The Structure of Sucrose Synthase-1 from Arabidopsis thaliana and Its Functional Implications

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Background: Sucrose transport is the central system for the allocation of carbon resources in vascular plants. During growth and development, plants control carbon distribution by coordinating sites of sucrose synthesis and cleavage in different plant organs and different cellular locations. Sucrose synthase, which reversibly catalyzes sucrose synthesis and cleavage, provides a direct and reversible means to regulate sucrose flux. Depending on the metabolic environment, sucrose synthase alters its cellular location to participate in cellulose, callose, and starch biosynthesis through its interactions with membranes, organelles, and cytoskeletal actin. The x-ray crystal structure of sucrose synthase isoform 1 from Arabidopsis thaliana (AtSus1) has been determined as a complex with UDP-glucose and as a complex with UDP and fructose, at 2.8- and 2.85-Å resolutions, respectively. The AtSus1 structure provides insights into sucrose catalysis and cleavage, as well as the regulation of sucrose synthase and its interactions with cellular targets.

Results: The crystal structure of sucrose synthase from Arabidopsis thaliana has been determined. The x-ray structures provide insights into the enzymology and regulation of sucrose synthase.

Conclusion: The structures suggest how sucrose synthase interacts with cellular targets, such as membranes, organelles, and cytoskeletal actin.

Significance: The structures show how sucrose synthase interacts with cellular targets, such as membranes, organelles, and cytoskeletal actin.

Sucrose transport is the central system for the allocation of carbon resources in vascular plants. During growth and development, plants control carbon distribution by coordinating sites of sucrose synthesis and cleavage in different plant organs and different cellular locations. Sucrose synthase, which reversibly catalyzes sucrose synthesis and cleavage, provides a direct and reversible means to regulate sucrose flux. Depending on the metabolic environment, sucrose synthase alters its cellular location to participate in cellulose, callose, and starch biosynthesis through its interactions with membranes, organelles, and cytoskeletal actin.

Sucrose is the primary photosynthetic in plants (1) that is transported to sites of growth, development, and energy storage (2). Yet, despite the vital nature of sucrose transport in carbon allocation, sucrose synthesis and cleavage are mediated by the activities of only four enzyme types (1): sucrose-phosphate synthase, sucrose-phosphate phosphatase, invertase, and sucrose synthase (SUS).3 By coordinating the levels of activity of these enzymes at different times during growth, in different plant organs, and in different cellular locations, plants create a spatial and temporal system of sucrose sources and sinks that effectively transports sucrose from one site to another (2–4). In fact, the balance between both sucrose flux and its metabolic utilization can be achieved simply by controlling sucrose cleavage via SUSs and invertases (2, 4).

Of the sucrose-cleaving enzymes, only SUS is capable of catalyzing the synthesis and cleavage of sucrose (Fig. 1) in a reversible and almost energy neutral manner (1, 2, 4). The SUS reaction therefore provides a direct and reversible link in sucrose metabolism between respiration, carbohydrate biosynthesis, and carbohydrate utilization (1), as well as allowing the rapid conversion of a sucrose sink into a sucrose source without the synthesis or degradation of enzymes (2, 4). Although invertases have a critical role in normal plant growth (5, 6), SUS is clearly involved in pollen tube growth (7, 8), the establishment of nitrogen fixation (9–12), biomass production (13, 14), and fruit and seed maturation (13, 15, 16), particularly during periods of abiotic stress (6, 17–19). SUS participates in the regulation of sucrose flux by rapidly altering its cellular location from the cytoplasm to sites of cellulose, callose, and starch biosynthesis (7, 20–22), and through its interaction with various organelle membranes (4, 23, 24) and cytoskeletal actin (25, 26). However, the mechanism by which SUS binds to cellular targets, the structural aspects controlling SUS partitioning, and the structural impact of partitioning on its catalytic function are unknown at a molecular level.

SUS is a member of the retaining GT-4 glycosyltransferase subfamily, within the larger metal-independent GT-B glycosyltransferase superfamily (27). From primary sequence and biochemical analyses, SUS is structurally and functionally similar to sucrose phosphate synthases and glycogen synthases (1). In some plants, more than one SUS isoform is expressed in vivo (3); six isoforms of SUS exist in Arabidopsis thaliana (6). The SUS1 isoform from A. thaliana (AtSus1) has the primary sequence structure of a typical sucrose synthase: residues 1 to 276 form an N-terminal “regulatory” domain involved in cellu-

HoSPS, H. orenii sucrose phosphate synthase; LCN, lichenan; NHF, 1,5-anhydrofructose; PDB, Protein Data Bank.
lar targeting (28) and residues 277 to 776 form the GT-B glycosyltransferase. Finally, a C-terminal extension, which is the most variable of the SUS domains, is only 31 residues long in AtSus1, but it can be much longer in other SUS isoforms (29). Within the N-terminal regulatory domain, two serine residues have been identified as sites for phosphorylation (30–32). In Zea mays, the phosphorylation of the SUS1 isoform at Ser-15 diminishes SUS binding to actin (4), increases membrane association (32, 33), and enhances SUS catalytic activity at acidic pH (33). In contrast, the phosphorylation of Ser-170 in Z. mays SUS1 promotes SUS turnover by enhancing SUS ubiquitylation (30, 34). Interestingly, SUS1 from Z. mays and Glycine max also bind the early nodulin 40 (ENOD40) peptides (30, 35), which act as hormone-like peptides in root nodule formation in legumes (36).

