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Inactivation of Highly Activated Spinach Leaf Sucrose-Phosphate Synthase by Dephosphorylation[1]

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ABSTRACT

Spinach (*Spinacia oleracea* L.) leaf sucrose-phosphate synthase (SPS) can be phosphorylated and inactivated *in vitro* with [γ-^{32}P]ATP (JLA Huber, SC Huber, TH Nielsen [1989] Arch Biochem Biophys 270: 681–690). Thus, it was surprising to find that SPS, extracted from leaves fed mannose in the light to highly activate the enzyme, could be inactivated in an ATP-*independent* manner when desalted crude extracts were preincubated at 25°C before assay. The "spontaneous" inactivation involved a loss in activity measured with limiting substrate concentrations in the presence of the inhibitor, Pi, without affecting maximum catalytic activity. The spontaneous inactivation was unaffected by exogenous carrier proteins and protease inhibitors, but was inhibited by inorganic phosphate, fluoride, and molybdate, suggesting that a phosphatase may be involved. Okadaic acid, a potent inhibitor of mammalian type 1 and 2A protein phosphatases, had no effect up to 5 micromolar. Inactivation was stimulated about twofold by exogenous Mg^{2+} and was relatively insensitive to Ca^{2+} and to pH over the range pH 6.5 to 8.5. Radioactive phosphate incorporated into SPS during labeling of excised leaves with [^{32}P]Pi (initially in the dark and then in the light with mannose) was lost with time when desalted crude extracts were incubated at 25°C, and the loss in radiolabel was substantially reduced by fluoride. These results provide direct evidence for action of an endogenous phosphatase(s) using SPS as substrate. We postulate that highly activated SPS contains phosphorylated residue(s) that increase activation state, and that spontaneous inactivation occurs by removal of these phosphate group(s). Inactivation of SPS *in vivo* caused by feeding uncouplers to darkened leaf tissue that had previously been fed mannose in the dark, may occur by this mechanism. However, there is no evidence that this mechanism is involved in light-dark regulation of SPS *in vivo*.

Regulation of SPS[2] activity is thought to be one of the components that controls the flux of carbon into sucrose, and hence translocation, *in situ* (for review see ref. 19). One level of control that has received considerable recent attention is covalent modification of the enzyme. Stitt *et al.* (20) demonstrated that light activation of spinach leaf SPS *in vivo* was only manifested as increased velocity, *v*, of the enzyme assayed at limiting substrate concentrations and with the inhibitor, Pi. They also showed that the SPS activation state (defined as $v/V_{max} \times 100$) was increased in darkness by feeding mannose to leaf discs (20). Thus, the effect of light was clearly indirect, and was possibly mediated by changes in availability of cytoplasmic Pi.

The mechanism underlying the changes in SPS activity with light/dark transitions was unequivocally demonstrated to involve a covalent modification, as activity changed while the amount of SPS protein (measured immunochemically) remained constant (22). Reversible protein phosphorylation was implicated in control of the activation state of SPS. This was based on the observation that radioactive phosphate was incorporated into the 120-kD SPS subunit by feeding [^{32}P]Pi to excised leaves and the demonstration that partially purified SPS could be phosphorylated and inactivated *in vitro* using [γ-^{32}P]ATP (9). Thus, phosphorylation of SPS occurred both *in vivo* and *in vitro*, and in both systems, the more highly phosphorylated enzyme had a lower activation state. Attempts to study the biochemical mechanism involved have been successful because the spinach leaf enzyme is often relatively stable (for short periods of time) at room temperature.

The work we report here focused on the highly activated enzyme extracted from illuminated leaves that were fed mannose. We compared this SPS form with the relatively inactive (extracted from darkened leaves) and intermediate enzyme-forms (extracted from leaves illuminated without mannose). We observed that highly activated enzyme was not stable when desalted crude extracts were preincubated at 25°C. Our study of this loss in activation state indicates that the "spontaneous" inactivation is mediated by an endogenous phosphatase(s), which constitutes evidence for a novel regulatory covalent modification of SPS.

MATERIALS AND METHODS

Materials

Biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and phosphorous-32 radionucleotide[3] was obtained from New England Nuclear (Boston, MA).

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[2] Abbreviations: SPS, sucrose-phosphate synthase; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluromethoxyphenylhydrazone.

