Identification of sucrose synthase as an actin-binding protein

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Abstract Several lines of evidence indicate that sucrose synthase (SuSy) binds both G- and F-actin: (i) presence of SuSy in the Triton X-100-insoluble fraction of microsomal membranes (i.e. crude cytoskeleton fraction); (ii) co-immunoprecipitation of actin with anti-SuSy monoclonal antibodies; (iii) association of SuSy with in situ phalloidin-stabilized F-actin filaments; and (iv) direct binding to F-actin, polymerized in vitro. Aldolase, well known to interact with F-actin, interfered with binding of SuSy, suggesting that a common or overlapping binding site may be involved. We postulate that some of the soluble SuSy in the cytosol may be associated with the actin cytoskeleton in vivo.

Key words: Actin-binding protein; Actin cytoskeleton; Aldolase; Sucrose synthase; Phalloidin

1. Introduction

Sucrose synthase (SuSy; EC 2.4.1.13) is recognized as an important enzyme of sucrose (Suc) utilization in plant sink tissues [1]. In particular, the highest activity of SuSy often occurs during rapid growth (e.g. elongating maize leaves [2]) or during storage product deposition (e.g. developing seeds [3,4]). Thus, factors that regulate either the activity or the intracellular localization of this enzyme are of interest and may contribute to the control of assimilate utilization.

SuSy is a globular protein and thus is generally considered to be soluble in the cytosol. However, some of the enzyme is known to be associated with the plasma membrane [5,6], perhaps in a specific complex with glucan synthase(s) in the membrane. Recent evidence suggests that reversible protein phosphorylation of SuSy at serine-15 [7] may control the process, with the dephospho-enzyme primarily associated with the membrane [8].

The present investigation concerns the phosphorylated 'soluble' form of SuSy and addresses the question of whether SuSy may be an actin-binding protein. In the course of our studies examining the membrane association of SuSy, we found that some of the SuSy protein was also associated with the detergent-insoluble fraction of microsomal membrane preparations suggesting a possible cytoskeleton association. In the present manuscript, we present evidence that soluble phospho-SuSy binds to both G- and F-actin in vitro, as well as evidence that some of the SuSy may be associated with actin in situ. These results provide the first evidence that at least a portion of the phospho-SuSy may be bound to the actin cytoskeleton in vivo.

2. Materials and methods

2.1. Materials

Maize (*Zea mays* L. cv. Pioneer 3183) plants were grown in soil in a greenhouse and fertilized three times weekly with a modified Hoagland's solution. The leaf elongation zone of 2 week old plants or the elongating internodal pulvinus of graviresponding 6–8 week old plants was harvested directly into liquid nitrogen and stored at −80°C. In the experiment presented in Fig. 2, the leaf elongation zone was isolated and vacuum infiltrated with buffered phalloidin (50 mM MOPS, pH 7.5, 70 μM phalloidin, 7 μM microcystin-LR, 20 mM NaF) and extracted as described below. Phalloidin was purchased from Molecular Probes (Eugene, OR, USA), Complete (protease inhibitor cocktail) was from Boehringer (Mannheim, Germany), rabbit muscle actin was from Cytoskeleton (Denver, CO, USA), monoclonal mouse anti-actin antibody (clone C4) was from ICN (Aurora, OH, USA), and all other biochemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Extraction and purification of SuSy

Plant tissue was ground to a fine powder in liquid nitrogen and homogenized with extraction buffer (4 ml/g fresh weight tissue), containing 100 mM MOPS, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM EDTA, 2 mM dithiothreitol, 20 mM NaF, 2.5% (v/v) Complete, 0.1 μM microcystin-LR, 1 mM PMSF and polyvinylpolypyrrolidone (0.1 g/g fresh weight). The homogenate was filtered through Miracloth and centrifuged at $10\,000\times g$ for 20 min at 4°C. The supernatant was then ultracentrifuged at $100\,000\times g$ for 60 min to pellet the microsomal membrane fraction (containing cytoskeletal components) and the resulting supernatant was taken as the 'soluble' fraction. Lipid components in the $100\,000\times g$ pellet were solubilized by resuspension in extraction buffer containing 0.5% (v/v) CHAPS. The detergent-insoluble, cytoskeleton-enriched fraction was separated by ultracentrifugation at $100\,000\times g$ for 1 h at 4°C.