In this report, we present the x-ray crystal structures of AtSus1, as a complex with UDP-glucose at 2.8-Å resolution and as a complex with UDP and fructose at 2.85-Å resolution. The structures shed light on structure-function relationships involved in sucrose catalysis and cleavage, as well as revealed the structural relationships between retaining and inverting glycosyltransferases in the GT-B superfamily. The structure of AtSus1 also highlighted features that may be involved in SUS regulation by phosphorylation, thiolation, and its interaction with cellular targets.

**EXPERIMENTAL PROCEDURES**

**Cloning**—The open reading frame of AtSus1 was PCR-amplified with High Fidelity Taq DNA polymerase (Invitrogen) using the first strand cDNAs prepared from A. thaliana (ecotype Columbia), a kind gift from Dr. E. Moehring, and the gene-specific primers AtSus1-FW, 5’-ACATGCAGGCAACGCTGAACGTATG-3’, and AtSus1-RV, 5’-GTGCAGATCATCTTTGTGCAAGAGGAA-3’. The primer sequences included appropriate restriction sites AflIII and SalI (underlined). The PCR product was cloned into pGEM®-T Easy vector (Promega). The excised fragment of the AtSus1 ORF was subcloned into expression vector pET17b (Novagen) using the compatible NcoI and XhoI sites to create a C-terminal fusion protein with a His6 tag. Constructs were confirmed by nucleotide sequencing with cellular targets.

**Protein Expression and Purification**—The transformed bacteria were cultured at 37 °C in 2YT medium containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol until reaching an A600 of ~1.0, and then gene expression was induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 12–16 h at 20–23 °C. Harvested cells (~4 g from 700 ml of cell culture) were resuspended in 50 ml of lysis buffer (30 mM Tris-HCl, pH 8.0, 200 mM NaCl, 150 mM sucrose, 2 mM EDTA, and 1 mM DTT) and lysed after two passes at 15,000–20,000 p.s.i on an Avestin EmulsiFlex C-3 homogenizer.

Following a centrifugation step at 20,000 × g (4 °C) for 20 min, the clarified lysate was fractionated by addition of (NH4)2SO4; the fraction that precipitated between 45 and 55% saturated (NH4)2SO4 was collected by centrifugation at 20,000 × g for 20 min. The precipitate dissolved in 50 ml of buffer A (lysis buffer without EDTA) was applied to a 10-ml nickel-nitrilotriacetic (Qiagen) gravity flow column, pre-equilibrated in buffer A. After extensive washing, fractions were eluted over 2 column volumes using buffer A containing 150 mM imidazole. Fractions containing AtSus1 were pooled and concentrated to about 1 ml, and 500-μl volumes were applied at 0.4 ml min⁻¹ to a Superose 6 gel filtration column (GE Healthcare) pre-equilibrated with either buffer A (1 mM EDTA) or buffer B (30 mM Tris-HCl, pH 8.0, 200 mM NaCl, 20 mM fructose, 1 mM EDTA, and 1 mM DTT). Peak fractions were concentrated to a protein concentration of ~15 mg ml⁻¹. The selenomethionine-labeled derivative of AtSus1 was expressed in the methionine auxotroph cell line B843(DE3) (Novagen) and purified in the same way as the native protein.

Analytical gel filtration chromatography was used to estimate the molecular size of purified AtSus1. A Superose 6 10/300 GL gel filtration column (GE Healthcare) was equilibrated in 30 mM HEPES, pH 7.4, 200 mM NaCl, 1 mM DTT, and either 20 mM fructose or 150 mM sucrose. The column was subsequently calibrated with gel filtration standards (Bio-Rad). AtSus1 samples were chromatographed in the presence of fructose or sucrose at a flow rate of 0.4 ml min⁻¹ at 4 °C.

