Mitochondrial Localization and Putative Signaling Function of Sucrose Synthase in Maize*

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In many organisms, an increasing number of proteins seem to play two or more unrelated roles. Here we report that maize sucrose synthase (SUS) is distributed in organelles not involved in sucrose metabolism and may have novel roles beyond sucrose degradation. Bioinformatics analysis predicts that among the three maize SUS isoforms, SH1 protein has a putative mitochondrial targeting peptide (mTP). We validated this prediction by the immunodetection of SUS in mitochondria. Analysis with isoform-specific anti sera revealed that both SH1 and SUS1 are represented in mitochondria, although the latter lacks a canonical mTP. The SUS2 isoform is not detectable in mitochondria, despite its presence in the cytosol. In maize primary roots, the mitochondrion-associated SUS (mtSUS; which includes SH1 and SUS1) is present mostly in the root tip, indicating tissue-specific regulation of SUS compartmentation. Unlike the glycolytic enzymes that occur attached to the outside of mitochondria, SH1 associates with the voltage-dependent anion channel in an isoform-specific and anoxia-enhanced manner and may be involved in the regulation of sucsolutes fluxes into and out of mitochondria. In several plant species, at least one of the SUS proteins possesses a putative mTP, indicating the conservation of the noncatalytic function across plant species. Taken together, these observations suggest that SUS has a novel noncatalytic function in plant cells.

In both plant and animal cells, a small number of catalytic and structural proteins play additional roles that in some cases are regulatory in nature (1–3). These “moonlighting” proteins possess the ability to assemble into multiprotein complexes and mediate sophisticated biological functions such as integrating signals. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the most studied among such versatile proteins (4). Besides its pivotal role in energy metabolism, GAPDH is shown to be involved in membrane fusion, microtubule assembly, RNA transport, DNA replication and repair, and cell death (5). The functional diversity of GAPDH is facilitated by its ability to interact with other proteins and translocate to multiple subcellular compartments.

Sucrose synthase (SUS) catalyzes the reversible conversion of sucrose and UDP into fructose and UDP-glucose and is a key player in plant sucrose catabolism. In maize, the following three genes encoding sucrose synthase are known: sus1, sus2, and shr1. The isoforms encoded by these genes are symbolized SUS1, SUS2, and SUS-H1, respectively (6). In this study, for the sake of simplicity, we call the SUS-SH1 isoform SH1. All the three isoforms are predominantly recovered as soluble (cytosolic) proteins from plant cells, in accordance with their predicted secondary structures. The well-documented function of this enzyme is in energy metabolism, by its catalysis of sucrose degradation. However, recent studies show that a portion of the protein can exist as a membrane-bound form in association with the cellulose/cellulase synthase complex, thus contributing to cell wall biosynthesis (7–9). In the roots of intact maize seedlings subjected to prolonged anoxia, SUS protein showed a steady accumulation in the root tip region, and much of the protein was associated with the membrane fraction. This increased membrane association of SUS correlated with an induction of callose accumulation in the root tip (9). A redistribution of SUS activity with a concomitant increase in the content of cell wall carbohydrates has also been observed in O2-deprived wheat roots (10). Mutant analysis indicated that this response may be paralog-specific. The SH1 isoform of the enzyme, encoded by the shrunk1 gene, is more responsive to O2 deprivation than the SUS1 protein encoded by the sus1 gene. Our interest is to elucidate the basis of the isoform-specific response of sucrose synthase in anoxic maize cells. An examination of the protein sequences revealed that the SH1 protein is marked by a putative mitochondrial targeting signal at its N terminus. In this study, we have investigated the significance of this sequence-based prediction. Our results show that two of the three maize SUS isoforms exist in the organelles of maize seedling tissues. In addition to this isoform specificity, the dynamic changes in the mitochondrion-associated SUS (mtSUS) levels in response to developmental as well as environmental cues, its interaction with the voltage-dependent anion channel (VDAC; an outer mitochondrial membrane protein, also known as mitochondrial porin), and an apparent conservation of mitochondria-targeted SUS among plant species indicate that the mtSUS may play an important role in the inter-compartmental signaling in plant cells.
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EXPERIMENTAL PROCEDURES

Plant Material and Genetic Backgrounds—The maize (Zea mays L.) inbred line B73/He was used for all studies, except when mutants were the subject of investigation. shi-ref, shi-b21-x1, sus1, and shi/sus1 double mutants used in the study were described in Subbaiah and Sachs (9). Maize seeds were germinated and seedlings grown as carried out previously (11). Seedlings used in the study had only the primary root (5–8 cm) with two small seminal roots (∼1 cm) and a pre-emergent shoot (where the primary leaves were still enclosed in the coleoptile) of 5–8 cm. The terms “tip” and “axis” apply to the 1-cm apical region and the distal portions of the primary root, respectively (12). Anoxia was imposed by submerging the seedlings in “flooding buffer” as detailed in Subbaiah et al. (13).

Antibodies—Mono-specific polyclonal antisera were raised against peptide sequences unique to SH1 (EFDALFDDSKEKYAP), SUS1 (MGEAGGDRVLSRL), and SUS2 (HHILDALDEVQSGGGR) in rabbits, affinity-purified, and used as described in Duncan et al. (14). The specificity of the antibodies was tested using recombinant SUS1 and SUS2 proteins. Results with maize SUS mutants presented in this study further confirm their specificity. A monoclonal antibody (mAb) that detects all maize SUS isoforms with maize SUS mutants presented in this study further confirm their specificity. A monoclonal antibody (mAb) that detects all maize SUS isoforms was a gift from Prem Chourey (University of Florida, Gainesville) and was described earlier (9). Fumarase antibodies are from the David Oliver laboratory, Iowa State University, Ames. Monoclonal antibodies (mAbs) for alternative oxidase and VDAC are from Tom Elthon (University of Nebraska, Lincoln).

