+}), 10 mM Glc-6-P inhibited kinase activity approximately 70%. Inhibition by Glc-6-P could not be ascribed to contaminants in the commercial preparations. Other metabolites inhibited PK_{III} in the following order: Glc-6-P > mannose-6-P, fructose-1,6P_2 > ribose-5-P, 3-PGA, fructose-6-P. Inorganic phosphate, Glc, and AMP were not inhibitory, and free Glc did not reverse the inhibition by Glc-6-P. Because SNF1-related protein kinases are thought to function broadly in the regulation of enzyme activity and gene expression, Glc-6-P inhibition of PK_{III} activity potentially provides a mechanism for metabolic regulation of the reactions catalyzed by these important protein kinases.

Suc-P synthase (SPS) is a highly regulated enzyme that catalyzes the penultimate reaction in Suc synthesis in plants. One of the mechanisms for the regulation of SPS activity involves reversible protein phosphorylation. SPS is known to be phosphorylated on multiple seryl residues, with three of the sites potentially of regulatory significance (Huber and Huber, 1996; Toroser and Huber, 1997). The first site to be identified was Ser-158 of spinach SPS, which is the major site responsible for light/dark modulation of SPS activity (McMichael et al., 1993; Toroser et al., 1999). Ser-158 is potentially phosphorylated by several leaf protein kinases, one of which (the PK_{III} enzyme) (McMichael et al., 1995; Toroser and Huber, 1997) is now known to be a SNF1-related protein kinase (SnRK1) (Douglas et al., 1997; Sugden et al., 1999).

The control of the phosphorylation status of specific sites on SPS continues to be an area of considerable interest. With respect to Ser-158 and light/dark modulation of SPS activity, changes in leaf metabolite pools are thought to play at least some role. Inorganic phosphate (Pi) inhibits the PP2A that dephosphorylates and activates phospho-SPS-158

(Weiner et al., 1992, 1993). Thus, the decrease in cytosolic [Pi] that may occur in response to induction of photosynthetic metabolism would increase PP2A activity in vivo and therefore promote SPS activation. As cytosolic [Pi] decreases, the concentration of P-esters would increase proportionally. This could also contribute to SPS activation, as Glc-6-P has been shown to inhibit the phosphorylation/inactivation of SPS in leaf extracts (Huber and Huber, 1991; Weiner et al., 1992).

The effect of Glc-6-P on the ATP-dependent inactivation of SPS could involve an interaction with SPS and/or the requisite protein kinase. Glc-6-P is known to be an allosteric activator of SPS (Doehlert and Huber, 1983), so an effect on the protein substrate could be involved. However, the interaction of Glc-6-P at the allosteric site of SPS is antagonized by the inhibitor Pi (Doehlert and Huber, 1983), whereas the Glc-6-P inhibition of SPS inactivation was not affected by Pi (Weiner et al., 1992), suggesting that the metabolite was interacting with the protein kinase rather than the protein substrate. This notion was supported by subsequent studies that demonstrated Glc-6-P inhibition of synthetic peptide phosphorylation by PK_{III} (McMichael et al., 1995). However, a recent study with two highly purified members of the SNF1-related protein kinase family (HRK-A and HRK-C) did not show metabolite regulation (Sugden et al., 1999). Thus, the occurrence of Glc-6-P inhibition of protein kinase activity is still the subject of active controversy.

The overall goal of the present study was to reexamine Glc-6-P inhibition of PK_{III} (which corresponds to HRK-C of Sugden et al. [1999]) with re-

spect to the stage of enzyme purification and kinase assay conditions. We demonstrate that Glc-6-P inhibition of PK_{III} occurs, but show that under some conditions, Glc-6-P inhibition can be lost during storage at 0°C. This may explain why inhibition has not been reported in some studies (Sugden et al., 1999). We also document a new assay for the continuous measurement of PK_{III} activity utilizing a novel synthetic peptide substrate. Both the continuous fluorescence assay and the fixed-time-point ^{32}P-incorporation assay documented Glc-6-P inhibition of PK_{III} activity.

RESULTS AND DISCUSSION

Inhibition of Protein Kinase Activity by Glc-6-P in a 3% to 20% (w/v) PEG Precipitate

We initially undertook a series of preliminary experiments to characterize the reduction in PK_{III} activity by Glc-6-P using proteins that precipitated between 3% and 20% (w/v) PEG (i.e. essentially a concentrated crude extract devoid of low-M_r components). It is possible to measure PK_{III} activity rather specifically even in crude extracts because it is the primary kinase phosphorylating the SP1 peptide in the absence of Ca^{2+} (McMichael et al., 1995). Typically, the addition of 10 mM Glc-6-P to a 3% to 20% (w/v) PEG fraction resulted in 30% to 50% inhibition of SP1-peptide kinase activity (data not shown). Time-course experiments indicated that peptide phosphorylation was linear with time during assays in the presence and absence of Glc-6-P, and that preincubation of the enzyme preparation with Glc-6-P had no effect on the inhibition observed (data not shown). In addition, the inhibition of PK_{III} activity by Glc-6-P was not reduced by the inclusion of the phosphatase inhibitors NaF (up to 25 mM), microcystin-LR (0.25 μM), EDTA (1 mM), or EGTA (1 mM) in the assay mixture (data not shown). Collectively, these results suggested that endogenous phosphatase(s) that may target either the SP1 phosphopeptide product or PK_{III} directly were not responsible for the observed Glc-6-P inhibition of kinase activity. As will be discussed further below, additional purification of PK_{III} by ion-exchange and hydrophobic interaction chromatography did not abolish Glc-6-P inhibition (data not presented), nor could the inhibition be attributed to contaminants in the commercial preparation. Thus, it appeared that direct inhibition of PK_{III} activity by Glc-6-P was the simplest explanation for the effects observed. Consequently, experiments were conducted to characterize the Glc-6-P inhibition of PK_{III}.

