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Site-specific regulatory interaction between spinach leaf sucrose-phosphate synthase and 14-3-3 proteins

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Abstract We report an Mg^{2+}-dependent interaction between spinach leaf sucrose-phosphate synthase (SPS) and endogenous 14-3-3 proteins, as evidenced by co-elution during gel filtration and co-immunoprecipitation. The content of 14-3-3s associated with an SPS immunoprecipitate was inversely related to activity, and was specifically reduced when tissue was pretreated with 5-aminoimidazole-4-carboxamide riboside, suggesting metabolite control in vivo. A synthetic phosphopeptide based on Ser-229 was shown by surface plasmon resonance to bind a recombinant plant 14-3-3, and addition of the phosphorylated SPS-229 peptide was found to stimulate the SPS activity of an SPS:14-3-3 complex. Taken together, the results suggest a regulatory interaction of 14-3-3 proteins with Ser-229 of SPS.

Key words: Sucrose-phosphate synthase; 14-3-3 protein; Spinach; Protein:protein interaction; Surface plasmon resonance

1. Introduction

Sucrose-phosphate synthase (SPS; EC 2.4.1.14) is one of a few plant enzymes known to be modulated by reversible phosphorylation [1]. In addition, SPS is also regulated by allosteric effectors and protein turnover (see [2] and references therein). Reversible phosphorylation of Ser-158 and Ser-424 is thought to be responsible for light/dark modulation [3] and osmotic-stress activation [4], respectively. The light/dark modulation is one of the common regulatory similarities that SPS shares with nitrate reductase (NR; EC 1.6.6.1) [5]. However, the inactivation of NR not only requires phosphorylation of its major regulatory phosphorylation site, Ser-543 in spinach [6], but also an additional protein factor that is now known to be a 14-3-3 protein [7,8].

In mammalian systems, 14-3-3s interact with target proteins in both a sequence-specific and phosphorylation-dependent manner and may function as components of signal transduction pathways [9]. In plants, NR [7,8] and the plasma membrane H^+-ATPase [10] are the only two enzymes that have been shown to bind 14-3-3s. In the case of NR, it is known that formation and maintenance of the inactive phospho-NR:14-3-3 complex require divalent cations at millimolar concentrations [11]. Since at least one plant 14-3-3 has been shown to bind $^{45}Ca^{2+}$ [12] and more recently Tb^{3+} (Athwal et al., manuscript in preparation), a fluorescent analog used to detect divalent-cation binding sites on proteins [13], the cation dependence of NR inactivation may be associated specifically with the 14-3-3 protein.

The initial batch of proteins recognized to interact with 14-3-3s all contained the conserved motif RSXpSXP (where X is any amino acid and pS is phosphoserine) [14], but subsequently it has become apparent that at least half of all known 14-3-3 binding proteins do not have this sequence. Recently, it was shown that peptides that deviate slightly from the aforementioned motifs also bind to human 14-3-3τ, with varying degrees of affinity [15]. There is also evidence for binding of 14-3-3s at novel serine-based sequences not containing the Pro residue at +2 [16,17].

Sequence analysis of spinach leaf SPS reveals several candidate binding sites for 14-3-3 proteins. Of these, only the putative site involving Ser-229 is conserved across species boundaries. However, the sequence surrounding Ser-229 (RQVSAP) is not an exact match for the known 14-3-3 binding motifs (see above), and thus it was necessary to determine whether this putative site could actually function in 14-3-3 binding. In this paper we present real time kinetic measurements to demonstrate that the phosphorylated SPS-229 synthetic peptide can function in 14-3-3 binding, and furthermore, that this site may be involved in a regulatory interaction with 14-3-3 proteins.