[3] Mention of a trademark or proprietary product does not consti-

Plant Material and Experimental Treatments

Spinach (*Spinacia oleracea* L. cv Dark Green Bloomsdale) was grown in soil in a growth chamber with a 12-h photoperiod (400 μmol·m^{-2}·s^{-1}) and 22°C day/18°C night temperature regime. Maize (*Zea mays* L. cv Pioneer 3184) was used in one experiment (Table VI). Maize plants were grown in soil in a greenhouse as previously described (8).

Sorbitol and mannose were supplied to leaves according to two different protocols. In one experiment, leaf discs (1-cm diameter) were floated on solutions (0.2 M) in darkness. In all other experiments, mannose (50 mM) was fed to excised leaves in the light through the transpiration stream.

Extraction and Assay of SPS

Leaf extracts were prepared in grinding buffer containing 50 mM Mops-NaOH (pH 7.5), 15 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, and 0.1% (v/v) octyl phenoxypolyethoxyethanol (Triton X-100) as previously described (8). The supernatant fluids were immediately desalted on Sephadex G-25 columns equilibrated with grinding buffer minus the Triton X-100. Extracts were kept at 0°C or preincubated at 25°C prior to assay as indicated in the text. SPS activity was assayed under either limiting substrate concentrations plus the inhibitor Pi ("limiting assay") or V_{max} substrate concentrations without Pi ("V_{max} assay") as the time-dependent formation of sucrose plus sucrose-P from UDP-Glc and Fru-6-P. For the limiting assay, 45 μL of tissue extract was incubated 10 min at 25°C with 10 mM UDP-Glc, 10 mM Pi, 3 mM Fru-6-P, 12 mM Glc-6-P (an activator of SPS), 50 mM Mops-NaOH (pH 7.5), 15 mM $MgCl_2$, and 2.5 mM DTT in a total volume of 70 μL. In the V_{max} assay, Pi was omitted, and the concentration of Fru-6-P was increased to 10 mM and Glc-6-P to 40 mM. Reactions were terminated by addition of 70 μL of 30% KOH and the tubes were placed in a boiling water bath for 10 min. After cooling, 1 mL of 0.14% anthrone in 13.8 N H_2SO_4 was added, and the tubes incubated at 40°C for 20 min prior to measuring absorbance at 620 nm.

Dephosphorylation of ^{32}P-SPS *In Vitro*

Radioactive phosphate was incorporated into the 120-kD subunit of spinach leaf SPS by placing the cut petiole of an excised leaf into 1 mL containing 50 μM Pi + 1 mCi [^{32}P]Pi. The liquid was replaced with degassed water as it was depleted by transpiration. After 2 h in the dark, the leaf was transferred to the light (400 μmol·m^{-2}·s^{-1}) and the leaf was fed mannose (50 mM). After 3 h of mannose-feeding, a leaf extract was prepared and desalted to remove low mol wt components and unincorporated radioactive Pi. The desalted extract was incubated at 25°C in the presence or absence of 20 mM KF. At 0, 30, and 60 min, SPS protein was precipitated by addition of a solution containing monoclonal antibodies (23), 10 mM KF, and 1 mM Na_3VO_4 (final concentrations). Immune complexes formed in 2 h at 0°C were precipitated by Immunoprecipitin (BRL, Bethesda, MD). After an additional 30 min on ice, the pellets were collected and washed (9) and the proteins in them dissociated and fractionated on 10% SDS polyacrylamide gels (9). The 120-kD subunit of SPS was excised and radioactivity determined by liquid scintillation counting.

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RESULTS

Inactivation of SPS *In Vitro*

Light activation of spinach leaf SPS is apparent as an increase in activity measured using the limiting assay while activity in the V_{max} assay remains relatively constant (Table I) (9, 20, 22). Hence, light activation involves an increase in the activation state of the enzyme, calculated as the ratio of the two activities. As previously reported (9), the light enzyme can be inactivated *in vitro* by preincubation with Mg·ATP as a result of phosphorylation catalyzed by an endogenous protein kinase(s). The ATP-dependent inactivation achieved *in vitro* was qualitatively and quantitatively similar to that which occurs during light modulation *in vivo* (Table I). In the absence of ATP, the enzyme was relatively stable when preincubated at 25°C (ref. 9; Table I).