Characterization of Glc-6-P Inhibition of Partially Purified PK_{III}

Kinases from spinach leaves were extracted in the presence of the phosphatase inhibitors microcystin-

LR, NaF, EDTA, and EGTA, as described in "Materials and Methods" and resolved on a 2-mL Resource-Q column. This method separated the majority of the total protein from the PK_{III} fractions in a convenient single-step procedure resulting in a 112-fold purification. Using the [γ-^{32}P]ATP-based synthetic peptide assay with the SP1 peptide, PK_{III} was strictly Ca^{2+} independent, as expected (McMichael et al., 1995), and 10 mM Glc-6-P inhibited activity 47% (data not shown). Further purification of PK_{III} on phenyl-Sepharose Cl-4B hydrophobic chromatography resulted in additional purification (691-fold relative to the PEG precipitate), and Glc-6-P inhibited activity about 45% (Fig. 1). Maintenance of Glc-6-P inhibition during purification supports the notion that the metabolite directly inhibits PK_{III} activity.

For most experiments, PK_{III} purified by ion-exchange chromatography (FPLC-RQ) was used. As discussed above, the partially purified PK_{III} was inhibited to approximately 50% by 10 mM Glc-6-P when assayed immediately after preparation. However, when column fractions were stored at 0°C for 24 h, there was usually some loss in peptide kinase activity and an even greater reduction in Glc-6-P inhibition (Table I). In some preparations, Glc-6-P
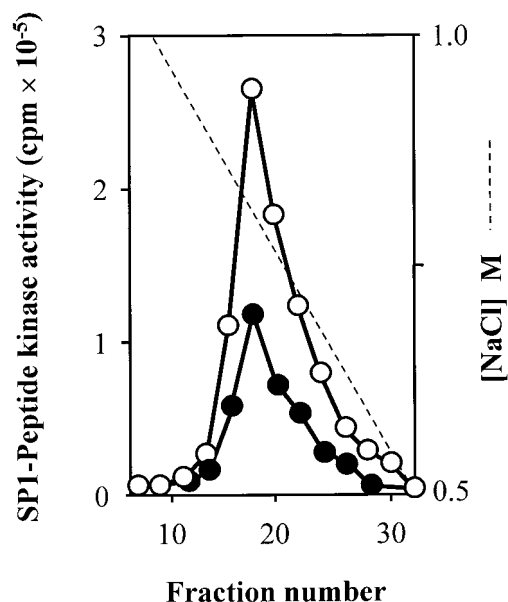
Figure 1. Inhibition of phenyl-Sepharose CL-4B purified PK_{III} in the presence of 10 mM Glc-6-P. Separations were performed by sequential ion-exchange and hydrophobic interaction chromatography (Resource-Q followed by phenyl-Sepharose CL-4B) following a 3% to 20% (w/v) PEG-8000 fractionation of total spinach leaf proteins as described in "Materials and Methods." Resource-Q fractions were immediately assayed for SP1 kinase activity and fractions containing PK_{III} were further purified by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B. Kinase fractions were assayed in the presence (●) and absence (○) of 10 mM Glc-6-P using the filter-binding assay. The experiment was performed three times to confirm reproducibility. Data were essentially identical in each experiment and representative results are presented.

Table I. *Stabilization of PK$_{III}$ and its inhibition by Glc-6-P in the presence of DTT, Glc-6-P preincubation, and glycerol*

Leaf proteins were resolved by Resource-Q chromatography and the PK$_{III}$ activity peak was collected. As indicated, aliquots were supplemented with additional DTT, glycerol, or Glc-6-P. Kinase activities in the presence and absence of Glc-6-P (10 mM) were determined immediately after chromatography (t$_0$) and after 24 h (t$_{24}$) using the SP1 synthetic peptide and the filter-binding assay. SP1 data are shown (ND, not determined).

Addition	PK$_{III}$ Kinase Activity		Glc-6-P Inhibition	
	t$_0$	t$_{24}$	t$_0$	t$_{24}$
	cpm × 10^{-3}		*% of control*	
DTT (1 mM)	6.65	5.46	49	25
DTT (10 mM)	ND	6.66	ND	53
50% Glycerol + DTT (1 mM)	ND	5.56	ND	41
Glc-6-P + DTT (1 mM)	ND	4.59	ND	54

inhibition was reduced to 5% to 10% after storage for 24 h. As shown in Table I, when column fractions were supplemented with increased DTT (10 mM), 50% (v/v) glycerol, or 10 mM Glc-6-P, the Glc-6-P inhibition of PK$_{III}$ activity was substantially retained. The three stabilizing agents differed in their ability to preserve absolute peptide kinase activity, with increased DTT being the most effective (Table I). Consequently, in subsequent experiments, column fractions were routinely supplemented with 10 mM DTT and 50% (v/v) glycerol.

A series of experiments were conducted assaying PK$_{III}$ with two different synthetic peptide substrates. In addition to SP1, which is based on the SPS-Ser-158

sequence, we also used SP46, which is a shorter peptide with several modifications to the sequence, including substitution of a Trp residue at the P-1 position (numbered relative to the phosphorylated Ser at position zero). The SP46 peptide was produced in an attempt to utilize a continuous fluorescence-based assay for PK$_{III}$ (described below), following a protocol originally developed for cAMP-dependent protein kinase (PKA; Wright et al., 1981). The SP1 and SP46 peptides were initially compared as substrates for PK$_{III}$ using the standard [γ-^{32}P]ATP assay while varying different components of the reaction mixture to characterize Glc-6-P inhibition.