**Crystallization and Cryoprotection**—UDP or UDP-glucose were added to the AtSus1 protein solution to a 5 mM final concentration. Crystals were grown by the hanging-drop method at 20 °C using 2 μl of protein solution mixed with an equal volume of reservoir solution. AtSus1 crystallized under a number of different conditions in monoclinic and tetragonal habits at 1.8–2.0 M (NH4)2SO4. The crystals of AtSus1 in complex with fructose and UDP grew in 80 mM sodium citrate, pH 5.8, 150 mM potassium sodium tartrate, and 1.86 M (NH4)2SO4. The crystals of the UDP-glucose complex grew in the same condition except the pH of the citrate buffer was 6.1. Prior to flash freezing in liquid nitrogen, all crystals were cryoprotected by sequential addition of increments of mother liquor supplemented with 2.0 M sodium malonate, pH 5.8. Native AtSus1 crystals diffracted to 2.6–2.9 Å for the C2 space group (Table 1) and 3.4 Å for the I422 space group. Interestingly, reducing agents may play a role in determining the crystal space groups. In the absence of DTT, AtSus1 crystals are about equally divided into the two space
**Structure of Sucrose Synthase-1 from A. thaliana**

**TABLE 1**

Data collection, phasing, and refinement statistics for AtSus1 structures

| Data collection | AtSus1-fructose-UDP (SeMet) | AtSus1-fructose-UDP | AtSus1-UDP-glucose |
|-----------------|-----------------------------|---------------------|---------------------|
| Space group     | C 2                         | C 2                 | C 2                 |
| a, b, c (Å)     | 276.2 263.7 159.7            | 277.2 261.5 161.1   | 276.8 261.9 160.2   |
| α, β, γ (°)     | 90.0 108.7 90.0              | 90.0 109.3 90.0     | 90.0 108.7 90.0     |
| Resolution (Å)  | 50–2.90                     | 50.00–2.85          | 50.00–2.80          |
| (2.95–2.90)     | (3.0–2.85)                  | (2.95–2.80)         |
| R_work          | 0.186(0.72)                 | 0.130(0.68)         | 0.110(0.62)         |
| R_free          | 0.217(1.3)                  | 0.161(1.1)          | 0.111(1.4)          |
| Completeness (%)| 99.9(89.7)                  | 99.4(99.5)          | 99.6(99.2)          |
| Redundancy      | 7.3                         | 2.6(2.6)            | 3.0(3.0)            |

| Refinement      | AtSus1-fructose-UDP         | AtSus1-UDP-glucose  |
|-----------------|-----------------------------|---------------------|
| No. reflections | 214,680                     | 214,680             |
| R_work/R_free   | 0.186/0.237                 | 0.185/0.237         |
| Protein         | 50,240                      | 50,240              |
| Ligand/ion      | 480                         | 480                 |
| Water           | 50                          | 50                  |
| B-factors       | 499                         | 499                 |
| Protein         | 46.29                       | 47.90               |
| Ligand/ion      | 48.47                       | 40.93               |
| Water           | 31.67                       | 32.34               |
| Root mean square deviations | 0.011 | 0.011  |
| Bond lengths (Å) | 1.136 | 1.136  |
| Bond angles (°) | 90.0 108.7 90.0             | 90.0 109.3 90.0     |

* The structure of seleniummethionine(SeMet)-labeled AtSus1 complexed with UDP and fructose was only partially refined, due to its lower resolution, and just used to phase the other two complexes.

* Values in parentheses are for highest-resolution shell.

Groups, whereas if 2 mM DTT is added, the population of Sus1 crystals is predominately monoclinic.

**Phasing, Structure Determination, and Refinement**—The native and single anomalous dispersion data were collected at LS-CAT (Advanced Photon Source, Argonne, IL) beamlines 21-ID-D and 21-ID-G and processed using XDS (37) and SCALA (38); the single anomalous dispersion data were collected at the observed wavelength (0.9794 Å) for the absorption peak of selenium. Positions for 94 of the 112 selenium sites in the selenomethionine-AtSus1-UDP-fructose complex were located at 3.1 Å using AutoSol in the Phenix (version 1.6.2) to minimize biasing the overall R_free. For both complexes, ~95.5% of the residues are in the most favored, ~4.4% are in the additionally allowed region of the Ramachandran plot, and ~0.1% are in disallowed regions. The figures representing the crystal structures were prepared with PyMol.

**Structural Superpositions and Multiple Sequence Alignments**—The three-dimensional superposition of AtSus1 with a variety of structurally characterized GT-B glycosyltransferases was done using programs LSQAB or TOPP in the CCP4 program suite (38). The SUS sequences from a variety of plant species were obtained from the NCBI, and aligned with T-Coffee (41) with respect to the different folding domains. Supplemental Fig. S1 shows the alignment of the CTD and EPBD domains (residues 1–276) in AtSus1 with 11 other SUS homologs from 9 different plant species. The figure was prepared using the program ALINE (42).

The structure of selenomethionine(SeMet)-labeled AtSus1 complexed with UDP and fructose was only partially refined, due to its lower resolution, and just used to phase the native data set of the AtSus1 complexed with UDP and fructose. The chemical information needed to define the stereochemistry and refinement restraints for the glucosyl intermediates, lichenan (LCN, Chembase ID 439241) and 1,5-anhydrofructose (NHF, Chembase ID 126517), was obtained from PubChem. Iterative model building with COOT 6.1 (40) and refinement against the native data using Phenix Refine yielded a final model that accounted for 6,246 of the possible 6,528 residues.