Bioinformatics—Secondary structure predictions were done using several programs available in the public domain, but the representative results presented here are from the PELE tool available at the Biology WorkBench site. SUS proteins were queried in TargetP, version 1.01, a two-layered neural network method for predicting subcellular targeting of proteins based on their N-terminal sequences (15). We have also used Predotar, which is also a neural network-based prediction program for in silico localization of proteins to the chloroplast, mitochondrion, both, or neither of the organelles (16). Default settings were used for the Predotar. User-defined settings were selected for TargetP to indicate the plant origin of sequences and with “no cut-off” stringency for all sequences tested.

Cell Fractionation and Purification of Mitochondria—Three-day etiolated maize seedlings or indicated seedling parts were homogenized in 0.4 M sorbitol, 50 mM Tricine-KOH, pH 8.5, 5 mM EDTA, 2 mM EGTA, 1 mM MgCl2, 5 mM dithiothreitol, and inhibitors of proteases, phosphatases, and kinases (20 mM NaF, 10 mM Na3VO4, 1 mM Na2VO4, 0.5 μM microcystin-LR, 1 mM benzamidine-HCl, 1 μM 4-(2-aminoethyl)-bezenesulfonylfluoride, 5 mM 6-amino caproic acid, 2 μM N-(trans-epoxy succinyl)-Leu-4-guanidinobutylamide, and 5 μM leupeptin). Except in preparations intended for analyzing cytosolic proteins, 0.5% bovine serum albumin was included in the homogenization buffer. The nuclei were pelleted at 500 g for 10 min, and the supernatant was spun at 3,000 × g for 10 min to collect the etioplasts. Mitochondria were pelleted from the post-plastid supernatant by centrifugation at 12,000 × g for 15 min. The crude mitochondria were loaded onto a discontinuous Percoll gradient (15–35–53%) and centrifuged in a swing-bucket rotor at 13,000 × g for 45 min. Mitochondria banded between 15 and 35% Percoll were collected, diluted in >10-fold volume of wash buffer (0.4 M sorbitol, 50 mM Tricine, pH 8), and pelleted at 15,000 × g for 1 h. The wash was repeated three times to completely remove the Percoll and any contaminating cytosol. The post-mitochondrial supernatant was spun at 20,000 × g for 10 min and used as the cytosol. In some studies, microsomes were separated from the soluble fraction by ultracentrifugation at 100,000 × g for 30 min. The crude nuclear fraction was enriched further by washing in the homogenization buffer containing 0.5% Triton X-100, after resuspension and incubation on ice for >30 min. Plastids were partially purified by pelleting through a 10% Percoll cushion and washed three times.

Thermolysin Protection Assay—Intact maize mitochondria suspended in 0.4 M mannitol, 50 mM Tricine-KOH, pH 7.5, and 0.1 mM CaCl2 with or without 1% Triton X-100 were incubated in thermolysin (10 μg/mg mitochondrial protein) for 1 h at 25°C. Protease was removed by washing the intact mitochondria three times in the resuspension buffer where CaCl2 was replaced by 1 mM EGTA and protease inhibitors. Mitochondrial pellets were lysed in SDS-PAGE sample buffer and used for protein-blotting analysis. Mitochondria treated with thermolysin in the presence of Triton X-100 were supplemented with EGTA and protease inhibitors at the end of the incubation and resuspended in SDS-PAGE sample buffer.

Subfractionation of Mitochondria—Mitochondria were lysed and subfractionated essentially as described by Sweetlove et al. (17). The effects of sucrose or mannitol were tested by washing the intact mitochondrial pellets in iso-osmotic buffers containing the respective sugars. Carbonate wash of membranes was carried out as described in Roper and Smith (18), except that the washed membranes were collected by ultracentrifugation.

Protein Analysis— Protein gel electrophoresis, blotting, and immunological detection were carried out as described previously (9, 19). The washed organelle pellets were directly resuspended in SDS-PAGE sample buffer, and the clear supernatants were used for analysis. The soluble fractions were concentrated by trichloroacetic acid precipitation and resolubilized in SDS-PAGE sample buffer. The protein content was determined by using the Bradford assay (20). Equal amounts of proteins (5 μg, unless stated otherwise) were analyzed by SDS-PAGE. Except in two sets of experiments (presented in Fig. 1 and Fig. 7) where peroxidase-coupled secondary antibodies and the chemiluminescence-based detection system (Pierce) were used, the secondary antisera used in protein-gel blot analyses were conjugated to Alexa Fluor 680 (Molecular Probes) or IRdye 800 (Rockland Immunochemicals). The blots were scanned using the NightOwl CCD camera (Berthold Co.) or a commercial infra-red fluorescence imaging system (ODYSSEY; Li-Cor Biosciences).

In Organello Import Assay—All precursor proteins, including the soybean alternative oxidase (AOX1), maize xyloglucan endo-transglycosylase (XET1), both the full-length and a truncated version that includes the first 300 amino acids of SH1 and the full-length SUS1, were generated using T7-promoter driven gene constructs, a commercial rabbit reticulocyte system (TNT-coupled Transcription and Translation System, Promega, Madison, WI), and [35S]methionine. Mitochondrial import reactions and sample processing were carried out as described in Whelan et al. (21).