The Effect of Substrate Concentrations on the Glc-6-P Inhibition of PK$_{III}$

A series of preliminary experiments indicated that kinase assays with the SP1 or SP46 synthetic peptides were fully saturated at 100 μM ATP. We tested the effect of lower concentrations of ATP on Resource-Q-purified PK$_{III}$ activity in the presence and absence of 10 mM Glc-6-P (Fig. 2, A and B). Significant inhibition by Glc-6-P could be observed with all concentrations of ATP. In the absence of Glc-6-P, the apparent K_m (ATP) was about 6 μM and Glc-6-P was a non-competitive inhibitor with respect to ATP for both substrates (replots not shown).

The activity of PK$_{III}$ was determined with various concentrations of the synthetic peptides to investigate the nature of the inhibition by 10 mM Glc-6-P with respect to the polypeptide substrate. Inhibition by Glc-6-P was apparent at all tested concentrations
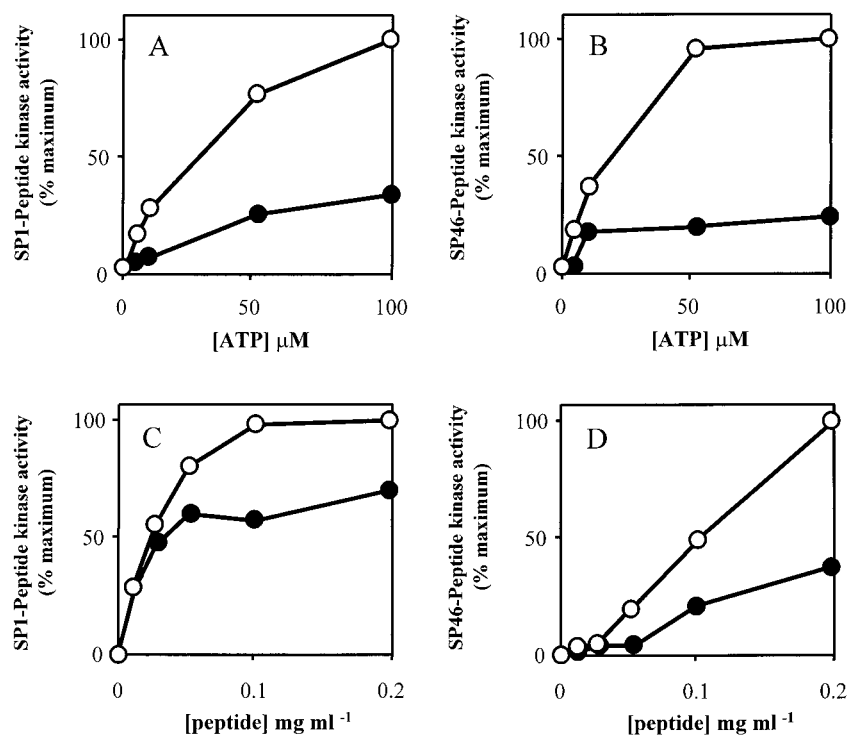
Figure 2. Characterization of the Glc-6-P inhibition of partially purified PK$_{III}$. Partially purified PK$_{III}$ was resolved by Resource-Q anion-exchange chromatography, and synthetic peptide kinase assays were performed with the partially purified PK$_{III}$ and the synthetic peptides SP1 and SP46, which are based on the spinach leaf SPS sequence. Experiments performed with various ATP (A and B) and synthetic peptide (C and D) concentrations are shown. Experiments were performed at least two separate times. Data were essentially identical in each experiment and representative results are presented. Kinase fractions were assayed in the presence (●) and absence (○) pf 10 mM Gk-6-P.

of the SP1 and SP46 synthetic peptides (Fig. 2, C and D). With both peptides, Glc-6-P reduced V_{max} without affecting the affinity for the peptide substrate and therefore was a non-competitive inhibitor. Apparent K_m values for SP1 and SP46 were 9 and 89 μM, respectively (replots not shown).

The Effect of [Mg^{2+}] on Glc-6-P Inhibition of PK_{III}

Characteristically, protein kinase assays in vitro require millimolar concentrations of Mg^{2+} for maximum activity, which is considerably in excess of that required to form the Mg^{2+}-ATP substrate complex (Morrison, 1979). Half-maximal stimulation of PK_{III} activity with either SP1 or SP46 as a substrate required approximately 1.5 mM Mg^{2+}, and this was unaltered by Glc-6-P. There was, however, a reduction in V_{max} activity, because slopes of the Hanes-Woolf replots were greater in the presence of 10 mM Glc-6-P (Fig. 3). These results rule out the possibility that Glc-6-P inhibition occurs as a result of Mg^{2+} chelation.