The activation state of SPS *in situ* can be further increased by feeding mannose to excised leaves in the light. SPS activated in this way often has an activation state that ranges from 50 to 80% (*cf.* Tables I, II). We observed that such highly activated enzyme preparations were labile when preincubated at room temperature before assay. As shown in Table II, preincubation at 25°C for 1 h resulted in a substantial reduction in enzyme activity measured in the limiting assay while V_{max} remained essentially constant; consequently, the activation state of the enzyme was reduced from about 65% to 40%. The inactivation that occurred was not dependent on ATP, as nucleotides were not added and the extracts were desalted to remove endogenous components. This "spontaneous" inactivation was not affected by addition of exogenous carrier proteins or a mixture of protease inhibitors (Table II). Thus, proteolytic modification of SPS was probably not involved in the inactivation.

The spontaneous inactivation of highly activated SPS at

Table I. *Changes in Spinach Leaf SPS Activity as Affected by Light/Dark Transitions and Preincubation of Extracts at 25°C*

Leaves were harvested in darkness or after exposure to light for 30 min. Extracts from light leaves were preincubated at 0 or 25°C for 30 min in the absence or presence of 2 mM Mg·ATP before assay.

Leaf Harvest	Enzyme Pretreatment	SPS Activity		Activation State[b]
		Limiting assay[a]	V_{max} assay	
		μmol·g^{-1} fresh wt·h^{-1}		%
Dark	0°C	10.3	69.9	15
Light	0°C	24.6	77.6	32
Light	25°C	22.8	75.6	30
Light	25°C + ATP	7.0	72.8	10

[a] Velocity, *v*, was determined under suboptimal substrate concentrations and in the presence of Pi. [b] Activation state is *v* (limiting assay), expressed as a percent of V_{max}.

Table II. *Protease Inhibitors and Exogenous Carrier Proteins do not Stabilize Highly Activated Spinach Leaf SPS*

SPS was extracted from spinach leaves fed 50 mM mannose in the light for 3 h. Some of the desalted extracts were amended to include exogenous proteins or a mixture of protease inhibitors (1 μg/mL leupeptin; 1 mM PMSF; 1 mM benzamidine; 1 mM antipain) before preincubation at 0 or 25°C for 1 h before assay. See Table I.

Additions to Pretreatment	SPS Activity		Activation State
	Limiting assay	V_{max} assay	
	$\mu mol \cdot g^{-1}$ fresh $wt \cdot h^{-1}$		%
None (0°C)	48.0	74.5	64
None (25°C, 1 h)	26.7	68.9	39
+ Protease inhibitors	32.0	71.6	45
+ Casein (0.8 mg/mL)	26.4	58.0	46
+ BSA (1 mg/mL)	25.0	69.0	36

25°C occurred in a time-dependent manner and could be substantially prevented by KF. As shown in Figure 1, in the absence of KF, the SPS activation state decreased progressively with time; maximal inactivation occurred after about 60 min at 25°C. Addition of 2 mM Mg·ATP after 60 min resulted in some additional inhibition of activity. The spontaneous inactivation was almost completely prevented by inclusion of 20 mM KF, whereas Mg·ATP-dependent inactivation was not affected (Fig. 1).

The effect of KF concentration on enzyme activity and the spontaneous inactivation of highly activated SPS is shown in Figure 2. Enzyme maintained at 0°C was stable for 60 min (Fig. 2A), and KF (up to 30 mM) had no effect on enzyme

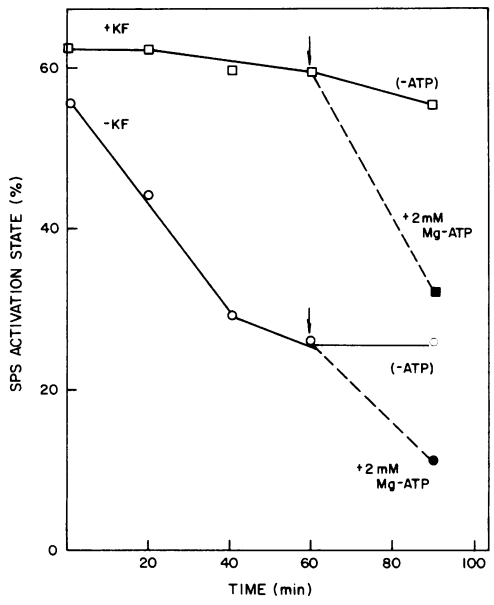

Figure 1. Spontaneous inactivation of spinach leaf SPS *in vitro*. Desalted extracts, prepared from leaves fed 50 mM mannose in the light, were preincubated at 25°C in the presence (squares) or absence (circles) of 20 mM KF. As indicated, preincubation mixtures were supplemented with 2 mM Mg·ATP after 60 min. Activation state is *v* (limiting assay), expressed as a percent of V_{max}.