Co-immunoprecipitation Analysis—SUS-VDAC interactions were detected using co-immunoprecipitation assay as described by Elion (22), with the following modifications. Purified mitochondria or liquid nitrogen powders of plant material were initially extracted in 2% Triton X-100 to improve the solubilization of membrane proteins. The extracts were later diluted to 0.1–0.2% Triton and pre-cleared by incubating with protein-G-Sepharose for an hour before antibody incubations. Pre-cleaning was necessary to remove nonspecific precipitation of VDAC, particularly from freeze-thawed extracts. The effects of sucrose or mannitol (0.1 M) and CaCl2 (1 mM) or EGTA (15 mM) were tested on the immunoprecipitability of SUS or porin by including the reagents in all buffers used.
the N terminus of SH1 forms more extensive helical structures (mitochondrial presequences and is critical for the recognition by the TargetP program (Table 1) (15). A similar prediction is also made by "Predotar," another program frequently used to predict the intracellular targeting of plant proteins (16). Although plant mTPs are not conserved in their primary sequence, certain structural features have been reported to be essential for targeting the cognate proteins to mitochondria (24, 25). A comparison of the N-terminal sequences of maize SUS proteins indicates that the N terminus of SH1 has features required for targeting proteins to the mitochondria, whereas SUS2 has N-terminal features that are unfavorable for mitochondrial import. Furthermore, the N terminus of SUS1 shows a few features favorable for mitochondrial targeting. The N terminus of SH1 is characterized by a greater abundance of Arg, Ala, Leu, and Ser (52% compared with 36% of SUS1 and SUS2 proteins) and is more basic (pI of 9.38) in its overall charge than SUS1 or SUS2 (pI of 7.93 and 7.63, respectively). The α-helix is the most native-like structure for mitochondrial presequences and is critical for the recognition by the import apparatus (26–28). Predicted secondary structures indicate that the N terminus of SH1 forms more extensive helical structures (>60%) than those of other two isoforms (30–40%).

Although not yet considered as selection criterion by the targeting programs, the binding of Hsp70 chaperones to organellar transit peptides is important for the translocation of proteins. 97% of plant mTPs have an Hsp70-binding site (27). Potential Hsp70-binding sites were predicted using a program based on the in vitro affinities of short peptides to the bacterial Hsp70 homolog DnaK (29). Strong HSP-binding sites are present in the N termini of both SH1 and SUS1, whereas they are distal to the N terminus in SUS2 (data not shown).

SUS Association with Mitochondria and Nuclei—To test the mitochondrial localization of SUS, maize seedling extracts were fractionated by differential and Percoll gradient centrifugation. All fractions obtained were tested for the presence of sucrose synthase by protein blotting experiments using a mAb for maize SUS (9). More than 90% of the cellular SUS was recovered as a cytosolic protein. However, SUS-specific signals were also present in purified mitochondria as well as the enriched nuclear fraction (Fig. 1). The nuclear preparation used here was only washed in 0.5% Triton X-100 without further purification. However, our subsequent analyses with purified nuclei consistently showed the presence of SUS in these organelles as well. Alternative oxidase (AOX), as detected by a mAb specific for the mitochondrial protein, was used as a marker for the distribution of mitochondria in these fractions. AOX was present also in a fraction that banded above the 15% Percoll layer (Fig. 1, lane Mix), marking the presence of mitochondria in this thylakoid-rich fraction. However, SUS did not co-localize to this band, indicating that the mitochondria banded here do not contain SUS. We hypothesized that the fraction contains intracellular light mitochondria and not fragments of "heavy" mitochondria that generally band between 15 and 35% Percoll layers (see below and "Discussion"). We infer from this observation that SUS association is specific to a subpopulation of mitochondria in seedling tissues and may be dependent on the developmental status of the organelles. Mitochondria from germinating seeds of maize have been shown to partition on sucrose density gradients into light and heavy subpopulations, distinct in their ultrastructure, protein composition, and activities. Only the heavy mitochondria showed an active tricarboxylic acid cycle after imbibition and developed further during germination (30). Similar mitochondrial functional heterogeneity associated with density differences has been reported from other species as well (31).

Isoform-specific Association of SUS with Mitochondria—Of the three SUS paralogs in maize, only the SH1 is predicted to have an mTP at its N terminus (Table 1). We tested whether this in silico prediction is valid in vivo. Isoform-specific antisera, generated against peptide sequences unique to each of the three SUS isoforms in maize (14) were used to address this question. As shown in Fig. 2, both SH1 and SUS1 were detected in the mitochondrial preparations. In most experiments, we have also used the pS15 peptide antisera (raised against the N terminus of SUS1 containing phosphoserine 15; see Ref. 19). These antisera detect mainly the phospho-form of SUS1 with some cross-reactivity against phospho-SH1. Despite lacking a predictable mTP at its N terminus, SUS1 was consistently detected in mitochondrial preparations from all the maize genotypes tested, except in the case of a sus1 mutant (Fig. 2). Although SH1 and SUS1 are known to exist as oligomers in vivo (32), the presence of SUS1 in the mitochondrial of the deletion mutant (sh1-bz1-x1; see Fig. 2B) indicated that SUS1 can directly associate with mitochondria, independent of its oligomerization with SH1. Some mitochondrial proteins encoded in the nucleus do not carry an mTP at their N termini but have an internal sequence that confers mitochondrial targeting (33). To determine whether SUS1 belongs to this category, we sequenced overlapping fragments of the SUS1 sequence in the TargetP program. A putative internal mTP was identified very close to the N terminus (residues 8–72). Because no other potential mTP is detectable in the downstream sequence, the N-terminal motif may direct the protein to mitochondria.