The Glc-6-P Inhibition of PK_{III} Is Enhanced at Low Mg^{2+} Concentrations and Low pH

Cytosolic Mg^{2+} concentrations are thought to be considerably lower than the concentrations commonly utilized in standard peptide kinase assays (10–15 mM). Similarly, cytosolic pH is probably somewhat variable in vivo but lower than the pH 7.5 often employed in kinase assays (Heber et al., 1994). Therefore, we examined Glc-6-P inhibition of our partially purified PK_{III} activity under a range of physiologically pertinent conditions, and tested two concentrations of Mg^{2+} at a range of pH values (pH 5.5–8.5). Partially purified spinach leaf PK_{III} was active from pH 5.5 to 8.5, with maximal activity between pH 7.5 and 8.0 with 10 mM Mg^{2+} (Fig. 4A) or between pH 7.0 and 7.5 with 2 mM Mg^{2+} (Fig. 4B). In addition to slightly shifting the pH optimum, reducing the Mg^{2+} concentration to 2 mM also sharpened the pH profile. Kinase activity at the optimum pH was only slightly reduced when Mg^{2+} was decreased to 2 mM. At both concentrations of Mg^{2+}, inhibition of SP1-kinase activity by Glc-6-P increased as the pH was reduced from 8.5 to 5.5 (Fig. 4C), and inhibition was consistently greater at the lower concentration of Mg^{2+}. Qualitatively similar results were obtained with the SP46 synthetic peptide, even though phosphorylation of this substrate was approximately 10-fold lower than the SP1 peptide (data not shown).

These results suggest that at Mg^{2+} concentrations and pH values closer to those encountered by SnRK1s in vivo, inhibition by Glc-6-P may be considerably greater than that measured using typical kinase assay conditions. It was of interest to determine whether PK_{III} was inhibited by concentrations of Glc-6-P that occur in planta. Figure 5 shows that appre-
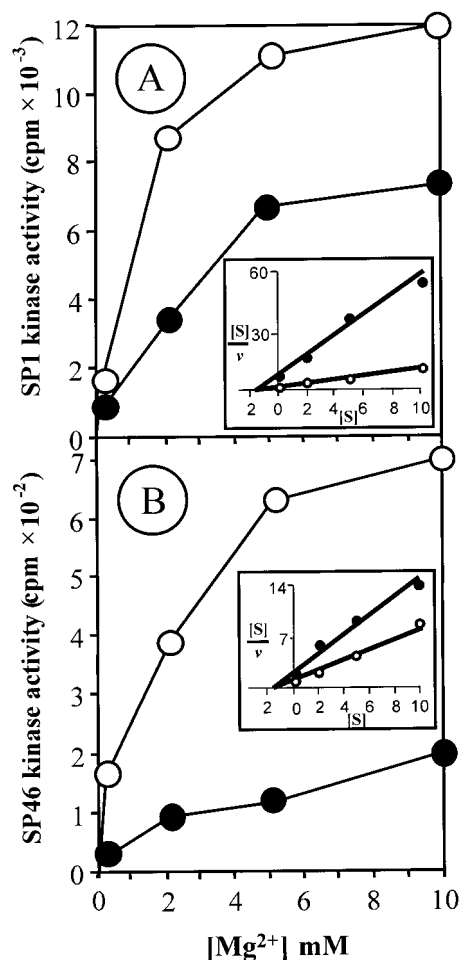

Figure 3. Glc-6-P inhibition of PK_{III} is non-competitive with respect to Mg^{2+} concentration. Partially purified PK_{III} was obtained by Resource-Q anion-exchange chromatography. Kinase assays were performed with the synthetic peptides SP1 (A) and SP46 (B) at the indicated free Mg^{2+} concentrations. Experiments were performed at least two separate times. Data were essentially identical in each experiment and representative results are presented. The insets are Hanes-Woolf replots of the data. Kinase fractions were assayed in the presence (●) and absence (○) pf 10 mM Gk-6-P.

ciable inhibition of PK_{III} occurred below 10 mM Glc-6-P, and that the observed inhibition was greater in the presence of 2 mM Mg^{2+} compared with 10 mM Mg^{2+}. Published data indicate that Glc-6-P concentrations in source tissues such as strawberry and spinach leaves can reach 8 mM in the cytosol (Stitt et al., 1987 and references therein). In sink tissues such as potato tubers, concentrations of Glc-6-P can be around 2 mM (Geigenberger et al., 1998).

Specificity of the Inhibition of Kinase Activities by a Range of P-Esters

There is some evidence that a range of metabolites may play a significant role in the regulation of protein kinase activities (Bachmann et al., 1995; McMichael et al., 1995). We decided to see whether any
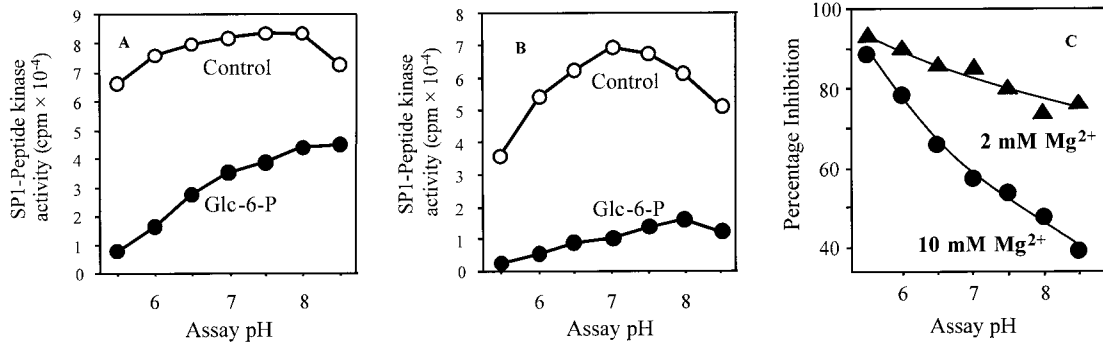
Figure 4. Glc-6-P inhibition of PK_{III} is affected by pH and Mg^{2+} concentration. Partially purified PK_{III} was obtained by Resource-Q ion-exchange purification and assayed using the SP1 synthetic peptide and filter-binding assay as described in "Materials and Methods." The pH of the reaction mix was adjusted between pH 6.0 and 8.5 using a buffer mixture containing 20 mM each of MES-KOH, MOPS-KOH, and Tricine-KOH. Filter-binding assays were then performed to test for Glc-6-P inhibition in the presence of 2 mM Mg^{2+} (A) and 10 mM Mg^{2+} (B). The percentage inhibition at various pH values and Mg^{2+} concentrations is presented in C. Experiments were performed at least two separate times. Data were essentially identical in each experiment and representative results are presented.