Data collection and refinement statistics for the AtSus1 structures are listed in Table 1. Although noncrystallographic symmetry restraints were used in the initial stages of model building, all noncrystallographic symmetry restraints were removed in the final stages of refinement. Because of the relatively high level of noncrystallographic symmetry in the asymmetric unit, the R_free test set was chosen, using the algorithms in Phenix (version 1.6.2) to minimize biasing the overall R_free. For both complexes, ~95.5% of the residues are in the most favored, ~4.4% are in the additionally allowed region of the Ramachandran plot, and ~0.1% are in disallowed regions. The figures representing the crystal structures were prepared with PyMol.
RESULTS AND DISCUSSION

Overall Structure of AtSus1—Iterative cycles of model building and refinement yielded well defined structures for AtSus1 complexed with either UDP and fructose or UDP-glucose, at resolutions of 2.85 and 2.80 Å, respectively. The resolved contents of the asymmetric unit revealed two 222-symmetric tetramers of AtSus1 (Fig. 2A, denoted as tetramers ABCD and EFGH). The AtSus1 polypeptide chain (residues 11–808) folds into a tri-lobed structure with four distinct domains (Fig. 2, B and C). The first two domains have been designated a cellular targeting domain (CTD, residues 11–127) and an ENOD40 peptide-binding domain (EPBD, residues 157–276). The final two domains comprise the GT-B glycosyltransferase with its Rossmann-fold domains (27).

The observed secondary structure of the CTD starts at residue 16 with α-helices α1 and α2 (Fig. 2B). Helix α2 is connected to a very short β-strand (β1), which is then followed by another two helices (helix α3 and α4). With β1, the next four β-strands create a 5-stranded, anti-parallel β-sheet, which has the appearance of a platform from which two α-helical loops (helices α1/α2 and α3/α4) dangle. Although the primary sequence in the helices of the CTD differs between SUS from different plant species, the core of the β-sheet (strands β2–β5) shows a high degree of sequence conservation (supplemental Fig. S1). The overall fold of the CTD bears no resemblance to previously known folds, as judged by the DALI program (43).

As the polypeptide chain exits helix α11, a short 5-residue stretch separates the EPBD from the first β-strand of a GT-B glycosyltransferase (Fig. 2B) and its two Rossmann-fold domains (27). As the structural topology and secondary structural elements of the GT-B enzymes are quite well conserved (27), we will refer to the N- and C-terminal domains of the GT-B glycosyltransferase as GT-BN and GT-BC, respectively. Likewise, the conserved secondary structural elements of the GT-B domain will be referred to by nomenclature defined by Ha et al. (44), e.g. Nα1 would refer to the first α-helix in the
GT-B\textsubscript{N} domain (supplemental Fig. S2). The initial portion of the GT-B\textsubscript{N} domain in AtSus1 extends from residues 277 to 526 and the GT-B\textsubscript{C} domain from residues 527 to 754.

As typical of all known GT-B glycosyltransferases (27), the interdomain hinge in AtSus1 consists of two linkers (Fig. 2C), one between the two domains and one between two final C-terminal \(\alpha\)-helices (Ca7 and Na6). As expected for a GT-B glycosyltransferase, the substrates (UDP-Glc or UDP and fructose) bound in the cleft between the GT-B\textsubscript{N} and GT-B\textsubscript{C} domains (Fig. 2D), and the GT-B domains appear in the “closed” and putatively active conformation. The polypeptide then ends with a helical, 37-amino acid C-terminal extension (Fig. 2, B and C).

From primary sequence analyses, the closest related GT-B glycosyltransferases to SUS are the sucrose-phosphate synthases (1). For the GT-B domains alone, the percent identity between SUS1 and SUSPs from \textit{A. thaliana} is about 26%. Recently, Chua et al. (45) determined the structure of a bacterial sucrosephosphate synthase from \textit{Halothermothrix orenii} (HoSPS). As the HoSPS structure was observed only in the open conformation and the AtSus1 structures are both in the closed conformation, the individual GT-B\textsubscript{N} and GT-B\textsubscript{C} domains were superimposed between AtSus1 and HoSPS. For the GT-B\textsubscript{N} domain, 196 topologically and spatially related residues superimpose well and yielded a root mean square deviation of 1.40 Å between paired C\textsubscript{a} atoms. For the GT-B\textsubscript{C} domains, 180 residues were identified for superposition, which yielded a root mean square deviation of 1.37 Å between paired C\textsubscript{a} atoms. Both superpositions reinforce the evolutionary relationship between the two enzymes involved in sucrose synthesis (1).