2. Materials and methods

2.1. Materials and growth, harvest and extraction of plant material

All experiments were performed with spinach (*Spinacia oleracea* L. cvs. Tyee and Bloomsdale) leaves. Plants were grown under standard green-house conditions [18]. Highly activated (dephospho-Ser-158) SPS was obtained by D-mannose feeding to excised leaves [19]. Highly inactivated (phospho-Ser-158) SPS was obtained from darkened spinach leaves. 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) was fed via the transpiration stream by placing leaves in a 10-mM solution for 2 h. Leaf tissue was harvested directly into liquid nitrogen prior to extraction [4]. The SPS-158 peptide (GRRJRRISSVEJJ; J = norleucine) was obtained from Dr. Kochansky and it was verified that replacement of Met residues with norleucine had no effect on phosphorylation in vitro. The phosphorylated SPS-229 peptide (CRVDLLTRQVpSAPGVDK) was obtained from Genemed Synthesis (San Francisco, CA, USA) and differs from the native sequence in the two terminal residues; the C-terminal Cys was added to allow coupling of this peptide to sensor chips (see below). The SPS-689 peptide (RIDEGSENSDTDSAKKK) was synthesized in-house on a Model Synergy 432A peptide synthesizer (Perkin Elmer) and corresponds to a Ser-rich region that could be involved in 14-3-3 binding. Peptides were purified by HPLC and stored in a desiccator at −20°C.

2.2. Immunopurification and assay of spinach leaf SPS

SPS was immunopurified from crude extracts with spinach leaf SPS-

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Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; MAbs, monoclonal antibodies; RU, response units; SPR, surface plasmon resonance; SPS, sucrose-phosphate synthase; ZMP, 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate

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specific monoclonal antibodies (MAbs) [20] and immunoprecipitin (Gibco-BRL) as the precipitating agent. The immunoprecipitin-MAb-SPS complex (immune complex) obtained was collected by centrifugation at 4°C (5000×*g*; 5 min) and then washed (as indicated) with 50 mM MOPS-NaOH, pH 7.5, 10 mM $MgCl_2$, 2.5 mM DTT, 0.5 mM PMSF, 2 mM benzamidine, 2 mM ε-amino-*n*-caproic acid and 0.25 μM microcystin-LR (buffer A). As indicated, the immune complexes were either used directly in SPS assays or, following fractionation by 10% SDS-PAGE, subjected to Western analysis as described below. Samples were assayed for SPS activity under selective (limiting substrates plus Pi; V_{lim}) and non-selective (V_{max}) conditions as previously described [18].

2.3. Partial purification of spinach leaf SPS, SPS protein kinase and 14-3-3 proteins by FPLC

Spinach leaf SPS and SPS protein kinase activities were partially purified by polyethylene glycol (PEG-8000) fractionation and fast protein liquid chromatography (FPLC), using Resource-Q resin (Pharmacia) as previously described [3]. Fractions of 1 ml were collected and assayed for SPS [18], SPS-kinase [3] and 14-3-3 proteins [6] as described. When 14-3-3 proteins devoid of SPS were required, fractions were further purified by Cibacron blue Sepharose affinity chromatography [6].

2.4. Size-exclusion chromatography of reconstituted SPS:14-3-3 complex on Fractogel TSK HW55 (S)

Fractions containing either SPS or 14-3-3 proteins, partially purified as described above, were pooled separately and then sequentially changed to buffer A using Centricon 10 concentrators (Amicon). Appropriate fractions were then mixed and after a 60-min preincubation on ice, applied to a Fractogel TSK HW55 (S) column (1.6×60 cm; Merck) as described [4]. Fractions were analyzed for SPS activity and 14-3-3 proteins by immunoblotting. The gel-filtration column was calibrated with thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.5 kDa) as molecular weight standards.

2.5. Immunoblotting

Fractions from the Fractogel TSK HW55 (S) chromatography or anti-SPS MAb immunoprecipitations were denatured by boiling with 2× Laemmli sample buffer [21] and separated by 10% SDS-PAGE. Proteins were then transferred onto Immobilon-P membranes (Millipore) and subjected to Western blot analysis with anti-spinach leaf 14-3-3 MAbs as described previously [7]. The band intensities were quantified on a Molecular Dynamics densitometer (model number 375).