2.3. In vitro polymerization of actin

G-actin (2 mg/ml) was prepared in low salt buffer (LSB; 5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP and 0.5 mM DTT) and denucleated by ultracentrifugation at $100\,000\times g$ for 1 h at 4°C. The pellet was discarded. Proteins to be tested for actin binding were desalted into LSB by centrifugal Sephadex G-25 filtration and then centrifuged at $100\,000\times g$ for 1 h at 4°C. Actin and test proteins were mixed in a final volume of 300 μl containing 20 mM MOPS, pH 7.5. The actin concentration was maintained >0.25 mg/ml with other proteins as indicated in the text. Polymerization was then induced by addition of 6 μl of polymerization inducer (PI; 20 mM MOPS, pH 7.5, 2 M KCl, 50 mM ATP and 100 mM $MgCl_2$). The mixtures were incubated at 30°C for 1 h. F-actin and associated proteins were pelleted by centrifugation at $100\,000\times g$ for 2 h at 15°C. The supernatants and pellets were separated for analysis by SDS-PAGE as described below.

2.4. Purification of SuSy

The soluble fraction of the leaf elongation zone was used for the affinity purification of SuSy following the procedure of Nguyen-Quoc et al. [2]. The final enzyme preparation was dialyzed against a buffer containing 50 mM MOPS, pH 7.5, 10 mM $MgCl_2$, and 2 mM DTT;

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Abbreviations: SuSy, sucrose synthase; Suc, sucrose

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10% (v/v) glycerol was then added and aliquots were stored frozen at −80°C.

2.5. Immunoprecipitation of SuSy

SuSy protein was immunoprecipitated using monoclonal antibodies [9] and Immunoprecipitin (Gibco-BRL, Grand Island, NY, USA) as precipitating agent.

2.6. SDS-PAGE and immunoblotting

Samples were subjected to SDS-PAGE and the gels were either stained with Coomassie or electroblotted onto Immobilon-P (Millipore, Bedford, MA, USA). Blots were probed with monoclonal antibodies against maize SuSy or rabbit actin, as indicated in the text. Immunodetection was performed with alkaline phosphatase-conjugated affinity-purified goat anti-mouse antibodies followed by chemiluminescence detection according to the manufacturer's protocol (Tropix, Bedford, MA, USA). In experiments where proteins were stained with Coomassie blue, protein bands were quantified by densitometry using a Personal Densitometer SI (Molecular Dynamics) and ImageQuant software (MicroSoft).

3. Results and discussion

3.1. SuSy binds to G-actin

Quantifying the distribution of SuSy between the soluble and microsomal membrane fractions, we found that the majority is in the soluble fraction as has been reported previously [5,6,8] and would be expected for a globular protein. However, a substantial amount of SuSy protein was also found in the detergent-insoluble pellet obtained from the membrane fraction (Fig. 1A). This fraction is known to be enriched in cytoskeleton polymers such as microfilaments and microtubules [10,11]. Under conditions that tend to stabilize F-actin we found increased amounts of SuSy in the detergent-insoluble pellet (data not shown).