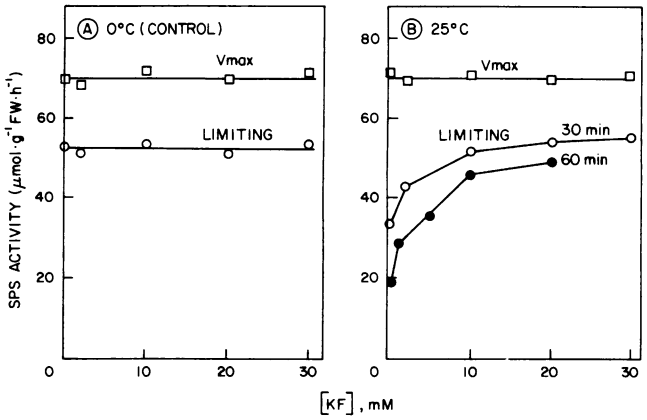

Figure 2. Prevention of the spontaneous inactivation of spinach leaf SPS by KF. Desalted extracts, prepared from leaves fed 50 mM mannose in the light, were amended to include the indicated concentration of KF and then preincubated at (A) 0°C for 60 min or (B) 25°C for 30 or 60 min prior to assay. In panel (B), V_{max} data are the means of the 30- and 60-min values.

activity in either the limiting or V_{max} assay. Thus, KF (carried over to the assay cocktail) had no effect on catalytic activity *per se*. As expected, when enzyme was preincubated at 25°C in the absence of KF there was a time-dependent loss in limiting activity while V_{max} remained constant. Millimolar concentrations of KF prevented the loss in activation state, and the maximum effect required about 20 mM KF (Fig. 2). Addition of KF to enzyme that had been preincubated at 25°C for 30 to 60 min had essentially no effect (data not shown); thus, KF prevented but did not reverse the spontaneous inactivation.

The effects of other phosphatase inhibitors and phosphorylated compounds on the spontaneous inactivation of SPS were tested. As shown in Table III, Na_3VO_4 (1 mM) and okadaic acid (5 μM), a type 1 and 2A protein phosphatase inhibitor (4, 12), had no effect, whereas $(NH_4)_2MoO_4$ (1 mM) blocked inactivation. Because certain phosphatase inhibitors were effective, we also tested several P-esters for their ability to stabilize SPS activity. Several P-esters were also effective, such as 3-P-glycerate and the nonphysiological compound *p*-nitro-

Table III. *Effect of Phosphatase Inhibitors and P-Esters on Stability of SPS Activation State* in vitro

SPS was extracted from spinach leaves fed 50 mM mannose in the light for 3 h.

Enzyme Pretreatment	SPS Activity		Activation State
	Limiting assay	V_{max} assay	
	$\mu mol \cdot g^{-1}$ fresh $wt \cdot h^{-1}$		%
None (0°C)	36.0	66.2	54
None (25°C, 1 h)	20.6	65.8	31
+ Na_3VO_4 (1 mM)	22.9	70.4	33
+ okadaic acid (5 μM)	19.0	65.2	29
+ $(NH_4)_2MoO_4$ (1 mM)	37.1	70.7	53
+ 3-P-glycerate (5 mM)	39.0	73.2	53
+ p-nitrophenyl-P (5 mM)	30.4	54.2	56

phenyl-P (Table III). Because P-esters are substrates for phosphatase action in crude extracts, we tested the ability of Pi to stabilize SPS activity. As shown in Figure 3, millimolar concentrations of Pi resulted in a near-complete stabilization of activity. Because Pi is an inhibitor of SPS activity, and there was some carryover of Pi (from the preincubation mixture into the assay cocktail), there was some inhibition of V_{max} activity by Pi. Nonetheless, these results indicate that low concentrations of Pi could effectively block the spontaneous inactivation of SPS, and that some of the apparent stabilization of P-esters may be attributed to liberation of Pi as a result of specific or nonspecific phosphatase action on the P-esters. However, it is unlikely that Pi liberation can completely explain the effect, and thus, some direct effect of the P-esters may be involved as well.