other sugar-phosphates could bring about the inhibition of PK_{III} observed using Glc-6-P. Figure 6 shows that at a concentration of 10 mM, Glc-6-P produced the greatest inhibition of PK_{III} activity. $Fru-1,6-P_2$, Mann-6-P, and Glc-1-P caused some inhibition, whereas a range of other sugar-phosphates had essentially no effect (Fru-6-P, Rib-5-P, and PGA). Similarly, Pi and Glc had no effect (not shown), nor did AMP, as has been reported previously (Hardie, 1999). The results suggest that significant specificity is apparent with respect to sugar-P inhibition of spinach leaf PK_{III} activity.
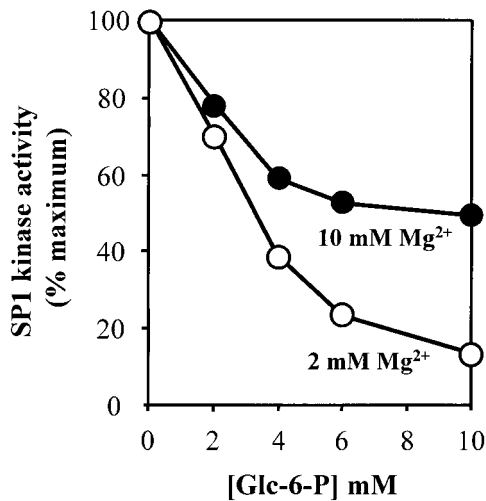
Continuous Kinase Assays Using the Fluorescent Synthetic Peptide SP46

The SP46 synthetic peptide, which has a Trp residue at P-1, was produced in an attempt to develop a continuous fluorescence-based assay for PK_{III}. Our strategy was based on the earlier report of Wright et al. (1981), who successfully developed a continuous assay for PKA. The SP46 synthetic peptide was found to be readily phosphorylated by PK_{III} and other protein kinases that phosphorylate the SP1 peptide. Elution profiles of kinase activity during Resource-Q chromatography were qualitatively similar when the two peptides were substrates in the filter-binding assay (Fig. 7). However, although SP46 was readily
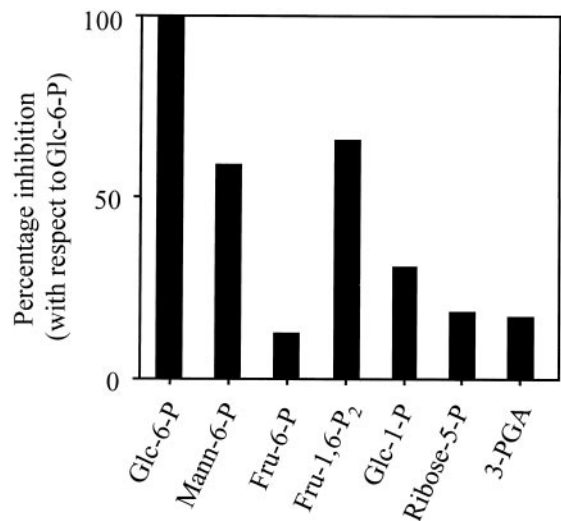

Figure 5. Inhibition of spinach leaf PK_{III} by several Glc-6-P concentrations and at 2 and 10 mM Mg^{2+}. Partially purified PK_{III} was obtained by Resource-Q ion-exchange purification and assayed using the SP1 synthetic peptide as described in "Materials and Methods." Filter-binding assays were then performed at various Glc-6-P concentrations in the presence of 2 mM Mg^{2+} and 10 mM Mg^{2+}. The experiments were performed two separate times to confirm reproducibility. Data were essentially identical in each experiment.

Figure 6. The effect of various sugar-phosphates on the kinase activity of PK_{III}. The indicated sugar-phosphates (10 mM) were added to a reaction mixture containing Resource-Q-purified PK_{III}. Synthetic peptide kinase assays were performed using SP1, as described in "Materials and Methods."
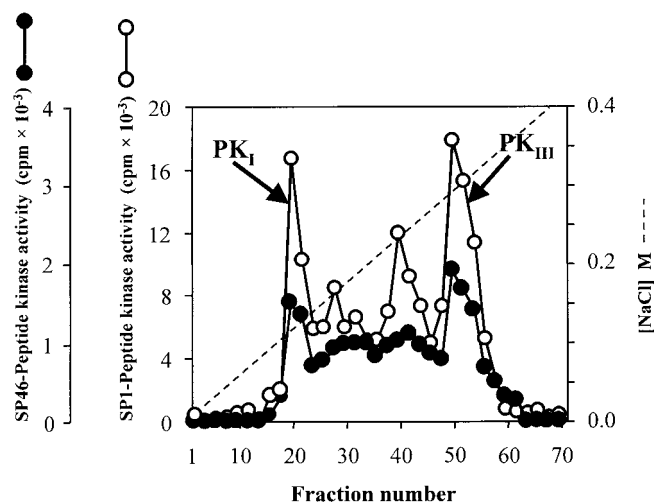
Figure 7. Partially purified spinach leaf PK_{III} kinase activity utilizes SP46 as a substrate. Proteins that precipitated between 3% and 20% (w/v) PEG-8000 were resuspended and fractionated by Resource-Q chromatography as described in "Materials and Methods." Synthetic peptide kinase assays were performed using the SP1 (GRMRRISS-VEMMDNWANTFK) and SP46 (RMKRKWSVEM) synthetic peptides using the filter-binding assay.