Cytosolic SH1 and SUS1 are heavily phosphorylated at their N termini in vivo. Dephosphorylation of the proteins seems to enhance their

### RESULTS

**Bioinformatics Analysis—**To determine the basis of isoform-specific functional diversity of maize sucrose synthases (9, 23), conserved functional domains and predicted structural properties of the three maize sucrose synthases were compared. An outcome of this analysis was the detection of a putative mitochondrial targeting peptide (mTP) at the N terminus of the SH1 protein as predicted by the TargetP program (Table 1) (15). A similar prediction is also made by "Predotar," another program frequently used to predict the intracellular targeting of plant proteins (16). Although plant mTPs are not conserved in their primary sequence, certain structural features have been reported to be essential for targeting the cognate proteins to mitochondria (24, 25). A comparison of the N-terminal sequences of maize SUS proteins indicates that the N terminus of SH1 has features required for targeting proteins to the mitochondria, whereas SUS2 has N-terminal features that are unfavorable for mitochondrial import. Furthermore, the N terminus of SUS1 shows a few features favorable for mitochondrial targeting. The N terminus of SH1 is characterized by a greater abundance of Arg, Ala, Leu, and Ser (52% compared with 36% of SUS1 and SUS2 proteins) and is more basic (pI of 9.38) in its overall charge than SUS1 or SUS2 (pI of 7.93 and 6.3, respectively). The α-helix is the most native-like structure for mitochondrial presequences and is critical for the recognition by the import apparatus (26–28). Predicted secondary structures indicate that the N terminus of SH1 forms more extensive helical structures (>60%) than those of other two isoforms (30–40%).

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Cytosolic SH1 and SUS1 are heavily phosphorylated at their N termini in vivo. Dephosphorylation of the proteins seems to enhance their
hydrophobicity and thereby membrane association (8, 9, 19). Analysis with pS15 peptide-antisera indicates that mtSUS1 is phosphorylated to a similar proportion as that of the cytosolic form (Fig. 2C), indicating that SUS1 localization in mitochondria may depend on N-terminal phosphorylation and therefore differ mechanistically from its association with microsomes.

Although SUS2 was present in the cytosolic fractions of both roots and shoots, this isoform was barely detectable in mitochondria purified from seedling tissues in all genotypes tested, including SUS mutants (Fig. 2, A and B). The sh1 mutant (reference allele) used in Fig. 2A showed anti-SH1 cross-reactive protein in roots and shoots. Nevertheless, the sh1 deletion mutant used in the study (sh1-bz1-x1)
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MtSUS Is Predominantly Associated with the Root Tip in Maize Primary Root—Previous analysis indicated that the SUS undergoes dynamic changes in the root tip of maize seedlings in response to anoxia (9). Here we determined whether there is any differential association of SH1 or SUS1 with mitochondria between the root tip and the axis. As shown in Fig. 3A, mtSUS (SH1 as well as SUS1) was restricted predominantly to the 1-cm tip of the primary root, with very little or no organellar SUS in the distal part of the root in all genotypes tested. A similar profile is observed in the inbred B73 either under aerobic or anoxic conditions (Fig. 3B). This profile of mtSUS distribution coincides with the oxygen uptake rates along the maize primary root, in that the 1-cm root tip has the peak O2 demand, with a sharp decline in the root axis (34). This pattern along the primary root may also be explained by other metabolic or developmental differences in the organelles from these two regions of the root. Furthermore, we noticed a loss of SUS association with root tip mitochondria in anoxic seedlings (Fig. 3B).

Three-day-old maize seedling shoots also contained mtSUS, comparable in amounts to that in root tips (Fig. 3, A and B). Young maize shoots, particularly mesocotyls, are known to contain highly active mitochondria (35). In some experiments, mtSUS levels were analyzed separately in mesocotyls and coleoptiles, by slicing the shoots at the coleoptile node. In contrast to the distribution in the root, the amount of mtSUS was found to be more uniform in these two shoot components (data not shown).

A sharp decline in mtSUS of root tips was evident during the first 15 h of O2 deprivation (Fig. 3). However, SH1 or SUS1 association with root tip mitochondria appears to recover under prolonged anoxia (Fig. 4).

SUS Is an Intrinsic Protein of Maize Mitochondria—In addition to their predominant localization in the cytosol, many of the glycolytic enzymes have been shown to be extrinsically attached to the OMM, as indicated by their susceptibility to an externally added protease (36). The authors proposed that this association may allow pyruvate to be provided directly to the mitochondrion, where it is used as a respiratory substrate. We analyzed the protease sensitivity of the SUS associated with mitochondria in maize to ascertain its location. MtSUS (SUS1 as well as SH1) is thermolysin-resistant in intact organelles but becomes labile after Triton X-100 lysis (Fig. 5A; data not shown). This indicated that mtSUS is integral to the mitochondrion and thus may not be involved in sucrose catabolism, considering the low abundance of the protein within the organelles (less than 1% of total cellular SUS) and the impermeability of mitochondria to sucrose (37). This analysis further ruled out cytosolic contamination as a source of mtSUS. The precise intramitochondrial location of SUS was probed by a stepwise osmotic and freeze-thaw lysis of organelles as described in Sweetlove et al. (17). In the experiment shown in Fig. 5B, mitochondria were initially lysed in hypo-osmotic sucrose buffer, and after 10 min, the osmoticum was

had no detectable SH1 protein and also showed a compensatory accumulation of SUS2 in the cytosol. Even in this mutant, SUS2 was absent in mitochondria (Fig. 2B). This observation is in conformity with the lack of an mTP-like sequence either at the N terminus or the distal parts of SUS2 (Table 1; data not shown). Our mutant data further confirmed the isoform specificity of the peptide antisera as originally demonstrated using recombinant proteins (14). We probed the mitochondrial preparations for the presence of sucrose-phosphate synthase, an enzyme structurally and functionally related to SUS to estimate the extent of cytosolic contamination. As shown in Fig. 2A, sucrose-phosphate synthase was found to be fairly abundant in the cytosolic fractions of the root tips but conspicuously absent from the mitochondria purified from same homogenates. This analysis further validated the differential intracellular distribution of SUS2.