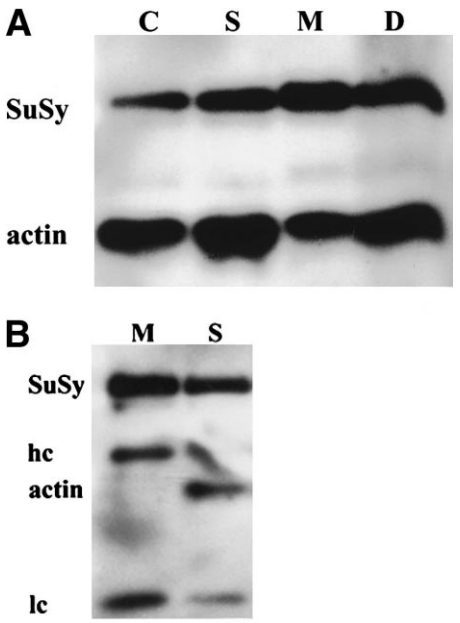

Fig. 1. Evidence for SuSy:actin interaction. A: Localization of SuSy in the detergent-insoluble fraction of microsomal membranes. A clarified crude extract (C) from the maize pulvinus was separated by centrifugation (100 000×*g*) into the soluble (S) and membrane fraction, which was then extracted with 0.5% CHAPS to yield the detergent-soluble membrane (M) and detergent-insoluble cytoskeleton (D) fractions. 15 μg protein of the crude, soluble and membrane fraction, and 50% of the entire detergent-insoluble fraction were loaded onto an SDS gel. The blot in A was probed with monoclonal antibodies against maize SuSy and rabbit actin. Note the presence of SuSy in all of the fractions, including the crude cytoskeleton fraction. B: Co-immunoprecipitation of actin with SuSy. Monoclonal antibodies were used to immunoprecipitate SuSy from the detergent-soluble membrane (M) and soluble (S) fractions of a pulvinus extract. The blot was probed with monoclonal antibodies against maize SuSy and rabbit actin. Note co-immunoprecipitation of actin with SuSy only from the soluble fraction (hc, antibody heavy chain; lc, antibody light chain).

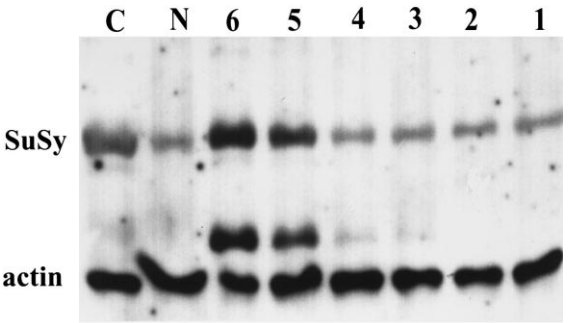

Fig. 2. Evidence for an in situ association of SuSy with actin filaments. The elongation zone of maize leaves was pretreated with phalloidin prior to extraction and the extract was then treated with and without anti-SuSy monoclonal antibodies prior to addition of Immunoprecipitin and ultracentrifugation (100 000×*g* for 20 min). The resulting pellets, and an aliquot of crude extract, were subjected to SDS-PAGE and Western blot analysis using monoclonal antibodies against SuSy and actin. C, crude extract; N, no antibody control; lanes 1–6: 0.1, 1, 5, 10, 50 and 100 μl of anti-SuSy antibody. The progressive increase in antibody heavy chain, the unmarked protein band just above actin, is evident.

Co-sedimentation of SuSy with the cytoskeletal fraction raised the possibility that SuSy might interact with one or more of the components found in the cytoskeleton. In order to obtain further evidence for a direct interaction, a co-immunoprecipitation experiment was performed. Immunoprecipitation of SuSy from the soluble (100 000×*g* supernatant) and the detergent-soluble microsomal membrane fraction resulted in co-immunoprecipitation of actin from the soluble fraction only (Fig. 1B). This suggested that the soluble, and more highly phosphorylated [8], form of SuSy has some affinity for G-actin. The membrane fraction contains actin (Fig. 1A, lane M), but none co-immunoprecipitates with SuSy (Fig. 1B, lane M). Thus, only soluble phospho-SuSy appears to interact with actin. An alternative explanation would be that the association between SuSy and actin in the membrane fraction was disrupted by the detergent extraction. However, this could be ruled out, because addition of Triton X-100 and CHAPS did not prevent co-immunoprecipitation of actin with SuSy from the soluble phase although the overall amount was reduced slightly. Based on these results, we propose that part of the SuSy:actin interaction involves the N-terminus of SuSy and that phosphorylation of Ser-15 [7] is necessary. Alternatively, phosphorylation of SuSy on Ser-15 may elicit conformational changes that expose an actin binding site elsewhere on the enzyme.

