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Sucrose-phosphate synthase phosphatase, a type 2A protein phosphatase, changes its sensitivity towards inhibition by inorganic phosphate in spinach leaves

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The activity of a type 2A protein phosphatase from spinach leaves was monitored using phosphorylated sucrose-phosphate synthase (SPS) as a substrate. After partial purification the overall activities of sucrose-phosphate synthase phosphatase (SPS-P) recovered from leaves harvested in the dark and in the light did not vary. However, SPS-P preparations from darkened leaves were more strongly inhibited by inorganic phosphate and certain phosphorylated compounds than preparations from illuminated or mannose fed leaves. We conclude, that activation of SPS involves an interconversion of multiple forms of SPS-P activity.

Protein phosphatase 2A; Sucrose-phosphate synthase; Phosphate inhibition; Protein phosphorylation; Light and mannose activation

1. INTRODUCTION

Protein phosphorylation is a key mechanism in the regulation of various cellular functions in plants [1] as in other eukaryotic organisms. Protein phosphatases are required to reverse the reactions directed by protein phosphorylation. Several classes of protein phosphatases have been identified in plants [2]. Of these, protein phosphatase 2A-type enzymes presumably dephosphorylate and modulate the activity of several important proteins in vivo including sucrose-phosphate synthase (SPS), nitrate reductase, phosphoenolpyruvate carboxylase and quinate dehydrogenase [2–5].

It is unclear at present how the activities of protein phosphatases of the type 2A are regulated and directed towards specific phosphoprotein targets. A type 2A protein phosphatase (SPS-P) has been shown to dephosphorylate and thereby activate sucrose-phosphate synthase in spinach leaves [6]. Recently, a pronounced increase in the apparent activity of SPS-P upon illumination after a longer dark period was reported [7]. This 'light activation' of SPS-P was accompanied by an increase in the in vivo activation state of its substrate protein SPS. It was concluded that SPS-P activity is a major determinant of the activation state of SPS in vivo. The observation that feeding mannose to leaves activates, and feeding inorganic phosphate (P_i) deactivates SPS in vivo [7,8] indicates that changes of P_i and metabolites could be involved in triggering these changes in protein phosphorylation (mannose leads to sequestration of P_i and phosphorylated metabolites as mannose 6-phosphate, see [9], and is a widely used tool to deplete P_i in plant tissues, see [10]).

In this paper we have performed experiments with partly purified SPS-P and phospho-SPS preparations. The objectives were: (a) to determine whether the apparent light activation of SPS-P observed in crude extracts is retained after purification; (b) to characterize its effects on the properties of SPS-P, in particular its sensitivity to inhibition by inorganic phosphate; and (c) to find out whether light per se is necessary to mediate the apparent activation of SPS-P in vivo.

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Abbreviations: SPS-P, sucrose-phosphate synthase phosphatase; P_i, inorganic phosphate; MOPS, 3-(*N*-morpholino)propane sulfonic acid; ATP, adenosine 5-triphosphate; CHX, cycloheximide; BSA, bovine serum albumin.

2. MATERIALS AND METHODS

2.1. *Materials*

Spinach (*Spinacia oleracea* cv. Hybrid 424) was grown in soil in growth chambers as described previously [11]. Biochemicals were obtained from Sigma, Deisenhofen and from Boehringer, Mannheim.

2.2. *Experimental treatment and partial purification of enzymes*

The following procedures were carried out essentially as described in [7]. (a) Feeding of mannose and cycloheximide (CHX) to leaf tissue; (b) preparation of desalted crude extracts; (c) partial purification and separation of SPS and SPS-P via fractionation by polyethylene glycol 8000 and anion-exchange chromatography on a Mono-Q column using a Pharmacia/LKB FPLC system. A mixture of protease inhibitors was present in all solutions (2 mM benzamidine, 2 mM ε-amino-n-caproic acid and 5 mg/l leupeptin); (d) assay of SPS with limiting substrates plus P_i ('limiting assay') or with saturating substrates ('V_{max} assay'). The ratio of the two activities multiplied by 100 is termed the activation state (in percent).

Unless otherwise mentioned the following buffer was used during purification and enzyme assays (buffer A): 50 mM MOPS/NaOH, 10 mM $MgCl_2$, 2.5 mM DTT, pH 7.5.