Although the GT-B domains in AtSus1 have all the conserved secondary structural elements of the GT-B glycosyltransferases, there are several structural elaborations not seen in other GT-B glycosyltransferases. In the GT-B\textsubscript{N} domain, the Rossmann-fold deviates from its canonical form by replacement of the Na2 helical return by the \(\beta\)-strand N\(\beta\)1*, which makes antiparallel interactions with the linker region between CTD and EPBD. Another unique feature of GT-B\textsubscript{N} is that helix Na1 is \(\sim 10\) Å longer than the homologous helix in any other known GT-B glycosyltransferase structure. As a result, the C-terminal end of Na1 sticks out of the GT-B\textsubscript{N} domain and abuts against the EPBD (Fig. 2C). As a consequence, the helix adjacent to the second hinge site (Na6, residues 757–776) is held in place by the Na1 helix of GT-B\textsubscript{N} and helices a6 and a11 in the EPBD domain. In contrast, the GT-B\textsubscript{C} domain generally follows the canonical Rossmann-fold seen in other GT-B glycosyltransferases.

**Conformational Variability in AtSus1 Tetramer**—The AtSus1 tetramers observed in the asymmetric unit display excellent noncrystallographic 222 symmetry with respect to all of the domains except for the CTDs. In the crystal, neighboring columns of tetramers are interdigitated (Fig. 3A), and this interdigitation impacts the order (supplemental Table S1) and conformation (Fig. 3B) of the N-terminal domains of each monomer. As a result, differing portions of the N termini could be resolved in each subunit. Only in the AtSus1-UDP-fructose complex could helix a1 of the CTD be resolved (Fig. 2, A and B): subunit H could be traced from residue 11 to 807 (supplemental Fig. S3) and subunit B from residue 14 to 807. The polypeptide chain could only be traced from residues 27 to 807 in the remaining subunits. In the AtSus1-UDP-glucose complex, the model included residues 28 to 807 for subunits B and H and residues 27 to 807 for the remaining six subunits, which meant that helix a1 was completely disordered in this complex.

The CTD domains also displayed significant conformational differences (Fig. 3C). In the crystal structures of both complexes, the CTD domain in the B and H subunits exhibited a rigid body rotation of \(\sim 6\) degrees with respect to the other domains compared with its orientation in the remaining 6 subunits. The helix a5 lies across the surface of the \(\beta\)-sheet, which seems to pivot on the helix surface (Fig. 3C). More precise measurements of domain movement are difficult because of the
high level of disorder in the CTD domains (supplemental Table S1).

Subunit Interactions in the AtSus1 Tetramer—The biophysical analyses on SUS from maize (34), mung bean (46), and potato (47) strongly suggested that its native oligomeric state is a tetramer, although SUS can exist in a dimer form (25). Results from analytical gel filtration of purified AtSus1, in the presence of 150 mM sucrose or 20 mM fructose, suggest that enzyme exists as a homogeneous molecular species with a size of ~360 kDa, which is close to the predicted value for a tetramer (~372 kDa).

The AtSus1 tetramer is a rather flat oligomer and displays two classes of subunit interfaces (A:B or A:D; Fig. 2A), which results in a large hole in the center of the oligomer. As other tetramer arrangements could be generated within the crystal, the monomer interactions were further analyzed through the use of the PDBEPIISA server. The analysis confirmed that the initial choice of the tetramer produced the most compact and plausible oligomer. The average surface area for the A:D interface is ~1280 Å² (about 4% of the total surface area of the monomer) with a predicted solvation free energy gain ΔG of ~18.8 kcal mol⁻¹. In contrast, the A:B interface covers less average surface area (~1076 Å²) and is much less hydrophobic (ΔG = −6.4 kcal mol⁻¹). Interestingly, the GT-B domains do not participate in subunit interaction in either of the A:B or A:D interfaces to any significant extent.

The proposed subunit interfaces were also examined to see if they were consistent with the published biochemical data. The N-terminal half of the CTD-EPBD domain linker (residues 131–142) and portions of the C-terminal extension (residues 778–796) create the A:D interface (Fig. 3D). Although the majority of contacts are between these two polypeptide segments, residues 131–134 of the linker also contact the surfaces of helices Na3 and Na4 in the GT-Bα domain. One prediction that can be made is that removal of the C-terminal extension would seriously compromise this subunit interface. Hardin et al. (28) demonstrated that C-terminal truncation of recombinant maize SUS1 resulted in the expressed protein forming dimers, a finding consistent with the A:D interface being part of the native AtSus1 tetramer.

In contrast, the A:B interface (Fig. 3E) is created entirely almost from interactions between adjacent EPBDS, although the C-terminal half of the CTD-EPBD domain linker (residues 147–154) also makes a few contacts with helix α10 of a neighboring EPBD. The A:B interface creates a long groove within Cys-266, which sits ~3 Å away from the dyad axis. Cys-266 is quite reactive; although heavy atom-derivatized crystals were not useful for phasing, Cys-266 can be quantitatively derivatized with mercurial compounds (data not shown). Röhrig et al. (48) demonstrated that the dodecapeptide ENOD40-A specifically thiolates Cys-264 of soybean SUS, which is equivalent to Cys-266 in AtSus1. ENOD40 peptides A and B are small, hormone-like peptides that are involved in root nodule organogenesis in legumes (36). Although A. thaliana is not a legume, it shares a high degree of sequence conservation with legumes around Cys-266 (supplemental Fig. S1). More surprisingly, the ENOD40 peptides bind tightly to SUS1 from maize (30), even though the cysteine is replaced by an alanine.