Our studies also indicate that the cross-reactivity with the SH1 antisera observed in sh1 mutants varies with the genetic background and appears to be due to a gene redundancy. The anti-SH1 antisera cross-reactive protein of ~90 kDa was observed also in sh1 deletion mutants of different genetic backgrounds as in the case of the sh1 reference allele (Fig. 2A).

FIGURE 3. Root axis mitochondria show poor or no SUS association. A, mitochondrial and soluble fractions from the root tip, the root axis, and shoots were probed by antisera specific for the SUS1 isoform. The genotype identities are as described in Fig. 2. The seedling picture shown indicates how the seedling parts are collected. SH1-specific antisera gave similar results (data not shown). B, etiolated 3-day-old B73 seedlings, either growing aerobically (Air) or submerged for 15 h (anox) were used. The seedling parts were separated as described in A. Mitochondrial and soluble proteins from aerobic and anox seedling parts were probed by antisera specific for maize SH1 or SUS1 isoform, as indicated on the left side of the panels.

FIGURE 4. SUS association with root tip mitochondria is maintained under prolonged anoxia. Etiolated 3-day-old B73 seedlings, either growing aerobically or submerged for 20 h, were used. Total proteins (extracted by tissue-homogenization in SDS-PAGE sample buffer) or mitochondria prepared from aerobic (Air) and anox (Anox) root tips (Tips) and shoots were probed by different antisera. The identity of antisera is given between the Total and Mitochondria panels. VDAC was used as a positive control and for normalizing mitochondrial protein loading, whereas sucrose-phosphate synthase (SPS) served as a negative control. Signal intensities in Total and mitochondrial protein preparations were used in computing the proportion of mtSUS relative to the total cellular SUS.

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raised to 0.3 M sorbitol. Under these conditions, the SH1 was predominantly associated with the OMM, whereas the SUS1 protein was more widely distributed (Fig. 5B). VDAC and fumarase served as markers for monitoring the extent of enrichment of different mitochondrial subfractions. To further distinguish the peripheral versus integral association of SUS to mitochondrial membranes, we subjected the membranes to a carbonate wash. The OMM-bound SH1 could not be destabilized by the high pH wash, indicating that the protein is integrated into the

![Diagram](image-url)

**FIGURE 5.** mtSUS is intrinsic to mitochondria. A, purified intact maize mitochondria were treated with thermolysin in the presence or absence of Triton X-100 and processed as described under “Experimental Procedures.” Additions (+) or omissions (−) are indicated at the top of the respective lanes. 30 μg of mitochondrial protein was used per lane for SDS-PAGE analysis, and the protein blot was probed by SUS1-specific antiserum. SH1-specific antisera gave similar results (data not shown). B, mitochondrial preparations were subfractionated by controlled hypotonic lysis (70 mM sucrose for 10 min and later raised to 300 mM sorbitol) and freeze-thaw disruption. Mitochondrial membranes were further washed in 0.1 M Na2CO3 as described under “Experimental Procedures.” 5 μg of protein from each fraction (except for the matrix, which was 3 μg) was probed by antiserum as indicated on the right side of each panel. Because of the limited amounts of protein available in submitochondrial fractions, same blots were reprobed with marker protein antisera. OM-CW, carbonate washed-out mitochondrial membrane; Pellet, membranes pelleted after the wash; Sup, supernatant of the washed membranes; IMS, intermembrane space fluid, IM-CW, carbonate washed-inner mitochondrial membrane. C, microsome-bound SUS is removed by carbonate wash. Microsomal pellets were washed in sodium carbonate and analyzed by protein blotting experiments. The antisera used are indicated on the right. The SH1 (light bars) and SUS1 (dark bars) signals were quantified using the ODYSSEY imaging system and shown in the bar graph. Mic, unwashed microsomes; CWP, pellet from the carbonate wash; CWS, supernatant of the carbonate wash. D, mitochondria were subfractionated as described in B, except that either sucrose or mannitol was used as an exclusive osmoticum. Carbonate wash was given only to the OMM in this experiment. The antisera used are indicated on the left. Reprobing the blots with fumarase or VDAC (data not shown) antisera indicated that the osmoticum did not alter the pattern of subfractionation. The SH1 and SUS1 signals from the carbonate-washed outer and inner mitochondrial membranes were quantified in three representative blots using the ODYSSEY imaging system, and the averages with standard errors are presented in the bar graph. Int, intact mitochondria; CW, carbonate wash; OMP, pellet from the carbonate wash of OMM; OMS, supernatant of the carbonate wash of OMM; IMS, intermembrane space fluid; IM, inner mitochondrial membrane.
membrane. Most of the SUS1 was also resistant to the carbonate treatment, but a part of this isoform was removed from the membranes into the supernatant of the wash. However, a similar alkaline wash of microsomes dislodged more than 70% of the SUS (Fig. 5C) (19), indicating that SUS may bind mitochondrial membranes by a different mechanism. Fig. 5D shows the effects of using sucrose or mannitol exclusively as an osmoticum during organellar lysis. Although there was some variability (most likely because of batch-to-batch variation in the purity of different subfractions), the distribution and affinity of SUS1 or SH1 clearly varied with the nature of osmoticum in the lysis medium. Sucrose apparently increased the specific interaction between SUS proteins and the OMM, with the nature of osmoticum in the lysis medium.