Dependence of Spontaneous Inactivation on Exogenous Bivalent Cations and pH

To further characterize the spontaneous inactivation, the dependence on bivalent cations and pH of the preincubation mixture was tested. In the absence of exogenous bivalent cations, some loss of enzyme activation state was observed (Table IV). However, addition of bivalent cations stimulated the loss of activation state. In particular, Mg^{2+} was more effective than Ca^{2+}. The enhancement of inactivation by bivalent cations, and their relative effectiveness, is reflected in the SPS activation states achieved after a 1-h incubation at 25°C. In the presence of $MnCl_2$, activity measured in the limiting assay (which contains 10 mM Pi) was extremely low whereas V_{max} activity was high, thereby giving a very low (8%) activation state. The effect of Mn^{2+} was not time-dependent, but was dependent upon the presence of Pi in the assay mixture. The low activity in the limiting assay was apparently caused by precipitation of manganese phosphate, which then bound soluble proteins in the crude extract in a rather nonspecific manner (data not shown). When "bound" to the insoluble manganese phosphate, SPS was apparently not enzymatically active. Thus, it was difficult to evaluate the effect of Mn^{2+} on the spontaneous inactivation reaction.

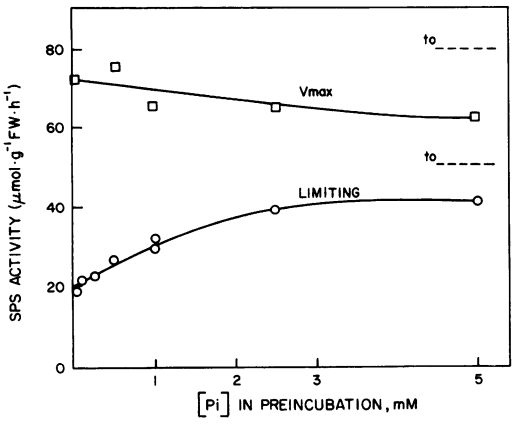

Figure 3. Prevention of the spontaneous inactivation of spinach leaf SPS by Pi. Desalted extracts, prepared from leaves fed 50 mM mannose in the light, were amended to include the indicated concentrations of Pi. The extracts were preincubated at 25°C for 60 min before assay. The dashed lines at right (t_0) are control (0 mM Pi) values obtained without extract preincubation (zero-time).

Table IV. *Effect of Exogenous Bivalent Cations on Stability of SPS Activation State* in vitro

SPS was extracted from spinach leaves fed 50 mM mannose in the light for 3 h. Extracts were desalted into a medium devoid of exogenous bivalent cations and containing 1 mM EDTA. Thus, the Mg^{2+} treatment in this table is equivalent to the control (25°C, 1 h) in Table III.

Enzyme Preincubation	SPS Activity		Activation State
	Limiting assay	V_{max} assay	
	$\mu mol \cdot g^{-1}$ fresh $wt \cdot h^{-1}$		%
0°C, 1 h	37.0	66.0	56
25°C, 1 h	29.6	65.0	46
+ $MgCl_2$ (5 mM)	20.6	61.0	34
+ $CaCl_2$ (5 mM)	26.8	65.9	41
+ $MnCl_2$ (5 mM)	5.4	69.4	8

The pH dependence of inactivation was tested over the range of pH 6.5 to 8.5. Relative to the extent of inactivation observed at pH 7.5 (standard preincubation condition), approximately 25% less inactivation occurred when the pH was lowered to pH 6.5 or raised to pH 8.5. Thus, the spontaneous inactivation was relatively insensitive to pH.