phoshorylated, the presence of the Trp residue at the P-1 position resulted in a reduction in the total incorporation of ^{32}P into the peptide. Under standard assay conditions, the rate of phosphorylation was about one-tenth that of the SP1 peptide. The Trp residue at the P-1 position (which replaced a Ser residue in the native sequence) was found to act as a phosphorylation reporter group for the phosphorylated Ser at position 0. Figure 8 shows some representative fluorescence emission spectra using the SP46 (RMKRKWSVEM) synthetic peptide. When PK_{III}, the SP46 peptide, and ATP were mixed, the emission spectrum displayed a peak at 356 nm that decreased in intensity with time (Fig. 8, curves 3, 4, 5, and 6). Thus, phosphorylation of the Ser residue in this peptide caused significant quenching of the intrinsic fluorescence of the Trp residue at P-1. The time-dependent decrease in fluorescence at 356 nm required kinase, ATP, and the SP46 synthetic peptide. There was also a linear increase in the rate of intrinsic fluorescence quenching with increasing additions of PK_{III} enzyme to the assay, as would be expected (Fig. 8, inset).

As discussed above, our preliminary experiments indicated that the inhibitory effect of Glc-6-P on PK_{III} was relatively rapid (on the order of <1 min) and did not require preincubation of the kinase with Glc-6-P. However, as shown in Figure 9, Glc-6-P inhibition of PK_{III}-catalyzed phosphorylation of SP46, as observed by changes in fluorescence intensity, had a slight delay (approximately 20 s) before inhibition was observed. Within approximately 1 min, the new steady-state rate was achieved. The continuous fluorescence assay was useful in observing this small delay in

Glc-6-P inhibition of PK_{III} activity, which would be difficult to identify using the standard radioactive assay. These results with the fluorescence-based kinase assay are significant for several reasons. First, they confirm the occurrence of Glc-6-P inhibition of PK_{III}, and second, they establish that metabolite regulation may not be instantaneous. Rather, the hysteresis observed may reflect some conformational change in PK_{III} that results in reduced catalytic activity.

Ba^{2+} Contamination of Commercial Samples of Glc-6-P Is Not the Cause of Glc-6-P Inhibition of PK_{III}

In a recent report, Sugden et al. (1999) suggested that the Glc-6-P inhibition of kinase activities observed by a number of investigators (Bachmann et al., 1995; McMichael et al., 1995) may have resulted from impurities (possibly Ba^{2+}) present in certain commercial preparations of hexose phosphates. Consequently, we were interested in determining whether impurities contributed to the inhibition of kinase activities observed in the present work. Two strategies were employed to test this possibility. First, Glc-6-P preparations were obtained from three different sources. When compared at a concentration of 10 mM, Glc-6-P from Sigma-Aldrich, Fluka (Milwaukee, WI), and ICN (Costa Mesa, CA) produced inhibition
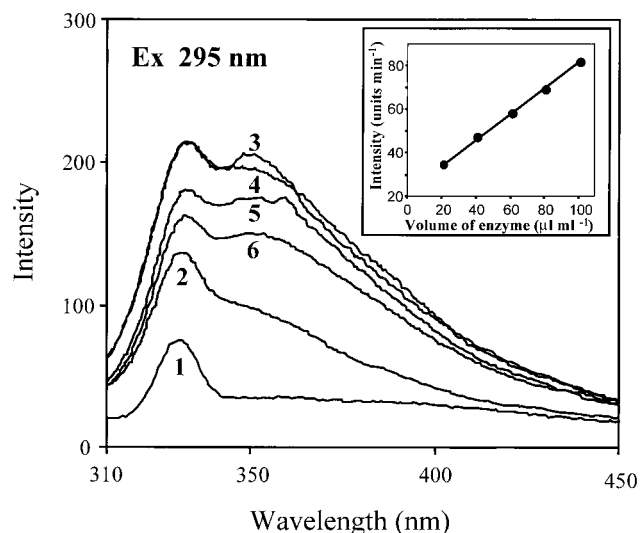