In Vitro Synthesized SUS Is Bound and Internalized by Isolated Mitochondria—We have tested the ability of isolated maize mitochondria to import in vitro synthesized SH1 and SUS1 proteins. Soybean AOX1 and maize XET1 (38) were used as positive and negative controls, respectively (Fig. 6A). In the initial experiments, a truncated version of the SH1 protein (the first 300 amino acids; roughly similar to AOX1 in size) was tested as the input to alleviate the problems because of the large size of SUS proteins, in particular when they form tetramers. Maize mitochondrial preparations that were proficient in importing and processing soybean alternative oxidase also bound the truncated SH1 efficiently in a protease-resistant manner (Fig. 6B). However, there was no evidence of further processing of the import protein. Similar to maize Rps10, a mitochondrial ribosomal protein encoded by the nucleus (24), SH1 seems to possess a noncleavable N-terminal mTP. The kinetics of SH1 binding to mitochondria were also different from that of the AOX1. For example, disruption of the membrane potential by the ionophore valinomycin did not disrupt the binding, instead it enhanced the stability of the SUS protein (Fig. 6C). Membrane potential independent import/translocation of other proteins into mitochondria has been reported in plants as well as other organisms (39–42). We have also tested the full-length SUS proteins for their import by isolated mitochondria. In vitro translated SH1 and SUS1 proteins bound to isolated mitochondia but to a much lesser extent than the AOX1 or the truncated form of SH1 (Fig. 6D). Nevertheless, SH1 was resistant to protease K added to intact mitochondria but was degraded by the protease after Triton X-100 lysis. In contrast, SUS1 incubated with mitochondria was proteinase K-sensitive, as indicated by the truncation of the protein (Fig. 6D).

**SUS Interacts with the Voltage-dependent Anion Channel (VDAC)**—Our intra-mitochondrial localization studies indicated that the association of SUS with mitochondria, in particular its carbonate-resistant binding to OMM, may partly be explained by specific protein-protein interactions with mitochondrial (membrane) proteins. Using the yeast two-hybrid approach, Holmgren et al. (43) recently reported that maize SUS proteins may bind VDAC. We tested whether this interaction demonstrated in yeast cells is valid in planta, using co-immunoprecipitation analysis. We found that the mAb of VDAC works in immunoprecipitation assays with high efficiency and specificity. Of the SUS antiserum available to us, only the SUS1 and pS15 antiserum immunoprecipitate their cognate antigenic proteins. However, the light chain of SUS1 antisera co-migrated with VDAC and cross-reacted against the secondary antisera, thus interfering with the analysis. pS15 antisera did not give this interfering signal and therefore were used in the co-immunoprecipitation analysis. Results show that pS15 or VDAC-specific antisera can reciprocally co-immunoprecipitate the other protein from maize seedling extracts (Fig. 7), indicating the existence of SUS1-VDAC protein complexes in vivo. SH1 is also co-immunoprecipitated by VDAC antisera, whereas SUS2 is not pulled down by the same antisera (Fig. 7). This isoform-specific interaction of SUS with VDAC is consistent with our observations from cell fractionation experiments but contradictory to the yeast two-hybrid results, where VDAC was found to be a putative partner of the SUS2 isoform (43). The ability of anti-pS15 antisera to co-immunoprecipitate VDAC indicated that N-terminal dephosphorylation is not needed for SUS1 interaction with VDAC. This is consistent with the data presented in Fig. 2C and the inference that SUS localization to mitochondria is distinct from its association with microsomes.

We also examined whether VDAC-SUS interactions are responsive to oxygen deprivation. Although anoxia did not considerably alter the levels of VDAC in total protein extracts of maize tissues (Fig. 4; data not shown), a greater amount of VDAC was consistently immunoprecipitated from anoxic extracts under nonnaturating conditions (Fig. 7 and Fig. 8). The relative signal intensity value in the anoxic samples is significantly greater than the average aerobic sig-
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(94 versus 58; p = 0.001; Student's t test). This increase is also associated with consistently greater amounts of SUS1 co-precipitated by the VDAC antisera (increase from an average intensity of 71 in the aerobic extracts to 87 in the anoxic ones; Fig. 8). In cotton, Ca\(^{2+}\) was shown to increase the affinity of SUS to membranes and to its interacting proteins that occur in the particulate fraction (44). We observed no trend in the data on Ca\(^{2+}\) effects, except for a mild increase in the precipitation of VDAC in the presence of Ca\(^{2+}\) and sucrose. The presence of sucrose consistently enhanced the co-immunoprecipitation of SUS and VDAC by the reciprocal antisera (Fig. 8). However, these effects were statistically insignificant (as revealed by the Student's t test).

FIGURE 7. Direct interaction between SUS and VDAC. A, total proteins were extracted from root tips and shoots of aerobic (Air) or 12-h anoxic (Anox) maize seedlings as described under “Experimental Procedures” and subjected to immunoprecipitation (IP) and immunoblot (IB) analysis. The antisera used in the immunoprecipitation reaction were VDAC (maize mitochondrial porin, mAb clone PMO35) and pS15, as indicated at the top of the figure. The additions made to extraction and co-immunoprecipitation buffers are shown at the bottom of the figure. The individual antisera tested in the immunoblot reactions are shown on the left. B, efficiency of VDAC immunoprecipitation by different antisera is shown. Antisera used in IP are identified at the top of the panels. 10 \(\mu\)g of protein from anaerobic seedling tissue extract made in the presence of mannitol and EGTA were tested with 1 \(\mu\)g of anti-pS15 or anti-SUS1 and about 0.1 \(\mu\)g of anti-VDAC antisera. The signal in the SUS1 lane is partly due to the co-migrating immunoglobulin light chain (see text for details). C, control assay processed without antibodies.