2.3. *In vitro phosphorylation of SPS*

In spinach, phosphorylation of SPS has little effect on maximum enzyme activity (V_{max}) but affects affinity for substrates and effectors [8]. In particular, inhibition of phospho-SPS by P_i is increased relative to the dephosphorylated enzyme [8,12]. Thus, phosphorylation and dephosphorylation of SPS can be conveniently monitored as a change in the SPS activity measured with limiting substrate concentration in the presence of P_i [6,7]. To supply the phosphorylated and inactivated form of SPS as a substrate for the SPS-P, partly purified SPS preparations were in vitro phosphorylated and inactivated as following. Mono-Q fractions containing partly purified SPS preparations also contain the protein kinase which phosphorylates and inactivates SPS [12]. These fractions were supplied with 0.5% (w/v) BSA and desalted. Addition of BSA prior to desalting was essential to recover SPS activity. Desalted enzymes were incubated at 25°C in the presence of ATP to obtain SPS in the phosphorylated and inactivated form (see [7] for details). 10 mM levamisole was included in the incubation mix to prevent ATP-independent inactivation of SPS (unpublished results). The SPS preparation was desalted a second time to remove adenine nucleotides on Sephadex G-25 columns equilibrated with buffer A or buffer A minus $MgCl_2$ in the presence of 0.1 mM EGTA (the latter only for experiments in the absence of magnesium as indicated in section 3).

2.4. *Determination of SPS-phosphatase activity*

To determine SPS-P activity, up to 10–20 μl of partial purified and desalted (see section 2.3. for details of desalting) SPS-P fraction (free of SPS) or desalted crude extract were incubated with phospho-SPS preparation in the presence of 10 mM levamisole at 25°C in a total volume of 220 μl buffer A (see above). Because of the high dilution, the crude extracts themselves do not significantly contribute to total SPS activity (crude extract endogenous SPS activity was less than 15% of the total SPS activity in the final incubation mix on a V_{max} basis). At various time intervals aliquots were taken, brought to 10 nM okadaic acid and used to determine SPS activity in the presence of limiting substrates (3 mM fructose-6-P, 12 mM glucose-6-P and 10 mM UDP-glucose) plus 10 mM P_i. During the time course SPS activity increased 2.5- to 3-fold as a result of dephosphorylation. The activity of SPS-P was directly calculated from initial velocities during the time course of SPS activation, which were linear up to a 0.7-fold increase in SPS activity and were defined from at least 4 time points. There was a linear relationship between the rate of SPS activation and the amount of SPS-P preparations (up to 30 μl) included in the assay (data not shown).

2.5. *Casein phosphatase assay*

Casein phosphatase activity was quantified as described in [13] by measuring the release of phosphate from ^{32}P-labelled casein [14]. Assays contained 1.1 μM [^{32}P]casein with some exceptions stated individually in section 3. Casein phosphatase activity was terminated before 10% release of ^{32}P. Casein phosphatase activity quoted in this paper refers to the activity which was inhibited by 2 nM okadaic acid. Unless otherwise stated, assays were carried out in buffer A without $MgCl_2$ in the presence of 0.1 mM EGTA. Desalted phosphatase preparations (see section 2.4.) were diluted into the assay 1:100 for screening Mono-Q fractions and 1:20 for testing the effect of various compounds on phosphatase activity. One unit of enzyme activity catalyses the dephosphorylation of 1 μmol of [^{32}P]casein per minute. Using partly purified enzyme preparations this activity compromised almost the total casein phosphatase activity (>90%) measured in the absence of okadaic acid (irrespective of the presence or absence of $MgCl_2$ during assay). Thus, under our experimental conditions using phospho-casein as substrate we obtained a sufficiently accurate estimation of type 2A-dependent protein phosphatase activity without using additional inhibitors to differentiate between type 2A and other phosphatases [2].

2.6. *Fitting procedure for phospho-enzyme saturation curves*

Initial velocities of SPS activation varied hyperbolically with the concentration of phospho-SPS (see Results). This behaviour is summarized in the Michaelis-Menten equation and quantitatively defined by $V_{max\ app}$ and $K_{m\ app}$, which were equal to (in the absence of the inhibitor P_i), or functions of (in the presence of P_i), the apparent parameters that characterize a given saturation curve. Saturation curves were fitted independently to each set of data points as rectangular hyperbolas and were transformed into their double reciprocal forms using a computer program for non-linear regression as described in [15].