2.6. Surface plasmon resonance analysis

Real time binding experiments were performed using a BIAcore 2000 system (BIAcore, Piscataway, NJ, USA). The phosphorylated SPS-229 peptide was immobilized onto a CM-5 sensor chip via the N-terminal cysteine residue using sulfo-MBS (*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester; Pierce Chemical, Rockford, IL, USA) as the coupling reagent. The recombinant plant 14-3-3 protein, GF14ω [12], was passed over the coupled sensor chip (1 μM in 20 mM MOPS, pH 7.5, 50 mM NaCl, 0.005% (v/v) P20 surfactant (BIAcore), with either 5 mM $MgCl_2$ present or absent) at a flow rate of 20 μl/min for 2 min. The association was monitored by the increase in response units (RU), caused by a change in refractive index on the sensor chip surface, per unit time. Dissociation of GF14ω from the peptide was monitored after the association phase, with the above buffer minus the 14-3-3s. Generated sensograms were analyzed using the BIAevaluation software (version 3.0). The surface of the chip was regenerated using 0.5% (w/v) SDS in 20 mM MOPS, pH 7.5, 50 mM NaCl, with a contact time of 2 min.

3. Results and discussion

3.1. Evidence for an Mg^{2+}-dependent interaction of spinach leaf SPS with endogenous 14-3-3 proteins

Co-elution by gel-filtration analysis is a powerful method to demonstrate protein-protein interactions. It has previously been used to show that 14-3-3 proteins were part of a ternary complex which co-eluted following gel filtration [22]. We used a similar approach to reconstitute stable 14-3-3:SPS protein complexes on ice and then subjected the samples to Fractogel TSK HW55 (S) chromatography. Column fractions were then analyzed by SDS-PAGE size-fractionation and Western analysis with anti-14-3-3 MAbs. The results showed that significant amounts of the much smaller 14-3-3 proteins (~60 kDa) coeluted with spinach leaf SPS (Fig. 1A). Furthermore, we tested the effect of Mg^{2+} on the formation and/or stability of the SPS:14-3-3 complex. Using the gel filtration procedure, we observed that the coelution of SPS and 14-3-3 proteins was significantly reduced in the absence of Mg^{2+} (compare Figs. 1A and 1B). However, in the presence of Mg^{2+}, 14-3-3 proteins alone did not elute at the much higher molecular weights (data not shown). These experiments indicate that stable