FIGURE 8. Anoxia increases the levels of immunoprecipitable VDAC and SUS. The signals from Fig. 7A were quantified using WinLight software and presented in the bar graph. Results from pS15-IP are presented in A and from the VDAC-IP in B.
GenBank™ for their subcellular location using TargetP. In most species. We queried all the putative SUS sequences available in the bioinformatics prediction of mTP held true in the case of maize SUS, the six SUS genes present in species, where there is enough sequence information, we detected at least one SUS paralog with a putative mTP at its N terminus (Table 2), and only these two are induced when grown in high CO2.5 It is also noteworthy that none of the plant SUS sequences possesses a putative chloroplast targeting sequence. Furthermore, two of the six SUS genes present in Arabidopsis (45) encode proteins with putative mitochondrial targeting (At3g43190 and At4g02280; Table 2), and only these two are induced when grown in high CO2. It is also noteworthy that none of the plant SUS sequences possesses a putative chloroplast targeting sequence.

**DISCUSSION**

**SUS Is an Integral Protein of Maize Mitochondria**—The bioinformatics prediction and the follow-up analyses suggest that SUS is a component of mitochondrial proteome in maize (Figs. 1–4). Most likely, this is the case in other plant species, based on the conservation of the targeting information (Table 2). Thus, our studies have uncovered a new facet of plant SUS biology in that the protein may have novel functions in addition to its role in sucrose catabolism. The three maize SUS isoforms differ in their association with mitochondria. These differences are reflected to a large extent by the differences in their N-terminal sequences. SH1 has features of a canonical mTP at its N terminus that are easily recognizable by TargetP and Predotar programs (Table 1). This isoform also showed stronger affinity to the OMM with or without the presence of sucrose (Fig. 5D). We hypothesize that the N terminus may be involved in this membrane interaction of SH1. Despite not having a predictable mTP at its N terminus, the unequivocal presence of SUS1 in mitochondria indicates that it may have an internal mTP that allows its import into mitochondria. Carrier proteins located on the OMM and even a few matrix proteins (e.g. Mtflp) are imported by mitochondria despite lacking any known targeting information at their N termini (41, 47). Analogous to SUS1 or SH1, adenylate kinase is predominantly cytosolic, with a minor fraction (only up to 6–10%) distributed also in mitochondria (48, 49). Adenylate kinase has an internal targeting sequence that is critical for organellar import (48). SUS1 possesses a putative targeting sequence very close to the N terminus (residues 8–72). Results from carbonate wash analysis and in organello import studies (Figs. 5 and 6) indicate that the mitochondrial association of this isoform is not as strong as that of SH1. SUS1 is quite distinct from SH1 and SUS2 based on the phylogenetic analysis of their glucosyltransferase domains (45). Our analysis indicates that the N terminus of SUS2 is also quite different from those of SUS1 and SH1. Consistent with this observation, SUS2 is excluded from mitochondria. However, under certain experimental conditions, this isoform may also associate with mitochondria as suggested by the interaction of this protein with the VDAC in yeast two-hybrid analysis (43), although our co-immunoprecipitation analysis gave a contradictory indication (Fig. 7).

**Regulation of SUS Mitochondrial Localization**—Besides the paralog specificity, we observed developmental or post-translational regulation of SUS compartmentalization. Tissue or developmental specificity is indicated by two lines of evidence. A light subpopulation of mitochondria banding between 0 and 15% Percoll lacked the SUS protein, whereas the heavy one that banded between 15 and 35% Percoll contained SUS (Fig. 1). Because SUS is present both in the soluble and membrane subfractions of the heavy mitochondria (Fig. 5B), the light fraction may represent an intact but distinct subpopulation of mitochondria rather than fragments of heavy mitochondria. A sharp decline in the amount of mtSUS is also observed above the 1-cm tip of primary roots (Fig. 3). With equal amounts of protein from heavy mitochondria of both root regions used in the analysis, these differences in the SUS association between the tip and the axis organelles indicate the involvement of either a spatially restricted component regulating SUS (e.g. a protein kinase) or a specific developmental/metabolic property of mitochondria in the localization of SUS to organelles.

SUS1 or SH1 association with mitochondria appears to mechanistically differ from their binding to microsomes. Our earlier work indicated that the dephosphorylation of SUS promotes its membrane binding (8, 9, 19). Current analysis using phospho-SUS1-specific antisera suggests that SUS1 associated with mitochondria is heavily phosphorylated, similar to the cytosolic component (Fig. 2C; see also Fig. 5B). In addition, SH1 or SUS1 bound to mitochondrial membranes is resistant to carbonate wash unlike the microsomal SUS (Fig. 5C).

**SUS May Have a Noncatalytic and Yet Important Novel Function**—Sucrose synthase and invertase are the two key enzymes of sucrose utilization in plants. The pathway of sucrose degradation by SUS is favored particularly under energy-limiting conditions because of the lower overall energy costs. This is reflected by the intricate regulation of the enzyme under O2 deprivation (9, 50). Although its association with mitochondria is also responsive to anoxia, the presence of SUS in mitochondria cannot be reconciled with its involvement in sucrose metabolism. The rationale is as follows. The low abundance of the protein in mitochondria is unlikely to make any significant contribution toward sucrose utilization/synthesis. This is further improbable considering its location as an intrinsic protein within the mitochondrial, its high $K_m$ value for sucrose (51), and the particularly limited levels of sucrose expected in mitochondria (37). However, two of the three isoforms of maize SUS are localized to mitochondria. Furthermore, this seems to be a more pervasive element of SUS regulation in plants, as indicated by our bioinformatics analysis (Table 2). Unlike the mitochondria-bound glycolytic enzymes, mtSUS is not in contact with the cytoplasm, indi-