3. RESULTS AND DISCUSSION

3.1. *The overall activity of partly purified SPS-phosphatase does not change after illumination*

In earlier experiments we observed an apparent light activation of SPS-P when it was assayed in desalted crude extracts. This light activation could reflect a tight binding effector, or some modification of SPS-P and/or SPS. Thus, it was of interest to further characterize and compare partially purified enzymes from darkened and illuminated leaves.

SPS-P was partly purified by fractionation with polyethylene glycol and Mono-Q chromatography. During the Mono-Q step several type 2A-dependent casein phosphatase activities were resolved (Fig. 1). The SPS-P activity eluted as one single peak, which coeluted with a casein phosphatase peak. The activities in individual fractions of the SPS-P peak were completely inhibited by 2 nM okadaic acid, which is reminiscent of a type 2A protein phosphatase [16]. SPS-P is apparently only a part of the total 2A type protein phosphatases present in leaf extracts.

No differences in the elution profile of SPS-P were observed during the Mono-Q step with different enzyme preparations from darkened, illuminated or mannose

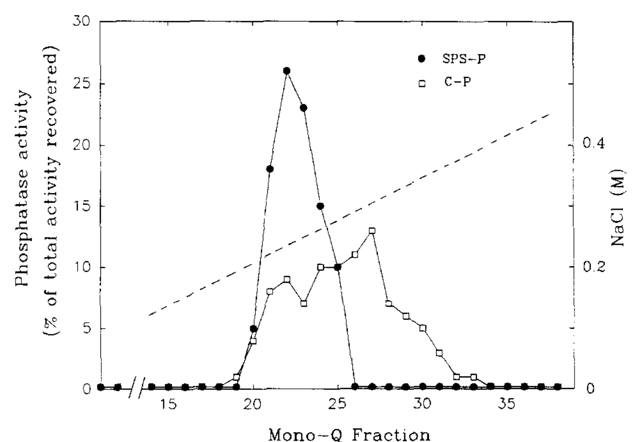

Fig. 1. Mono-Q anion-exchange chromatography of type 2A protein phosphatases. The polyethylene glycol cut (5–12%) was applied to a Mono-Q column and fractionated (details are given in section 2). The closed and open symbols refer to sucrose-phosphate synthase phosphatase (SPS-P) and casein phosphatase (C-P) respectively. The salt gradient is denoted by the dashed line. The data correspond to enzyme preparations from 'light-mannose' tissue. Very similar results were obtained with enzymes from 'dark' tissue (data not shown).

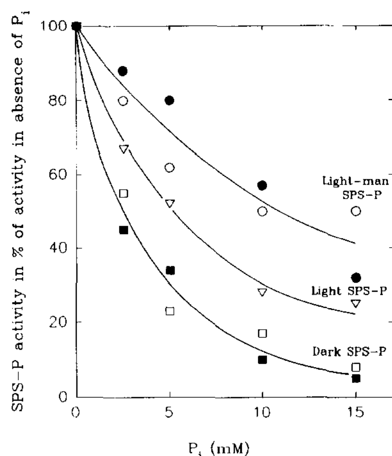

Fig. 2. Inhibition of SPS-P by P_i. Partially purified preparations of SPS-P from darkened (■,□), illuminated (▽) and illuminated mannose fed leaves (●,○) were assayed as indicated either with phospho-SPS preparations from darkened (closed symbols) or illuminated mannose fed leaves. SPS-P was measured using in vitro phosphorylated SPS as a substrate. The activity of SPS-P is expressed as initial velocity of the increase in the activity of SPS (mU · ml^{-1} · min^{-1}) during the preincubation of both enzymes with rising concentrations of P_i. Each incubation mix contained about 0.9 units · ml^{-1} SPS (V_{max}). The data are based on three independent experiments: 1, ■,●; 2, □,○ and 3, ▽,○. The ○-data are mean values of the results of experiment 2 and 3.

fed leaves. The amount of SPS-P activity in the summed fractions of a Mono-Q peak was also very similar in these preparations (see below for data). Obviously the apparent light activation of SPS-P previously observed in crude extracts [7] cannot be attributed to a major increase in the total SPS-P activity in leaves.