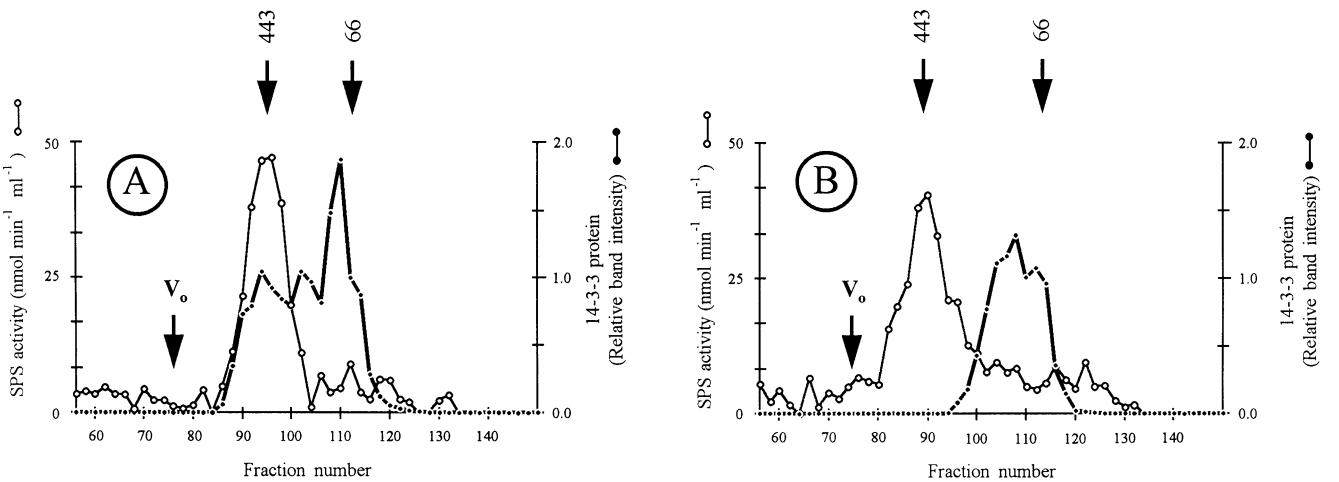

Fig. 1. Evidence for in vitro reconstitution of a spinach-leaf SPS:14-3-3 protein complex by gel-filtration chromatography on Fractogel TSK HW55 (S) resin. Anion-exchange purified spinach leaf SPS and 14-3-3 proteins were preincubated on ice and the mixture was applied to a Fractogel TSK HW55 (S) column (1.6×60 cm) equilibrated with buffer A containing 50 mM NaCl and Mg^{2+} as indicated. The column was developed against gravity at a flow rate of 0.2 ml/min and 0.5-ml fractions were collected for further separation by SDS-PAGE followed by Western analysis with anti-14-3-3 MAbs and also V_{max} SPS assays; ○—○, V_{max} SPS activity; ●—●, 14-3-3 protein (relative band intensity). The vertical arrows indicate elution of sizing standards. Reconstitution of complexes and size fractionation carried out in the (A) presence and (B) absence of Mg^{2+}.

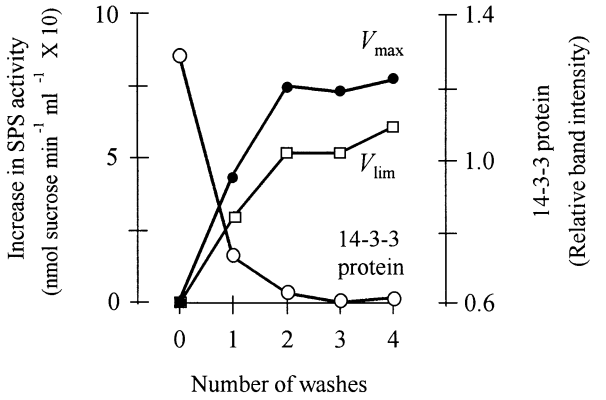

Fig. 2. Co-immunoprecipitation of endogenous 14-3-3 proteins with spinach leaf sucrose-phosphate synthase and reciprocal relationship between SPS activity and 14-3-3 binding. Spinach leaf crude extracts were incubated with anti-spinach leaf SPS MAbs [20] for 30 min. Following addition of a one-tenth volume of immunoprecipitin (relative to MAb), the immunocomplexes were removed by centrifugation ($3000\times g$; 5 min). Pellets were resuspended and assayed for SPS activity and 14-3-3 proteins by immunoblotting. □—□, V_{lim} SPS activity; ●—●, V_{max} SPS activity; ○—○, 14-3-3 protein content (relative band intensity).

SPS:14-3-3 complexes could be reconstituted in vitro only in the presence of Mg^{2+} ions.