3.2. Phalloidin pretreatment leads to SuSy precipitation with F-actin

To further reduce depolymerization of microfilaments during the extraction process, we vacuum-infiltrated young maize leaves with buffer containing phalloidin, a drug known to

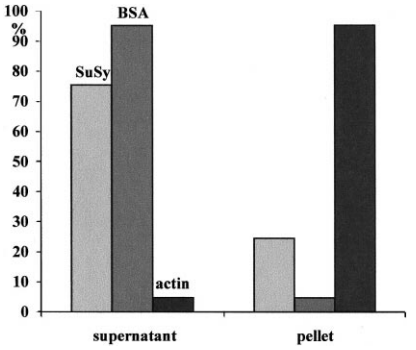

Fig. 3. BSA does not bind to F-actin and does not prevent the binding of SuSy. Binding of affinity-purified SuSy and BSA to F-actin, polymerized in vitro, was assessed by sedimentation followed by SDS-PAGE analysis of the pellet and supernatant. Binding of SuSy to F-actin in the absence of BSA was identical to that shown (data not presented).

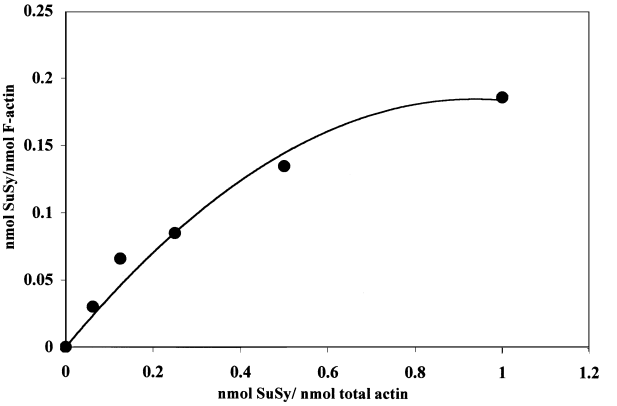

Fig. 5. Saturation of SuSy binding to F-actin. The amount of SuSy bound to F-actin, polymerized in vitro, was assessed by sedimentation and SDS-PAGE analysis of the pellets ($n = 2$).

stabilize F-actin, prior to extraction. The clarified crude extract from phalloidin-treated tissue was treated with and without anti-SuSy antibodies (plus Immunoprecipitin as precipitating agent) prior to ultracentrifugation. The amount of actin in the pellets was high and constant and generally independent of anti-SuSy antibody concentration, as would be expected. However, recovery of SuSy in the high speed pellets was quite different. The most important point is that there was significant SuSy protein pelleted in the absence of antibody (Fig. 2, lane N) or with low concentrations of antibody (lanes 1 and 2). At higher concentrations of anti-SuSy antibody, additional SuSy protein was immunoprecipitated and recovered in the pellet (lanes 3–6). The results suggest that some of the 'soluble' SuSy may be associated in situ with phalloidin-stabilized actin filaments. Comparison of the amount of SuSy pelleted with and without antibodies (Fig. 2, lanes N and 6) suggests that approximately 15% of the total SuSy protein may be associated with the actin cytoskeleton.