3.2. *SPS-phosphatase activity apparently occurs in multiple forms which differ in their sensitivity towards inhibition by P_i*

Previous studies have shown that the apparent SPS-P is inhibited by inorganic phosphate concentrations, in the physiological range [7,17]. It was concluded that a reduction of the cytosolic P_i pool after illumination of leaves or after feeding leaves with the P_i-sequestrating sugar mannose might play a role in signalling light/dark changes to SPS-P and, in turn, to SPS. Therefore, it was of interest to characterize the inhibition of SPS-P by P_i in more detail. As shown in Fig. 2, pronounced differences in the sensitivity of SPS preparations towards P_i inhibition were obtained. Preparations from darkened leaves were more strongly inhibited by P_i (IC_{50} = 2 mM) than preparations from illuminated leaves (IC_{50} = 5 mM) or from mannose-fed leaves (IC_{50} = 11 mM). No differences in P_i sensitivity were obtained when phospho-SPS preparations from darkened leaves, or illuminated and mannose fed leaves were used as substrate for a given SPS-P preparation. Thus, the P_i sensitivity depends on SPS-P rather than on the phospho-SPS preparation used. We conclude that SPS-P activity apparently exists in multiple forms which differ in their sensitivity towards inhibition by P_i.

These results at least partly explain the apparent diurnal changes in SPS-P activity reported earlier [7]. Spinach leaves contain up to 30 μmol P_i per gram fresh weight depending on the supply of nutrients during growth (data not shown). The desalting efficiency with viscous crude extracts is often less than 80% (data not shown). Such extracts prepared with a low ratio of volume of extraction buffer to fresh weight of leaf tissue therefore often contained up to 2 mM P_i even after desalting, which would lead to a large inhibition of SPS-P activity in the dark-extract sample, but not in an extract from mannose-fed leaves (see Fig. 2). Further, leaf extracts apparently contain additional inhibitors other than P_i which are not removed by desalting on a Sephadex G-25 column (H. Weiner and S.C. Huber, unpublished results).

To investigate the mode of P_i inhibition, we determined the apparent affinity of SPS-P towards phospho-SPS in the presence and absence of P_i. In partially purified enzyme preparations from mannose-fed leaves, P_i was a competitive inhibitor with respect to phospho-SPS (Fig. 3A). A secondary plot of slopes of the lines in the double reciprocal plot versus P_i concentration revealed a linear competition (not shown) and an apparent dissociation constant of 3 mM for the P_i–SPS-P complex, assuming that P_i binds to the substrate binding site of SPS-P. A more detailed kinetic analysis is necessary to differentiate whether the apparent P_i inhibition of SPS-P is due to binding of P_i directly to SPS-P, or to binding of P_i to phospho-SPS. In contrast, P_i decreased the V_{max} activity of SPS-P from darkened leaves (Fig. 3B). Secondary plots of the slopes of the lines and their intercepts with the y-axis versus P_i gave rise to curves plots (not shown) indicating a complex inhibition type. The two SPS-P activity forms also differed slightly in their apparent K_m values for the target phosphoprotein; in two independent experiments values were 0.36 and 0.43, and 0.55 and 0.70 units · ml^{-1} for the SPS-P forms from illuminated and darkened mannose-fed leaves, respectively. Thus, dark enzymes seem to have a slightly lower affinity towards phospho-SPS than enzymes from mannose-fed leaves. The striking difference regarding inhibition of the two activity forms of SPS-P by P_i shown in Fig. 2 therefore appears to involve a change in the mode of inhibition, with P_i being a much more potent inhibitor for the dark-form than for the light-form of SPS-P activity, especially in the presence of near saturating or saturating concentrations of phospho-SPS.

3.3. *Light per se is not likely to be involved in the apparent 'light activation' of SPS-phosphatase*

Recently we observed that pretreatment of leaves in the dark with cycloheximide (CHX) reduced the apparent 'light activation' of SPS-P in vitro, and dramatically