3.2. Spinach leaf SPS activity is inhibited by 14-3-3 proteins

To confirm the SPS:14-3-3 interaction, co-immunoprecipitation experiments were performed using anti-spinach leaf SPS MAbs [20]. Proteins in immunoprecipitates were separated by SDS-PAGE and then subjected to Western blot analysis with anti-14-3-3 MAbs [7]. Significant amounts of endogenous 14-3-3 proteins co-immunoprecipitated with the anti-SPS MAbs as revealed by densitometric analysis of the blot (Fig. 2, open circles). Furthermore, even after significant washing of the immunoprecipitated SPS:14-3-3 protein complex a notable residual 14-3-3 component could still be detected (Fig. 2). The results provided additional independent evidence for a relatively stable interaction between SPS and 14-3-3 proteins. Fig. 2 also shows that removal of the 14-3-3 proteins from the immune complex by successive washing was associated with an increase in both the V_{lim} and V_{max} activity of SPS. These results clearly suggest that 14-3-3s may have a role in the modulation of SPS activity. To our knowledge, this is the first report of an SPS modification that results in changes under both V_{max} and V_{lim} assay conditions. We propose that an association between SPS and 14-3-3s proteins could be occurring at site(s) where substrate access to SPS may be directly impeded.

In addition to the stimulation of SPS activity observed with the disassembly of the SPS:14-3-3 complex (Fig. 2), we wanted to determine whether in vitro reconstitution of the complex would result in a corresponding decrease in SPS activity. SPS was immunopurified and extensively washed to remove bound proteins and the immobilized SPS was pretreated with the protein kinase PK_I [4] and ATP to ensure that putative binding sites were phosphorylated. Reconstitution experiments were then performed with increasing amounts of 14-3-3 proteins. Fig. 3 shows that, as expected, addition of increasing amounts of 14-3-3 proteins to SPS resulted in an inhibition of SPS activity. To further test whether the reconstitution of the SPS:14-3-3 complex requires the presence of requisite phosphoserine residue(s) on SPS, we used alkaline phosphatase to dephosphorylate immobilized SPS. Following alkaline phosphatase treatment, inhibition of SPS activity by addition of 14-3-3 proteins was strongly reduced. The results are consistent with the notion that serine phosphorylation of spinach-leaf SPS is necessary for its association with 14-3-3.

The phosphorylation requirement for 14-3-3 binding has been shown for nitrate reductase [7] and BAD [23]. However, in some cases, the requirement for phosphorylation may not be absolute and the mere presence of a (dephospho-)binding motif may increase the affinity between 14-3-3 proteins and their ligands (e.g. see [24]).

3.3. Binding of 14-3-3s to the phosphorylated SPS-229 peptide

Surface plasmon resonance (SPR) is a powerful technique to measure real time binding and has been used in previous studies to demonstrate sequence-specific and phosphorylation-dependent binding of 14-3-3s to immobilized peptides [14,15]. In the present study, we wanted to determine whether the sequence surrounding Ser-229 of SPS could constitute a 14-3-3 binding site. To do this, immobilized phosphorylated SPS-229 peptide on a sensor chip was tested for its ability to bind recombinant *Arabidopsis* GF14ω in the presence and absence of Mg^{2+}. Typical results are shown in the sensograms that are presented in Fig. 4. Buffer was flowing continuously over the immobilized peptide and from time zero to 120 s, buffer containing GF14ω was passed over the chip. Binding is indicated by an increase in the SPR signal (expressed in arbitrary response units). At 120 s, the sample was replaced with buffer devoid of 14-3-3 proteins to examine the dissociation of the complex. Two fundamental conclusions can be drawn from these results. Firstly, the phosphorylated SPS-229 peptide was clearly able to bind the recombinant *Arabidopsis* 14-3-3 protein and thus, the RQVpSAP sequence may be added to the list of known 14-3-3 binding motifs. Secondly, binding of GF14ω was stimulated several-fold by Mg^{2+}, confirming the results obtained with native SPS protein (Fig. 1A,B). Most importantly, the results suggest that Mg^{2+} may be exerting