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**TABLE 2**

List of plant SUS genes with putative mTP as predicted by TargetP program

| Species            | Gene   | mTP score | Specificity | Reliability class |
|--------------------|--------|-----------|-------------|------------------|
| Z. mays            | sh1    | 0.822     | 0.90        | 3                |
| Hordeum vulgare    | sus1   | 0.830     | 0.90        | 3                |
| H. vulgare         | sus2   | 0.635     | <0.90       | 4                |
| Triticum aestivum  | sus1   | 0.823     | 0.90        | 3                |
| Oryza sativa       | sus1   | 0.737     | 0.90        | 3                |
| Saccharum officinarum | sus2 | 0.783     | 0.90        | 4                |
| Bamboosa oldhamii  | sus1   | 0.707     | <0.90       | 4                |
| Tulipa gesneriana   | sus2   | 0.647     | <0.90       | 5                |
| Solanum tuberosum  | sus2   | 0.809     | 0.90        | 3                |
| S. tuberosum        | sus3   | 0.743     | <0.90       | 4                |
| Arabidopsis thaliana| sus2  | 0.725     | <0.90       | 4                |
| A. thaliana         | sus4   | 0.723     | <0.90       | 5                |
| Daucus carota       | sus2   | 0.656     | <0.90       | 3                |
| Pyrus pyrifolia     | sus1   | 0.730     | 0.90        | 5                |
| Citrus sinensis     | sus1   | 0.58      | <0.90       | 5                |
| Lycopersicon esculentum | sus4  | 0.859     | 0.90        | 3                |
| L. esculentum       | sus2   | 0.804     | 0.90        | 4                |

a The recommended specificity is 0.90.

Conservation of mTP across Plant Sucrose Synthases—Because the bioinformatics prediction of mTP held true in the case of maize SUS, we analyzed the potential compartmentation of SUS in other plant species. We queried all the putative SUS sequences available in the GenBank™ for their subcellular location using TargetP. In most species, where there is enough sequence information, we detected at least one SUS paralog with a putative mTP at its N terminus (Table 2). This indicates that the association of SUS to mitochondria is not unique to maize but is conserved among plants. Furthermore, two of the six SUS genes present in Arabidopsis (45) encode proteins with putative mitochondrial targeting (At3g43190 and At4g02280; Table 2), and only these two are induced when grown in high CO2. It is also noteworthy that none of the plant SUS sequences possesses a putative chloroplast targeting sequence.
cating that its role is related to a pathway localized in mitochondria. But much like the glycolytic enzymes, both SH1 and SUS1 are predominantly cytosolic in their distribution. This entails the possibility of SUS participating in inter-compartmental signaling events. Our observations on nuclear localized SUS also indicate this may be a likely scenario.7

To functionally characterize the organellar association of predominantly cytosolic proteins, such as SH1 and SUS1, presents logistical hurdles, because the effects of available mutants are not specific to organelles. This becomes particularly challenging, when the organelle is not connected with the primary function of the enzyme. Even in well studied examples such as GAPDH or hexokinase, the biological significance of their compartmentation is still being unraveled (5). Our co-immunoprecipitation studies (Fig. 7) and recent results from the yeast two-hybrid analysis (43) provide some hints about the biological role of mtSUS. The VDAC is the core component of the permeability transition pore and acts as a gatekeeper of solute movement between mitochondria and the cytosol (52, 53). SUS interaction with VDAC may be analogous to hexokinase interaction with the latter in animal cells (54, 55). Hexokinase is associated with mitochondria (although extrinsically) via its interaction with VDAC in a tissue-specific, isoform-dependent, and metabolically regulated manner. This specific localization of hexokinase seems to enable a tight coupling between glycolysis and oxidative phosphorylation as well as to regulate the opening of mitochondrial permeability transition pore, nutrient/sugar signaling, and apoptosis (54, 55).

mtSUS Sheds New Light on the Phylogeny of Plant SUS—Plant SUS proteins have prokaryotic ancestors. Based on the presence of homologous sequences to SUS and other proteins related to sucrose metabolism in proteobacteria, it has been proposed that sucrose synthesis originated in proteobacteria or in a common ancestor of proteobacteria and cyanobacteria (56). However, Salerno and Curatti (57) argued that the cyanobacteria could be a more probable progenitor of the enzymes involved in sucrose metabolism. The new information presented here provides a different perspective on the origin of plant sucrose synthases.

Although there are rare examples where proteins of cyanobacterial origin are targeted to mitochondria (e.g. NDC1; see Ref. 58), the following observations indicate that eukaryotic SUS genes may have their origin in the proteobacterial progenitors of mitochondria: (a) the presence of SUS inside maize mitochondria (Fig. 5) and possibly of other plant species (Table 2); (b) its absence in plastids (Fig. 1) (Ref. 59 and references therein; also see Ref. 60 for a report of SUS association with unpurified maize etioplasts during greening); (c) nonrepresentation of chloroplast targeting sequences in eukaryotic SUS proteins; and (d) presence of putative mitochondrial targeting information in proteobacterial SUS homologs as against poor representation of chloroplast targeting information in cyanobacterial SUS genes (data not shown).

The evolution of mitochondria from an endosymbiotic proteobacteria involved the transfer of genes from the bacterium to the host cell nucleus. Often the encoded protein made in the host cytosol is translocated back into the organellae. Many of the electron transport chain proteins fall into this category. However, there are other gene products that have been integrated into the host cell metabolism and were entirely lost by the endosymbiont (reviewed in Ref. 61). SUS may represent a distinct third category of genes that were transferred from the endosymbiont genome, where the gene product exists in both the host and the symbiont compartments. Is mtSUS simply an evolutionary relic or does it still serve a useful function in the plant cell? We do not yet have a clear answer. However, the interaction with mitochondrial porin and its dynamics during plant and organelle development indicate that mtSUS has a significant biological role.

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