Figure 8. Fluorescence emission spectra of the SP46 synthetic peptide indicates its phosphorylation status. The SP46 synthetic peptide (RMKRKWSVEM) was phosphorylated in a total volume of 500 μL and a time course study was performed at the indicated time intervals in a scanning fluorimeter using an excitation wavelength of 295 nm as described in "Materials and Methods." The six spectra shown represent: the background signal from the buffer (1), PK_{III} in buffer (2), and SP46, ATP plus PK_{III} at time zero (3), 30 s (4), 180 s (5), and 600 s (6). An emission peak at approximately 335 nm was apparent with the buffer only, whereas the Trp containing SP46 synthetic peptide showed an emission peak at 356 nm. Inset, Linear relationship between the rate of fluorescence decrease and the volume of PK_{III} present in the continuous assay. The results are representative of three or more separate experiments.
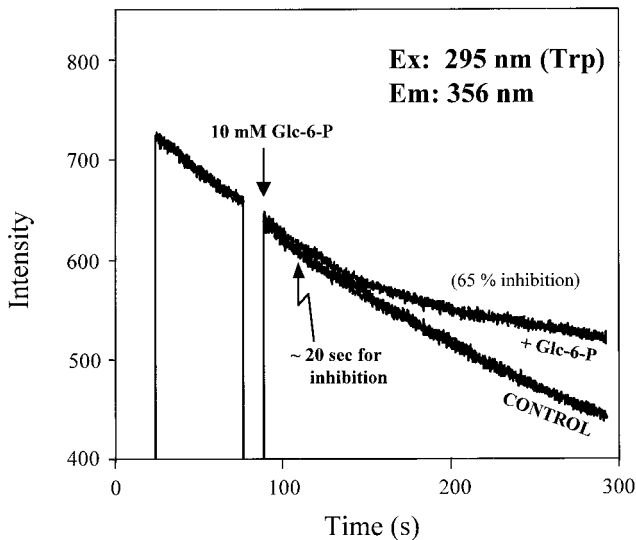
Figure 9. The effect of Glc-6-P on the phosphorylation-dependent fluorescence quenching of SP46. A complete reaction mixture containing SP46, ATP, and PK_{III} was allowed to attain a steady-state rate of intrinsic fluorescence change, and 10 mM Glc-6-P was then added to the cuvette, as indicated by the arrow. Measurement was then immediately restarted. Inhibition of PK_{III} first became apparent after approximately 20 s, and a new steady-state rate of fluorescence decrease was attained after approximately 1 min.

of PK_{III} activity of 51%, 51%, and 58%, respectively. In the second strategy, a Glc-6-P preparation that produced 51% inhibition of PK_{III} activity was enzymatically dephosphorylated using alkaline phosphatase and tested for loss of the inhibitory effect. Following dephosphorylation of Glc-6-P, the inhibition was reduced to 17%, with the remaining inhibition being explainable by residual 3.5 mM Glc-6-P in the reaction mixtures. Thus, the inhibition by Glc-6-P cannot be attributed to contaminants in the commercial preparations.

CONCLUDING REMARKS

The most important conclusion of the present study is that Glc-6-P inhibits the catalytic activity of PK_{III}, a member of the plant SNF1-related family of protein kinases (SnRK1s). The inhibition is not the result of breakdown products (Pi or Glc) or contaminants in the commercial preparations (e.g. Ba^{2+}, as suggested by Sugden et al. [1999]). There is no evidence for involvement of additional enzymes or protein factors in the inhibition: for example, a Glc-6-P-stimulated protein phosphatase that would be analogous to the xylulose-5-P-activated protein phosphatase that acts on Fru-6-P,2-kinase:Fru-2,6-Pase in mammalian liver (Nishimura and Uyeda, 1995). The Glc-6-P inhibition of PK_{III} occurs rapidly, although with the continuous assay system a short lag (approximately 20 s) was observed before inhibition was apparent. This may suggest that Glc-6-P causes a slow conformational change in the protein kinase

that reduces catalytic activity. Using the standard ^{32}P-incorporation assay, it was difficult to detect this slight hysteresis. At present, we do not know where on the protein kinase Glc-6-P is binding (catalytic or regulatory subunits). Regardless of the exact binding site, we conclude that Glc-6-P directly inhibits the catalytic activity of PK_{III} in vitro with synthetic peptides as substrates. We suggest that this is responsible, at least in part, for the effect of Glc-6-P on ATP-dependent inactivation of SPS.

Metabolite regulation of plant SnRK1s is controversial. Sugden et al. (1999) reported that effects of Glc-6-P on HRK-C (thought to be equivalent to PK_{III}), when observed, could be traced to a contaminant in certain commercial preparations. Although the putative contaminant was not identified, Ba^{2+} was suggested as a possibility. We suggest that Glc-6-P inhibition is a property that can be lost during preparation/storage of the enzyme. While conditions required for maintaining regulatory properties have not been rigorously established, maintenance of a high concentration of reductant (e.g. 10 mM DTT) in the presence of glycerol seemed to be at least partially effective. The molecular basis for the loss of regulatory properties remains to be determined, but Glc-6-P inhibition of PK_{III} in vitro could be significant, and thus be an important mechanism for regulation of SnRK1 activity in vivo. This could be important physiologically, because SnRK1s are thought to function broadly in the regulation of gene expression (Bhalerao et al., 1999) and in the control of enzyme activity (Halford and Hardie, 1998; Hardie, 1999). The Glc-6-P inhibition of SnRK1 activity could be part of the mechanism underlying sugar regulation of genes (e.g. encoding pathogenesis-related proteins), in which metabolism (i.e. Glc-6-P formation) is required (Sheen et al., 1999). Further work will be required to test these postulates.

MATERIALS AND METHODS

Growth and Harvest of Plant Material

Spinach (*Spinacia oleracea* L. cv Bloomsdale and cv Tyee) plants were grown in a soil mixture under standard greenhouse conditions as previously described (Huber et al., 1989). Leaves were harvested directly into liquid nitrogen at midday and stored at −80°C until required for experimentation.