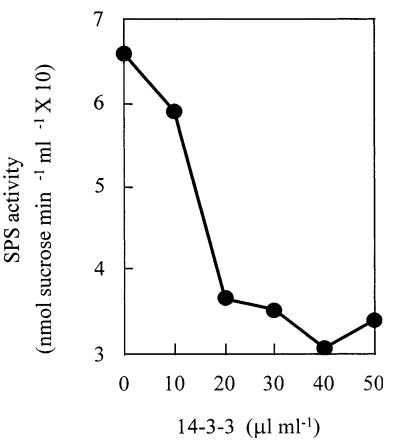

Fig. 3. Inactivation of spinach leaf sucrose-phosphate synthase by 14-3-3 proteins. Immunopurified spinach leaf SPS was preincubated with the protein kinase PK_I, ATP (1 mM) and increasing concentrations of endogenous 14-3-3 proteins (from a 98 μg/ml stock). Following a 30-min preincubation, the 14-3-3-dependent decrease in SPS activity was determined under V_{lim} conditions.

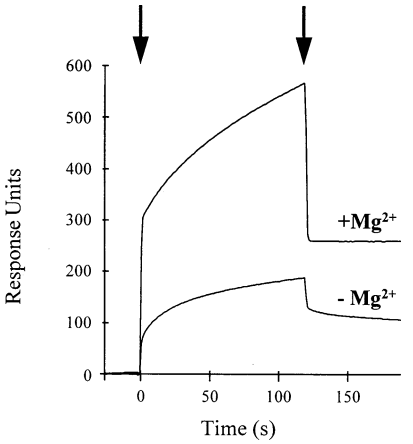

Fig. 4. Overlay plot of sensograms showing the Mg^{2+}-stimulated interaction between the recombinant *Arabidopsis* GF14ω protein and immobilized phospho-SPS-229 peptide. The sensograms were corrected for non-specific binding observed in the absence of 14-3-3 protein. At the first arrow, buffer containing 14-3-3 protein was passed over the chip and at the second arrow the solution was changed to buffer devoid of 14-3-3s. Response units (RU) are an arbitrary measure of refractive index changes on the sensor chip surface, per unit time.

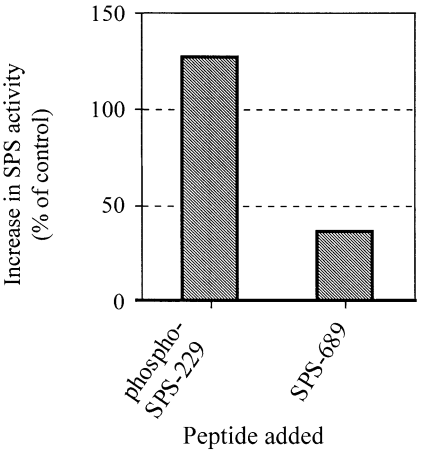

Fig. 5. Stimulation of spinach leaf SPS activity by the phosphorylated SPS-229 peptide. A phosphorylated SPS:14-3-3 immune complex was preincubated with the SPS-229 peptide or the unphosphorylated SPS-689 peptide (at 0.2 mg/ml) for 30 min, followed by assay of SPS activity. The percentage increase in V_{lim} activity, relative to the control that was preincubated in the absence of peptide, is shown.

its effect on 14-3-3 binding by an interaction with 14-3-3 rather than SPS. Calculated binding of the 14-3-3 protein at equilibrium was 5 times higher in the presence of Mg^{2+} compared to the control. Preliminary kinetic analysis suggests that the 'on-rate' was similar in the presence and absence of Mg^{2+} but that the presence of Mg^{2+} caused a large reduction in the 'off-rate'. These and other aspects are under continued study.

3.4. The phosphorylated SPS-229 peptide stimulates SPS activity

Synthetic phosphopeptides, based on binding site sequence, have been shown to disrupt the complex of 14-3-3s with Raf-1 [14] and NR [6,8]. Consequently, we speculated that the phosphorylated SPS-229 peptide might disrupt the SPS:14-3-3 complex and thereby increase SPS activity similar to the removal of 14-3-3s from an immune complex by washing (Fig. 2). Fig. 5 shows that this was indeed observed. In control experiments with the unphosphorylated SPS-229 peptide there was no increase in SPS activity (data not shown). Thus, our findings define a novel phosphoserine-based putative 14-3-3 binding motif represented by the consensus sequence RXXpSXP.