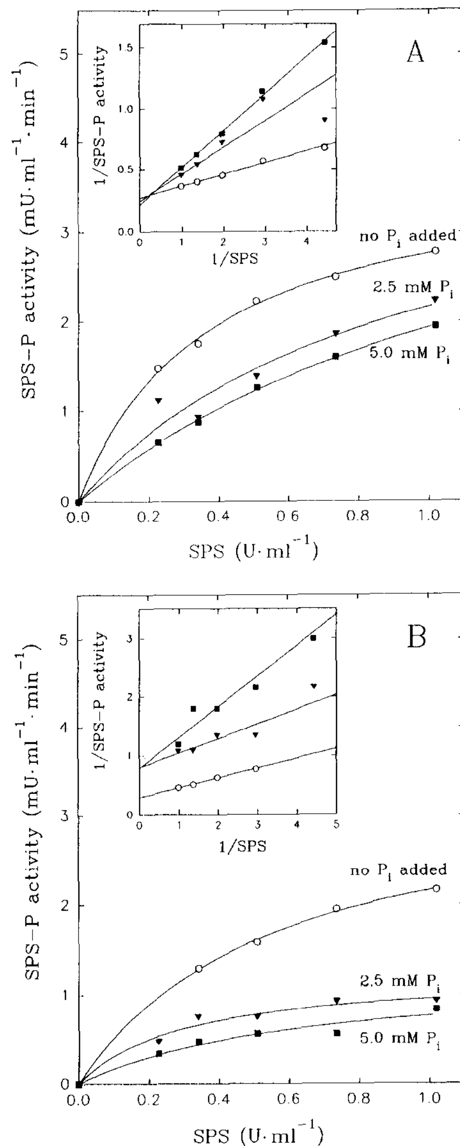

Fig. 3. Kinetic analysis of the P_i inhibition of the activity of the partially purified SPS-P preparations from illuminated and mannose fed leaves (A) and from darkened leaves (B). Phospho-SPS preparations (units · ml^{-1}, V_{max}) from darkened leaves were used as a substrate for SPS-P (activity of SPS-P as explained in Fig. 2). The experimental data points are presented as the phospho-SPS saturation curves with double reciprocal plots shown as insert. Assuming Michaelis-Menten kinetics, these curves were fitted to the data points using non-linear regression. The results of one experiment are shown, which were confirmed in a second independent experiment (data not shown).

reduced the activation state of SPS in vivo [7]. This suggests that a protein synthesis step may be involved in this activation of SPS-P by light. The results of our present work explain this apparent 'light activation' of SPS-P as a change in SPS-P, involving decreased sensitivity towards P_i (and certain phosphorylated metabolites) during illumination of leaves. Thus, it was of interest to see whether pretreatment of leaves with CHX prior to feeding mannose prevents: (a) the decrease in the sensitivity of SPS-P towards P_i and (b) the mannose activation of SPS in vivo.

As shown in Fig. 4, feeding mannose to leaves leads to the activation of SPS in vivo in the dark, as well as in the light. This is paralleled by a decrease in the P_i-sensitivity of SPS-P in the dark as well as in the light (Fig. 5). Feeding CHX prevented the mannose-dependent rise in SPS activation in the dark and in the light (Fig. 4). Cycloheximide had no effect on the accumulation of mannose 6-phosphate in leaves during the mannose feeding (data not shown), showing that CHX is not acting by interfering with the sequestration of P_i. Instead, CHX blocked the decrease in the sensitivity of SPS-P towards P_i inhibition during the mannose treatment (Fig. 5). These results show, that light per se is not necessary to mediate the 'activation' of SPS-P, and that the trigger is more likely to involve changes in phosphate status. They also indicate that protein synthesis could be involved in this 'activation' of SPS-P.

3.4. *Effect of phosphorylated compounds on SPS phosphatase activity*

We next investigated whether the inhibition of SPS-P is specific for P_i. As already mentioned, preparations from dark or light-mannose pretreated leaves contain similar overall SPS- and similar casein-phosphatase activities (Table I). Both phosphatase preparations were inhibited by P_i, 3-*P*-glycerate (3PGA), dihydroxyacetone phosphate (DHAP), but not by glucose-6-phosphate (G6P). The differences in the sensitivity of SPS-P from dark or light-mannose tissue towards inhibition by P_i were also found for 3PGA and DHAP. During the assay of SPS-P we observed a release of phosphate from DHAP and 3PGA equivalent to 40% of the compound added. The inhibition of SPS-P by these compounds cannot be solely attributed to this phosphate release.