Loss of Radiolabel from ^{32}P-SPS

The results suggested that highly activated SPS was the substrate for an endogenous phosphatase(s) that removed one or more phosphate groups that increase activation state. To test directly for action of a phosphatase, radioactive phosphate was incorporated into the subunit of SPS by feeding [^{32}P]Pi to excised spinach leaves. After an initial labeling period of 2 h in the dark, the detached leaves were fed 50 mM mannose in the light (for an additional 3 h) to achieve a high activation state. After labeling with ^{32}P, leaf extracts were prepared, desalted, and incubated at 25°C in the presence or absence of 20 mM KF. In the absence of KF, substantial spontaneous inactivation was observed as activation state decreased from 72 to 50% during the 60-min time period (Fig. 4A). As expected (Figs. 1, 2), the inactivation was completely prevented by KF (Fig. 4A). Incubation of extracts at 25°C resulted in a substantial reduction in the amount of radiolabel associated with the SPS protein. In the absence of KF, approximately 54% of the ^{32}P was removed from the SPS protein during the 60-min period; in the presence of KF, loss of ^{32}P was substantially reduced (about a 20% decrease in the initial level). These results confirmed that SPS was substrate for one or more endogenous phosphatases. Moreover, the loss of ^{32}P from SPS, and the inhibition by fluoride, generally corresponded with the changes in enzyme activation state (Fig. 4, A and B). Apparently, there is more than one phosphatase involved, and/or more than one phosphorylation site because a 20% loss of ^{32}P from SPS occurred even in the presence of KF but inactivation was completely prevented.

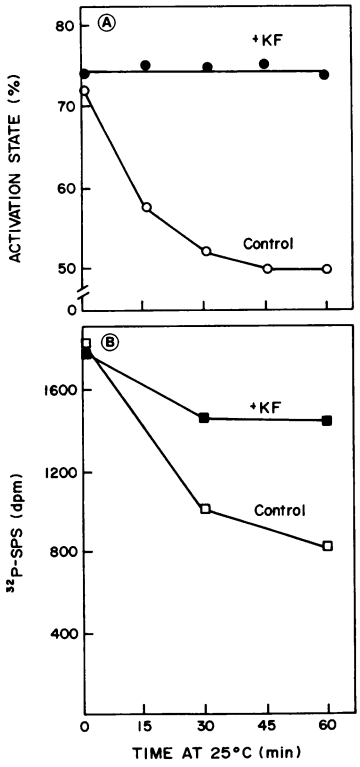

Figure 4. Dephosphorylation of ^{32}P-labeled SPS by endogenous phosphatase(s). Excised spinach leaves were fed [^{32}P]Pi in the dark for 2 h followed by mannose-feeding in the light for an additional 3 h. An extract was prepared, desalted, and incubated at 25°C in the presence and absence of 20 mM KF. At various times, aliquots were assayed for (A) SPS activation state and (B) radiolabel associated with the SPS subunit (approximately 3 μg SPS protein per aliquot).

Table V. *Uncouplers Reverse the Mannose Activation of SPS in Spinach Leaf Discs in the Dark.*

Leaf-Disc Treatment		SPS Activity		
Step 1 dark, 12 h	Step 2 dark, 3 h	Limiting assay	V_{max} assay	Activation State
		$\mu mol \cdot h^{-1} \cdot (g\ fresh\ wt)^{-1}$		%
Mannose	Sorbitol	31.1	38.2	81
	Sorbitol + CCCP (50 μM)	10.5	38.8	27
	Sorbitol + FCCP (5 μM)	11.1	44.9	25
Sorbitol	Sorbitol	10.0	42.1	24
	Sorbitol + FCCP (5 μM)	9.5	40.8	23

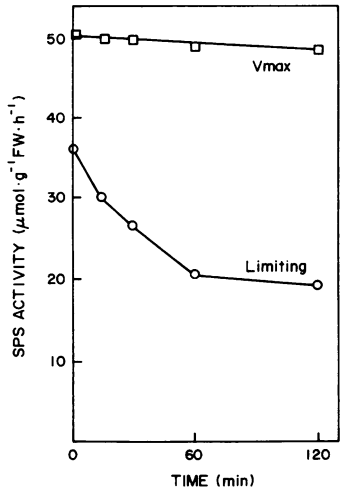

Figure 5. Uncoupler-induced inactivation of SPS *in situ*. Spinach leaf discs were fed 0.2 M mannose in the dark for 12 h. Then, the discs were transferred to sorbitol (0.2 M) that contained 5 μM FCCP. After the indicated times in darkness, extracts were prepared, desalted, and SPS was assayed (limiting and V_{max} assays). SPS in control leaf discs, transferred to sorbitol solution without uncoupler, was stable over the time period tested (data not shown).