Hardin and colleagues (30) also observed that ENOD40 peptides binding to SUS1 also inhibited the phosphorylation of Ser-170 in maize. When Ser-170 is phosphorylated, SUS turnover is enhanced by increased ubiquitinylation (30, 34). Serine 167, the equivalent residue in AtSus1, lies within the A:B interface at the N-terminal end of α6. In the intact native tetramer, Ser-167 is inaccessible to phosphorylation, but would be accessible if the tetramer dissociates into dimers upon disruption of the A:B interface, a reasonable hypothesis given that this interface is predicted to be much less hydrophobic than the A:D interface. The dissociation into dimers would then allow its phosphorylation and increase the potential for ubiquitinylation and turnover. Hence, the in vitro effects of ENOD40 peptides on SUS phosphorylation can be completely explained by interactions at the A:B interface.

The Active Site of AtSus1—No significant global conformational differences with respect to the GT-B domains were observed at the AtSus1-UDP-glucose and AtSus1-UDP-fructose complexes. In the AtSus1-UDP-glucose complex, UDP and d-fructose were clearly resolved in each active site within the asymmetric unit; what appeared to be UDP-glucose was also well resolved in each active site of the AtSus1-UDP-glucose complex. As monomer F has the lowest average B-factor for both complexes among the eight monomers (supplemental Table S1), the discussion will refer to this subunit.

The UDP moiety is bound in an identical manner in both complexes (Figs. 4A and 5), primarily through interactions with the GT-Bα domain (loops Cβ1 → Cα1, Cβ3 → Cα3, and Cβ4 → Cα4, as well as helices Cα3 and Cα4); the only interactions with the GT-Bγ domain involve hydrogen bonds from the backbone amide nitrogen of Gly-303 to O1B and O3B of the β-phosphate. The face of the uracil ring stacks against the methyl group of Met-578, whereas the edge of the ring makes three hydrogen bonds: the uracil atoms O4, N3, and O2 to the amide nitrogen of Gln-648, to the carbonyl oxygen of Gln-648, and to Nδ2 of Asn-654, respectively. The O2’ and O3’ hydroxyls of the ribose ring hydrogen bond to the carboxylate oxygens of the Glu-685. The α-phosphate of UDP is bound to the protein through hydrogen bonds from the O1A and O2A atoms to backbone amide nitrogens of Thr-680 and Leu-679, respectively. However, the O1A atom also participates in a hydrogen bond chain with the amide nitrogen of Arg-580 that is mediated by two conserved water molecules in the active site. Finally, the Nη1 atom of Arg-580 contacts the β-phosphate via the O1B and O2B oxygens, whereas the Nζ nitrogen of Lys-585 interacts with O2B and O3A atoms. Overall, the UDP moiety binds in a similar manner to AtSus1 as ADP in E. coli glycogen synthase (EcGS), although the ribose ring of ADP is liganded by Asp-21 from the GT-Bγ domain (49) and not by a residue from the GT-Bα domain.

In the AtSus1-UDP-fructose complex, fructose is firmly bound in the β-furanose form within a pocket formed exclusively by residues from the GT-Bγ domain (Figs. 4A and 5). The 8 fructose molecules in the asymmetric unit tend to be in the 5E envelope conformation or in the two neighboring twist conformations, as defined by French and Tran (50). Each hydroxyl of fructose is hydrogen bonded, which may help optimize substrate specificity. The O1 hydroxyl interacts with the amide
The O3B β-phosphate atom of the UDP and the C1 anomic carbon of the glucose moiety in all monomers within the asymmetric unit. Attempts to fit the glucose moiety of an intact UDP-Glc into the rather flat region of electron density always resulted in marked distortion of the stereochemistry at the anomic carbon and the appearance of significant negative electron density in \( F_o - F_c \) difference maps.

The proposed mechanisms for saccharide transfer in the retaining glycosyltransferases fall into three different types (27): 1) a double-displacement mechanism that predicts the formation of a covalent enzyme-glycosyl intermediate, 2) an \( S_{N,i} \) mechanism that predicts a transition state with a transient oxocarbenium ion pair, and 3) an \( S_{N,i} \)-like mechanism that combines a transient ion pair intermediate with a conformational shift as the incoming acceptor attacks. The \( S_{N,i} \)-like catalytic mechanism (Fig. 6) has been proposed for several retaining glycosyltransferases from the metal-dependent GT-A and the metal-independent GT-B enzyme families (27, 51), although a preponderance of evidence favoring one mechanism over the others is still lacking. Against this background of possible catalytic mechanisms, we evaluated several different scenarios for UDP and glucose within the AtSus1 active site.