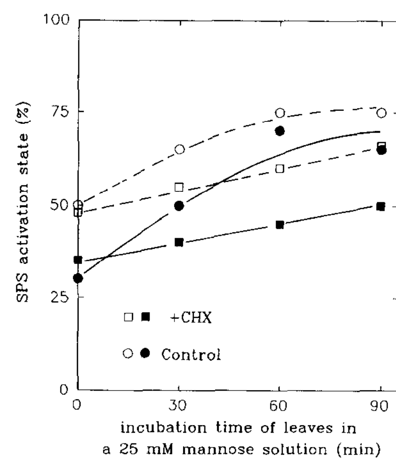

Fig. 4. Cycloheximide (CHX) reduces mannose activation of SPS in vivo. Detached spinach leaves were fed with 10 μM CHX or with water (control) in the light (open symbols) or in the dark. After 2 h the leaves were transferred into a mannose solution (25 mM) with and without illumination as before. At the times indicated, leaf material was removed for crude-extraction and assay of SPS activity (expressed as % activation state). The data are based on two independent experiments (□,○ and ■,●).

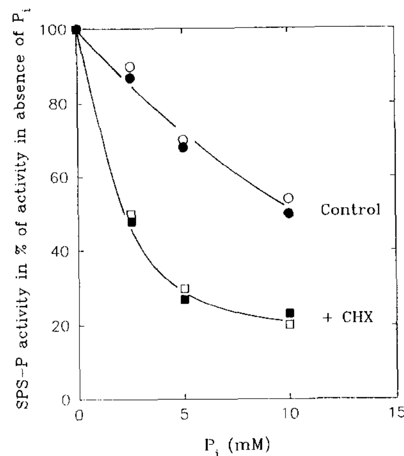

Fig. 5. CHX blocks the mannose induced decrease of the sensitivity of SPS-P towards inhibition by P_i. Leaves were fed with and without CHX pretreatment as described in Figure 3. After 90 minutes of mannose feeding, leaves were extracted to obtain partially purified SPS-P. SPS-P activity was determined in the presence of rising P_i concentrations as described in Fig. 2. Two independent experiments are shown (■,● and □,○) using enzyme preparations from darkened (closed symbols) and illuminated leaves.

We tentatively conclude that the effects of P_i on SPS-P activity is at least in part non-specific and that certain other phosphorylated compounds (with some exceptions like G6P) inhibit SPS-P activity in a manner similar to P_i.

The SPS phosphatase measurements shown in Table I were repeated with diluted crude extract instead of partly purified preparations (see section 2) and very similar results were obtained (data not shown). This indicates that the differential inhibition by P_i and phosphate esters on SPS-P activity is not generated due to changes occurring during purification. The data on Table I were not effected by the presence or absence of $MgCl_2$ during measurements of protein phosphatase activities (data not shown). Thus, it seems to be unlikely that magnesium ions, which are known to interfere with P_i and phosphorylated compounds have contributed to the apparent inhibition observed with these compounds.

3.5. *Comparison of casein- and SPS-phosphatase activities*

Next, we examined whether this inhibition by P_i is enzyme- or substrate-directed. To do this we investigated the effects of the compounds tested in Table I on the casein-phosphatase activity which coeluted with SPS-phosphatase from the Mono-Q gradient. Unlike SPS-P activity, casein dephosphorylation was only weakly inhibited by P_i and phosphorylated intermediates, and no difference was found between enzyme preparation from darkened, or illuminated and mannose treated leaves. Dephosphorylation of SPS and casein also differed in that vanadate at 1 mM almost completely inhibited SPS-P activity, whereas casein phosphatase was only slightly affected. No further inhibition of casein phosphatase was observed with up to 4 mM vanadate (data not shown). NaCl also inhibited SPS-P more strongly than casein phosphatase activity. Taken together it appears, that the differential inhibition by P_i of SPS-P in dark or light-mannose preparations is substrate-specific (i.e. restricted to dephosphorylation of phospho-SPS). Also, as indicated by the different responses to vanadate and NaCl, it appears possible that phospho-SPS and -casein may be dephosphorylated by different phosphatases of the type 2A.