Extraction of Plant Material and Partial Purification of Protein Kinase Activities

Frozen spinach leaf tissue was ground in a chilled mortar. Usually, 25 g fresh weight was extracted in 100 mL of extraction buffer containing 50 mM 3-(N-morpholino)-propanesulfonic acid (MOPS)-NaOH, pH 7.5, 2 mM EGTA, 2 mM EDTA, 5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ϵ-amino-*n*-caproic acid, 1 mM benzamidine, 25 mM NaF, 0.25 μM microcystin-

LR, and 0.1% (v/v) Triton X-100. The homogenates were filtered through four layers of Miracloth[3] (Calbiochem-Novabiochem, La Jolla, CA) and centrifuged at 10,000*g* for 15 min. To the supernatant, polyethylene glycol (PEG) was added from a 50% (w/v) solution to give an initial concentration of 3% (w/v). After stirring for 10 min, the solution was centrifuged at 38,000*g* for 10 min and the pellet discarded. The supernatant was then adjusted to 20% (w/v) PEG and stirred for 15 min. The precipitated protein pellet was harvested by centrifugation at 38,000*g* for 15 min, and resolubilized in buffer containing 50 mM MOPS-NaOH, pH 7.5, 2 mM EGTA, 2 mM EDTA, 0.25 μM microcystin-LR, 5 mM sodium pyrophosphate, 5 mM NaF, and 2.5 mM DTT (0.5 mL g^{-1} tissue used).

The solution was finally clarified (by centrifugation at 38,000*g* for 10 min) and the supernatant applied to a 2-mL Resource-Q column (Pharmacia Biotech, Piscataway, NJ). The column was washed with buffer A (50 mM MOPS-NaOH, pH 7.5, and 1 mM DTT) until the A_{280} decreased to baseline. The bound proteins were eluted with a 70-mL linear gradient from 0 to 500 mM NaCl in buffer A at a flow rate of 3 mL min^{-1}. Fractions (3 mL) were collected and assayed for peptide kinase activity as outlined below. For some experiments, appropriate Resource-Q fractions were pooled (usually five fractions) and further purified by phenyl-Sepharose CL-4B chromatography. The 2-mL column was equilibrated with 10-column bed volumes of 1 M NaCl in buffer A at a flow rate of 0.5 mL min^{-1}. Following application of the kinase sample in 1 M NaCl, the column was developed at the same flow rate with 30 mL of a linear gradient from 1 to 0 M NaCl in buffer A. Following purification, active fractions were supplemented with 10 mM DTT and stored at 0°C. Because kinase activity was greatly reduced following snap-freezing, all subsequent PK$_{III}$ preparations were stored at 0°C and used within a few days after preparation.

Synthetic Peptides

Synthetic peptides were designed from the regulatory phosphorylation site (Ser-158) of spinach leaf SPS (McMichael et al., 1993). The synthetic peptide SP1 (GRMR-RIS*S*VEMMDNWANTFK) was obtained from Dr. Jan Kochansky (U.S. Department of Agriculture-Agricultural Research Service, Insect Neurobiology and Hormone Laboratory, Beltsville, MD) and the variant peptide SP46 (RMKRKW*S*VEM) was synthesized using Fmoc chemistry on a peptide synthesizer (Synergy model 432A, Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The peptides were lyophilized and stored with dessicant at −20°C.

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[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 might also be suitable.

Assays for Peptide Kinase Activity

Using [γ-^{32}P]ATP

Typically, a 40-μL reaction mixture contained 0.1 mg mL^{-1} of synthetic peptide (SP1 or SP46) and 4 μL of partially purified (usually by Resource-Q and phenyl-Sepharose) protein kinase in buffer containing 0.1 mM [γ-^{32}P]ATP (S.A. 150–500 cpm pmol^{-1}) in 50 mM MOPS-NaOH, pH 7.5, and 10 mM MgCl$_2$. When the assay pH was varied from pH 5.5 to 8.5, the buffer contained 20 mM each of MES-KOH, MOPS-KOH, and Tricine-KOH. Reactions were started by the addition of ATP. Following a 10-min incubation at ambient temperature, a 20-μL aliquot of the reaction mixture was spotted onto a 2 × 2 cm square of phosphocellulose paper (P81, Whatman, Clifton, NJ). The papers were then immediately washed in 300 mL of 75 mM H$_3$PO$_4$ three times at 5 min per wash to remove unincorporated ATP. ^{32}P incorporation into the synthetic peptides was determined by scintillation counting on a liquid scintillation spectrometer (Tri-Carb 1500, Packard Instrument Co., Downers Grove, IL).

Using Trp Fluorescence

The fluorescent synthetic peptide based kinase assay was a modification of the method described by Wright et al. (1981). The synthetic peptide used in all fluorescence experiments was SP46 (RMKRKW*S*VEM). All experiments were performed using a spectrofluorophotometer (RF-5301 PC, Shimadzu, Columbia, MD). Trp was excited at 295 nm and emission spectra were recorded from 310 to 450 nm. A 10-mm path length quartz cuvette was used. After the initial mixing, magnetic stirring was continuous, and care was taken to prevent the presence of air pockets.

Dephosphorylation of Glc-6-P by Agarose-Conjugated Alkaline Phosphatase

Commercial sources of Glc-6-P were dephosphorylated using agarose-conjugated alkaline phosphatase (20 units; Sigma-Aldrich, St. Louis) by incubation at 37°C for 2 h. The alkaline phosphatase was removed from the mixture by centrifugation at 12,000*g* for 5 min at 4°C. The phosphatase-free supernatant was used for further experimentation. The extent of dephosphorylation of Glc-6-P was monitored by enzymatic analysis of residual P-ester. The Glc-6-P-detection assay (1 mL) contained 50 mM MOPS-NaOH, pH 7.5, 0.4 mM NAD, and 1 unit mL^{-1} of *Leuconostoc* Glc-6-P dehydrogenase. The increase in A_{340} was recorded following the addition of the metabolite sample.

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