The flat electron density around the C1 anomic carbon suggested a distorted glucosyl species with a missing O1 oxygen, similar to the glucosyl intermediate observed in EcGS (49). Two model compounds were chosen that mimic the oxocarbenium-phosphate ion pair intermediate predicted by the \( S_{N,i} \)-like catalytic mechanism (Fig. 6): 1,5-anhydrofructose (NHF), which is a natural product (52), and lichenan (LCN), a tautomer of NHF that is a C2 deprotonation product of the D-glucopyranosyl (oxocarbenium ion) and contains a C1-C2 double bond. The primary difference between NHF and LCN is the extent of the planar portion of the ring, i.e. O1-C1-C2-O2-C3 atoms lie in a plane in LCN, but C1-C2-O2-C3 atoms lie in a plane in NHF. After parallel cycles of refinement and model building using various combinations of UDP-Glc and UDP with the individual glucosyl analogs, the results unequivocally rule out the existence of an enzyme-glycosyl intermediate, even at a low level of occupancy, and also suggest that the glucose moiety of UDP-Glc or free glucose probably contributes to less than 10% to the electron density of the unknown glucosyl species. On the other hand, LCN, NHF, or a mixture of LCN and NHF can account for the observed electron density (Fig. 4B). Thus, in the absence of an acceptor, AtSus1 hydrolyzes UDP-Glc and traps an intermediate consistent with the oxocarbenium ion expected in \( S_{N,i} \)-like mechanism (step 3 in Fig. 6). For simplicity, interactions between the glucose moiety and AtSus1 will be described using LCN, although the NHF displayed identical intermolecular interactions with the protein and UDP. The protein hydrogen bonds LCN at four points (Fig. 4B): the Oe1 oxygen of Glu-675 and amide nitrogen of Phe-677 bind the O3’ hydroxyl, the amide nitrogen of Gly-678 binds the O4’ hydroxyl, and the Ne2 nitrogen of His-438 binds the 6-hydroxyl.

As both complexes are in the identical closed, ligand-bound form, the pairwise superimposition of the Ca atoms between the GT-B domains of the two complexes gives root mean square deviations of less than 0.23 Å. Given the high degree of confor-
mational homology between the two complexes, the structures were superimposed to provide a composite view of the ligands with respect to each other (Fig. 4C). The composite view shows that the C1 carbon is in close proximity to the backbone carbonyl oxygen of His-438 (3.2 Å), O2 hydroxyl of fructose (2.3 Å), and O3B of the UDP β-phosphate (2.9 Å). Moreover, the attacking group (O2 hydroxyl of fructose) and the leaving group (O3B phosphate oxygen) are both on the α face of LCN, whereas the carbonyl of His-438, which may stabilize the partial positive charge at the C1 carbon, is on the β face. The AtSus1-UDP-glucose complex also suggests that the stabilized glucosyl intermediate makes intimate interactions with the pyrophosphate of UDP (Fig. 4C): the O2’ hydroxyl interacts with O2B and the O4’ hydroxyl interacts with O2A.

At 2.8-Å resolution, it is difficult to provide a more detailed analysis of the AtSus1 complexes. Nonetheless, it is clear that there is no suitable protein residue in the immediate area surrounding LCN and fructose to act as a glucosyl acceptor, as...
would be expected for the \( S_{N}2 \) reaction mechanism and that UDP-glucose is cleaved into UDP and an unknown glucosyl species that is not glucose (Fig. 4B). Moreover, this glucosyl intermediate is also stabilized by interactions with the protein (His-438 and Glu-675) and the pyrophosphate of UDP (Figs. 4C and 5). Finally, O3B oxygen of UDP and the O2 hydroxyl of fructose are in a position to attack the same face of LCN.

Fig. 7A shows that the interactions within the active site are evolutionarily conserved in several retaining glycosyltransferases. AtSus1 and EcGS (Protein Data Bank code 3GUH) were superimposed with the UDP-glycosyltransferase OtsA (PDB 1UQU) from Mycobacterium tuberculosis (51). Each of these enzyme complexes was crystallized in the presence of UDP-glucose or ADP-glucose. Although the nucleosides and the pyrophosphate moieties overlapped well, the glucosyl intermediates observed in AtSus1 and EcGS (49) are displaced from the glucosyl moiety in OtsA due to its intact O3B-C1 bond. Although primary sequence alignments between AtSus1, HoSP5, EcGS, and OtsA exhibit very low levels of conservation, the glucosyl moieties are liganded in a conserved manner: the O3’ hydroxyl by a carbonylate and the O6’ hydroxyl by a histidine (Table 2). Moreover, conserved glycines (Gly-302 and Gly-303 in AtSus1), a conserved lysine (Lys-585 in AtSus1), and a conserved arginine (Arg-580 in AtSus1) also coordinate the pyrophosphate oxygens (Table 2 and Fig. 7A). A virtually identical arrangement is also seen in the active sites of other retaining GT-B glycosyltransferases (53, 54), despite levels of sequence identity that fall well below 20%.