3.3. SuSy binds to F-actin in vitro

In order to determine whether SuSy could bind directly to actin, we polymerized rabbit actin in vitro in the presence and absence of SuSy plus BSA. Binding of proteins to F-actin was then monitored by sedimentation. As shown in Fig. 3, actin polymerization was nearly complete with $>95\%$ of the actin protein recovered in the pellet. Importantly, the F-actin pellet also contained approximately 25% of the total SuSy protein but less than 5% of the BSA protein. The results demonstrate that SuSy binds directly to F-actin and the lack of binding of BSA suggests that the complex formation is specific. Earlier work in other laboratories has shown that under similar conditions, alkaline phosphatase and apoferritin do not bind to F-actin in vitro, ruling out non-specific charge interactions and entrapment as mechanisms to explain the observed binding of phosphofructokinase [12].

In contrast to BSA, alkaline phosphatase and apoferritin, it is well known that aldolase ([13] and references 1–6 therein) and several other glycolytic enzymes can interact in a specific manner with F-actin. Binding of SuSy and aldolase, alone and in combination, to F-actin is presented in Fig. 4. The steady-state binding to F-actin of aldolase and SuSy was similar when presented individually in equimolar amounts. When both enzymes were present, a five-fold excess of aldolase reduced SuSy association with actin about 30%, while binding of aldolase was not affected. The apparent competitive binding suggests that the two proteins may share a common (similar) binding site on actin. Alternatively, distinct sites on actin may be involved that are so close together that binding of aldolase can prevent binding of SuSy.

In order to characterize further the interaction between SuSy and F-actin, we examined the effect of SuSy protein concentration on binding to a fixed amount of actin. As shown in Fig. 5, increasing the total concentration of SuSy resulted in an increase in the amount of bound protein with saturation of binding at approximately 0.2 nmol SuSy monomer per nmol of actin (monomer). Assuming that SuSy binds as a tetramer, then one native molecule may be associated with F-actin equivalent to 20 monomers.

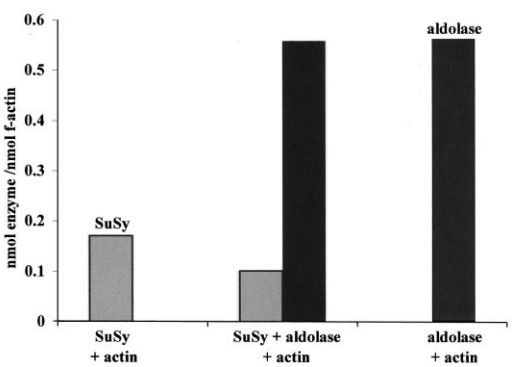

Fig. 4. Aldolase interferes with SuSy binding to F-actin. Binding of affinity-purified SuSy and aldolase, alone and in combination, to F-actin polymerized in vitro was assessed by sedimentation followed by SDS-PAGE and densitometric quantification. Note that in the mixture, aldolase binding was unaffected whereas SuSy binding was reduced.

4. Concluding remarks

The results obtained in the present study provide several lines of evidence suggesting, for the first time, that SuSy may be an actin-binding protein. Binding of SuSy to actin is apparently specific, because: (i) other proteins, such as BSA, do not bind and do not reduce SuSy binding; (ii) steady-state binding displays saturation, suggesting a finite number of

binding sites on actin; and (iii) aldolase, well known as an actin-binding protein, interfered with SuSy binding. Thus, the major conclusion of the present study is that some of the 'soluble' SuSy may actually be bound to the actin cytoskeleton in vivo. The significance of the association of SuSy with actin remains to be elucidated. By analogy with animal systems, where association of enzymes with microtubules and actin filaments is well documented [12,14,15], binding could function to regulate activity or provide a scaffold for juxtaposition of enzymes from the same pathway. It will also be important to identify the site(s) on the SuSy protein that interact with actin. Many actin-binding proteins contain specific sequences that constitute the actin-binding domain, and many of the proteins share similar amino acid motifs [16,17]. For example, the actin-binding domain of aldolase is close to its N-terminus because a synthetic peptide corresponding to residues 32–52 specifically competes with native aldolase for binding to F-actin [13]. Because aldolase competes with SuSy, a similar site may be involved. These and other aspects are currently under study.

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