Effect of Uncouplers on the Activation State of SPS *In Situ*

Having obtained evidence for spontaneous inactivation of highly activated SPS *in vitro*, we sought to determine whether a similar mechanism might occur *in situ*. As one approach to this question, spinach leaf discs were first treated with either 0.2 M mannose or sorbitol (control) for 12 h in the dark; the discs were then transferred to sorbitol in the presence or absence of an uncoupler for an additional 3 h in the dark. As shown in Table V, mannose activation was observed, relative to the sorbitol control, as increased activity in the limiting assays. The presence of CCCP or FCCP completely reversed the mannose-activation but had little effect on enzyme activity in the sorbitol control. Throughout all treatments and conditions, V_{max} remained essentially constant. These results suggested that ATP synthesis may be required to maintain the highly activated form of SPS. The inactivation that occurred in the presence of an uncoupler would not be expected to be an ATP-dependent process, as uncouplers would likely interfere with ATP production in the dark (5). It is possible that under these extreme conditions, spontaneous inactivation (*i.e.* non-ATP-requiring) was occurring *in vivo*.

The time course of uncoupler action is shown for FCCP in Figure 5. Addition of FCCP to leaf discs previously treated with mannose in the dark resulted in a time-dependent loss in activity of SPS measured in the limiting assay while V_{max} activity remained constant. The decrease in activation state was maximal after about 60 min. The SPS activation state of tissue not treated with FCCP was high and constant over the time period tested (data not shown). Other controls showed that there was no effect of adding FCCP or CCCP to the SPS assay cocktail (data not shown). Thus, the uncoupler-dependent changes in activation state are likely the result of changes in covalent modification of the SPS protein.

Evidence for Spontaneous Inactivation of Maize Leaf SPS

We also examined the stability of maize leaf SPS *in vitro* in order to determine whether the results obtained with spinach may apply to other species. Maize SPS is also activated by light (10, 18), but unlike spinach, light activation increases both the limiting and V_{max} activities (8). Another difference between the two species is that in maize, illumination of leaves (without mannose-feeding) is sufficient to highly activate the enzyme. Table VI shows that light-activated maize leaf SPS was labile in extracts at 25°C. Preincubation of the extract at 25°C for 60 min resulted in a substantial loss of activation state and also V_{max} activity. The spontaneous inactivation was substantially reduced by 20 mM KF (Table VI). Thus, light activated maize leaf SPS appears to behave similarly to light plus mannose-activated spinach SPS. However, we have been unable to completely prevent inactivation of maize SPS with KF. Nonetheless, the results suggest that the observations made with spinach may apply to other species as well.

DISCUSSION

The results obtained in the present study demonstrate that highly activated spinach leaf SPS rapidly loses activation state when preincubated at 25°C prior to assay. We refer to this inactivation as "spontaneous" to distinguish it from the ATP-dependent mechanism which involves protein phosphorylation (9). The spontaneous inactivation reported in this paper is apparently mediated by an endogenous phosphatase. This conclusion is based on two lines of evidence: (a) the inactivation was blocked by several classical phosphatase inhibitors [KF, $(NH_4)_2MoO_4$, and Pi]; and (b) *in vitro* loss of radiolabel from ^{32}P-SPS followed a similar time course and inhibitor sensitivity to that of loss of SPS activation state. The overall conclusion is that phosphorylation of certain site(s) increases the activation state of the enzyme. This implies that regulation of SPS by phosphorylation is complex, because phosphorylation of other site(s) results in inactivation of the enzyme (Table I; Fig. 1) (9).

Does the spontaneous inactivation occur *in vivo*? It may, but only under extreme conditions, as when mannose-treated tissue is challenged with an uncoupler. Under these conditions, SPS activation state declines rapidly in an apparently ATP-independent mechanism (Fig. 5). However, spontaneous inactivation during a normal dark-light transition remains undemonstrated. We postulate that it does not occur, because the light-activated enzyme is relatively stable when preincubated at 25°C (Table I). Moreover, spontaneous inactivation, when observed, does not lead to complete loss of activation state. Rather, activation state typically decreased from the initial high level (50–80%) to a minimum of about 30 to 40%. Furthermore, if this was the mechanism of dark inactivation (*i.e.* dephosphorylation inactivates the enzyme), then one would expect more radioactive phosphate to be incorporated in the activated enzyme. However, the reverse is true (9), which is consistent with inactivation *in vivo* occurring by a protein phosphorylation reaction.