3.6. *Properties of phosphatase forms of higher molecular weight and their catalytic subunits*

SPS can be dephosphorylated and activated by the isolated catalytic subunit of protein phosphatase 2A from rabbit skeletal muscle (unpublished results with enzyme preparations kindly provided by Dr. G. Mieskes; see also [6]). The catalytic subunits of type 2A protein phosphatases (about 36 kDa) are known to behave differently from native 2A phosphatases [18]. Further, catalytic subunits are known to be released from enzyme forms of higher molecular weight during purification under certain conditions [13]. We therefore

Table I

Comparison of the effects of P_i, phosphate-esters, vanadate and NaCl on the activities of SPS-phosphatase and the coeluting casein phosphatase activity in partly purified SPS phosphatase preparations from darkened or illuminated and mannose fed leaves

Additions to preincubation and assay	SPS phosphatase activity ($mU \cdot ml^{-1} \cdot min^{-1}$)		Casein phosphatase activity ($mU \cdot ml^{-1}$)	
	Dark	Light-mannose	Dark	Light-mannose
None	2.0	2.4	0.95	1.05
P_i (5 mM)	0.38	2.0	0.57	0.68
3-*P*-Glycerate (5 mM)	0.20	1.6	0.74	0.91
DHAP (5 mM)	0.80	2.0	0.60	0.75
Glucose-6-P (5 mM)	2.0	2.6	0.90	1.09
Vanadate (1 mM)	<0.1	<0.1	0.59	0.70
NaCl (100 mM)	0.96	1.2	0.68	0.80

DHAP: dihydroxyacetone-phosphate. Phosphatase preparations were preincubated with effectors prior to assay for 5 min at 4°C.

checked whether the changes in P_i sensitivity could be due to a selective release of the catalytic subunit during purification. During gelfiltration of the Mono-Q peak fractions of SPS-P obtained from dark and light-mannose preparations on a Sephacryl S200 HR column (Pharmacia), protein phosphatase activity completely eluted as higher molecular weight forms above 140 kDa (data not shown). This provides evidence that the changes in Fig. 2 are not due to the release of the catalytic subunits per se from protein phosphatase 2A holoenzymes during purification.

After treating partly purified SPS-P preparations with ethanol according to [3] to obtain the catalytic subunit of protein phosphatase(s) 2A we observed dramatic changes in the affinities to substrates and inhibitors. With phospho-casein as substrate, the apparent K_m and V_{max} values increased from 1.5 μM and 2.5 mU $\cdot$ ml^{-1}, respectively, prior to ethanol treatment, to 5 μM and 10 mU $\cdot$ ml^{-1} after ethanol treatment. No differences were found between dark and light-mannose enzymes prior to or after ethanol treatment. The casein phosphatase activity of the catalytic subunit seems to be suppressed by its interactions with other components of the native 2A phosphatase as has been reported for animal phosphatase 2A [13]. P_i was a much more potent inhibitor of casein phosphatase activity after the ethanol treatment (I_{05} = 0.8 mM after, compared to 8 mM prior to ethanol treatment).

In contrast to casein phosphatase activities, we observed a 5- to 10-fold decrease in the activity of SPS-phosphatase after ethanol treatment. Activity was too low for accurate estimation of apparent kinetic constants. These results indicate that the isolated catalytic subunit of SPS-phosphatase has a very low activity with SPS and/or that the activity of this catalytic subunit was not sufficiently recovered after the ethanol treatment. Currently, we are trying to differentiate between these options as a prerequisite for further studies to explain the mechanisms which determine the specificities of type 2A protein phosphatase towards different phospho-enzymes.

4. CONCLUDING REMARKS

The signal transduction pathway mediating SPS activation apparently consists of a cascade involving covalent modification of SPS and interconversion of different forms of SPS-P activity. This could provide a very sensitive system to modulate the rate of sucrose synthesis. Since interconversion of SPS-P (this paper) and SPS [8] alters their sensitivity to regulation by P_i and metabolites, this cascade will stimulate sucrose synthesis even when cytosolic metabolites remain relatively constant, as is sometimes the case in light-dark transitions [19]. The nature of the signal which triggers the interconversion of SPS-P still needs to be elucidated; it does not require light, but is linked directly or indirectly to changes in phosphate status, and may involve protein synthesis.

Our results provide evidence that the catalytic subunit of SPS phosphatase is likely to be complexed to other proteins in plant extracts [20] which, in analogy to animal phosphatases 2A, may function as 'targeting' and 'regulatory' factors (see [21] for references). In this context, it is highly significant that SPS-P exists in different kinetic forms in vivo in plants. The molecular basis for the interconversion of different kinetic forms of SPS-P is unclear at present, and the next step will be to determine whether an existing protein is being modified, possibly by exchange of such regulatory factors binding to SPS-P, or by phosphorylation of SPS-P [22,23].

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