In addition to the pSPS-229 peptide, we experimented with numerous synthetic peptides designed from possible 14-3-3 binding sites on the SPS molecule. For example, the SPS-689 peptide is taken from a region of spinach leaf SPS that nests in a serine rich region that shows sequence similarities to previously reported serine rich 14-3-3 binding regions [24]. However, addition of the SPS-689 peptide to an SPS:14-3-3 complex had only a small stimulatory effect on SPS activity suggesting that the peptide did not disrupt the complex.

3.5. AICAR activation of SPS in situ involves dissociation of 14-3-3s

The inactivation of phospho-NR by 14-3-3s can be reduced in vitro by addition of 5′-AMP [11]. The basis for the AMP activation has been unclear but it is now apparent that AMP interacts with 14-3-3s and directly reduces their binding to target ligands (Athwal et al., manuscript in preparation). This effect can be mimicked in situ by feeding AICAR, which is cell permeable and phosphorylated to ZMP, an analog of 5′-AMP [25]. When AICAR is administered to leaf tissue in the dark, NR is activated concomitant with the accumulation of ZMP in the tissue [26]. Importantly, this occurred without marked changes in the levels of adenine nucleotides [26], suggesting that general phosphate metabolism had not been grossly altered. These results with NR led us to ask whether AICAR feeding could also activate SPS in the dark. As shown in Table 1, AICAR feeding did result in SPS activation in the dark and the activation was associated with a reduction in the level of 14-3-3s associated with the initial SPS immune com-

Table 1
Treatment of spinach leaves in the dark with AICAR activates SPS and specifically reduces its association with 14-3-3 proteins

Tissue treatment	SPS activation state (%)	14-3-3 protein content (relative units)
Control	26	119 (100%)
AICAR	46	72 (60%)
Mannose	55	105 (88%)

Darkened spinach (*Spinacia oleracea* L. cv. Tyee) leaves were fed AICAR (10 mM), D-mannose (50 mM) or water as control for 2 h via the transpiration stream at ambient temperature. SPS was immunopurified as described in Section 2 and aliquots were assayed for enzymatic activity under V_{max} and V_{lim} conditions for activation state determinations. Samples were further subjected to SDS-PAGE analysis followed by Western blotting to determine the levels of endogenous 14-3-3s bound to SPS under the various treatments.

plex. It is important to note that mannose feeding, which also activates SPS in darkness [27] as a result of phosphate sequestration and Ser-158 dephosphorylation [3], had only a slight effect on the SPS:14-3-3 complex. These results are consistent with the notion that SPS and 14-3-3s are associated in vivo and suggest that the interaction may be influenced by metabolites that may be of physiological significance, such as 5′-AMP.

4. Concluding remarks

Results of the present study demonstrate that 14-3-3 proteins can associate with SPS in the presence of Mg^{2+}. Evidence for an SPS:14-3-3 interaction has recently also been obtained in other laboratories (M. Stitt and C. MacKintosh, personal communication). We suggest that the likely site of interaction is phospho-Ser-229 of SPS and that RQVpSAP be considered as a motif for 14-3-3 binding.

Furthermore, we have shown that bound 14-3-3s appear to inhibit SPS activity. In addition to a direct effect on activity, another possibility is that the 14-3-3 protein functions as a scaffold to mediate the interaction of SPS with another protein, which has not yet been identified. For example, one intriguing possibility is UDP-glucose pyrophosphorylase (UGPase; EC 2.7.7.9), which has recently been cloned [28], and from the deduced sequence we would predict binding of 14-3-3 proteins to the site $RFKS_{416}IP$. We are currently testing the postulate that UGPase is a 14-3-3 binding protein, and that it might be involved in a complex with SPS, since each 14-3-3 dimer has the potential to bind two target motifs [15].

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