We have recently identified a protein phosphatases(s) that dephosphorylates and activates SPS extracted from darkened spinach leaves (6). The spontaneous activation was inhibited by Pi, molybdate, vanadate and, most notably, okadaic acid (6). Okadaic acid is a specific inhibitor of type 1 and type 2A protein phosphatases (for review see ref. 4). Feeding okadaic acid to excised spinach leaves in the dark blocked subsequent light activation of SPS without affecting photosynthetic rate (6). These findings established that light-dark regulation of SPS *in situ* involves a phosphorylation (inactivation)/dephosphorylation (activation) cycle.

Although protein phosphorylation is well known to occur in plants, relatively few specific enzymes are known to be regulated by phosphorylation (1). Moreover, little is known about the plant protein kinases and protein phosphatases involved in the reversible phosphorylation and dephosphorylation processes. The first plant enzyme shown to be regulated by reversible phosphorylation was the mitochondrial pyruvate dehydrogenase complex [PDC (15, 16)]. Associated with the complex are the regulatory converter enzymes (pyruvate dehydrogenase kinase and phosphatase) which phosphorylate (inactivate) and dephosphorylate (activate) PDC (13, 17). The pyruvate dehydrogenase protein phosphatase associated with the complex requires millimolar concentrations of Mg^{2+} and the Mg^{2+}-dependent activation is inhibited by Ca^{2+} (2). Other protein phosphatases have been identified and partially purified from plant tissues. For example, the thylakoid membrane protein phosphatase has been partially purified from wheat leaves (21), and two histone/casein phosphatases have been purified from wheat embryos (14) and a histone phosphatase has been purified from soybean hypocotyls (11). Nonetheless, relatively little is known about the protein phosphatases that dephosphorylate specific plant enzymes and thereby modulate activity.

In animals, where much more is known, there are four major protein phosphatases that account for most of the serine/threonine-specific protein phosphatase activity (for review see ref. 4). The enzymes are placed into two groups (type 1 and type 2) depending on substrate specificity, inhibitor sensitivity, and Me^{2+} requirements. The type 2 phosphatases comprise three enzymes (type 2A, 2B, and 2C). Comparative biochemical studies on extracts of developing *Brassica napus* (oilseed rape) seeds have established the exclusive presence of types 1 and 2A protein phosphatases in this plant tissue (12). In addition, a type 2A protein phosphatase has recently been

Table VI. *Effect of KF on Spontaneous Inactivation of Maize Leaf SPS*

Desalted extracts, prepared from leaves harvested in the light, were preincubated at 25°C, in the presence or absence of KF (20 mM). Activity was then measured in the limiting and V_{max} assays. Values are means from two experiments.

Extract Incubation		SPS Activity		Activation State
Time at 25°C	KF	Limiting assay	V_{max} assay	
		$\mu mol \cdot g^{-1}$ *fresh wt* $\cdot h^{-1}$		%
0 min	–	73.0	159	46
	+	76.5	165	46
60 min	–	12.7	113	11
	+	45.0	147	31

partially purified from leaves of the CAM plant, *Bryophyllum fedtschenkoi,* and shown to dephosphorylate night-form phosphoenolpyruvate carboxylase and thereby alter its sensitivity to malate inhibition (3).

In summary, two phosphatases have been identified that act on spinach leaf SPS. The phosphatases differ in inhibitor sensitivity and apparently dephosphorylate different sites on SPS because the effects on SPS activation state are different [activation (6) *versus* inactivation, in the present study]. This is consistent with the recent finding that SPS is likely phosphorylated at multiple sites *in vivo,* as revealed by phosphopeptide mapping (7). The protein phosphatase(s) which activated SPS was inhibited by okadaic acid (6) and therefore is similar to either the mammalian type 1 or type 2A enzyme. In contrast, high levels of okadaic acid were totally ineffective as an inhibitor of the phosphatase(s) which inactivated SPS (Table III). The inactivating phosphatase is also not similar to the mammalian type 2B enzyme, because Ca^{2+} was not absolutely required for activity (Table IV). Similarly, the observed stimulation by Mg^{2+} (Table IV) is only remotely suggestive of the mammalian type 2C enzyme, which shows an absolute dependence on Mg^{2+} (4). Thus, it is quite possible that the phosphatase involved is not specific for protein substrates. These observations, of course, are only preliminary to purifying and characterizing the phosphatase(s) responsible for spontaneous inactivation of SPS.

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