These results lend credence to the hypothesis that a conserved enzymatic mechanism exists for most, if not all, retaining glycosyltransferases in the GT-B superfamily. Furthermore, the observations of stabilized glucosyl intermediates in AtSus1 and EcGS (49) favor the proposed \( S_{N}2 \)-like reaction mechanism (27, 51, 53) for sugar transfer. The long-lived nature of the glucosyl intermediate seen in AtSus1 is quite remarkable. Why would SUS generate a stable glucosyl intermediate in the absence of fructose is an open question. However, SUS can reversibly catalyze the transfer of glucose between UDP and fructose; virtually all other retaining glycosyltransferases catalyze the irreversible transfer of the sugar from a nucleoside diphosphate donor to an acceptor.

**Insights into the Evolution of the Retaining and Inverting GT-B Glycosyltransferases**—A major unanswered question concerns the evolution of the inverting and retaining reaction mechanisms within the GT-B superfamily (27). As noted by Martinez-Fleites et al. (55), the loop between \( C\beta4 \) and \( C\alpha4 \) differs markedly between the inverting and retaining glycosyltransferases in the GT-B superfamily (Fig. 7, B and C). In the retaining GT-B glycosyltransferases, the \( C\beta4 \rightarrow C\alpha4 \) loop is quite long and essentially blocks the pyrophosphate and glucosyl moieties of the NDP-sugar donor from entering this area (Fig. 7B). In fact, a portion of the \( C\beta4 \rightarrow C\alpha4 \) loop (residues 675–680) participates in the binding of the UDP-glucose in AtSus1 (Fig. 5). In contrast, the much shorter \( C\beta4 \rightarrow C\alpha4 \) loop in the inverting subfamily allows the NDP-sugar donor to bind across the front of the \( C\alpha4 \) helix (Fig. 7C), which may allow the positive end of the helix dipole to assist in liganding the pyrophosphate group (56, 57). An additional feature was noted when comparing AtSus1 with other inverting and retaining glycosyltransferases in the GT-B superfamily. The \( N\beta1 \rightarrow N\alpha1 \) loop in the inverting GT-B glycosyltransferases is also quite short and extends outward from \( N\beta1 \) before returning to begin the \( N\alpha1 \) helix (Fig. 7C). As a consequence, the acceptor molecule lies away from the mouth of \( N\alpha1 \) helix. In the retaining GT-B glycosyltransferases, however, the \( N\beta1 \rightarrow N\alpha1 \) loop is much longer, but extends up and over \( N\beta1 \) and \( N\alpha1 \) before beginning the first turn of the \( N\alpha1 \) helix (Fig. 7B). Now the acceptor molecule can lie at the mouth of the \( N\alpha1 \) helix, and in a position to be affected by the positive helix dipole of \( N\alpha1 \). The \( N\beta1 \rightarrow N\alpha1 \) loop (residues 302–304) also participates in the binding of fructose and the pyrophosphate (Fig. 5).
The conserved active site residues in retaining GT-B glycosyltransferases

| Enzyme | GT family | Site 1 | Site 2 | Site 3 | Site 4 |
|--------|-----------|--------|--------|--------|--------|
| AtSus1 | 4         | ypdG507qvvy | tiaH488alek | R280LdrvK285nls | palyE299algf |
| HoSPS  | 4         | hpdG181qhvy | fhgH151slpa | R276LdqG181ubv | tsfG300pgf |
| MaHA   | 4         | tgdG212mvny | htaH131ltaa | R231LqdK236gqq | pssfE313sfgl |
| EcGS   | 5         | liktG14ladv | ftvH141nlay | R200LtsqK205gld | psfeE177pgf |
| OtsA   | 20        | aasaG122lagv | fflH225lplp | R262LdykG267glp | tprD327gml |
| MalP   | 35        | algaG110lgvl | ytnH320lmp | R334LheyK340rgh | tskE417stg |

*Residue numbering were from structural superpositions of AtSus1 with HoSPS (PDB 2B60), MaHA (PDB 3C4V), EcGS (PDB 3G9U), OtsA (PDB 1UQ0), and MalP (PDB 2ASV).

*As defined in the CAZy database.

Functional Implications of the AtSus1 Structure—The mechanisms by which the presence of sucrose (25, 28), the thiolation of Cys-266 (48), and the phosphorylation of Ser-13 (25, 30, 33, 46) seem to alter SUS binding to cell membranes and F-actin are unknown. In maize SUS1, the phosphorylation of Ser-15 (25, 33) also results in additional and particularly interesting biochemical effects. First, it shifts the oligomerization of SUS1 from dimers to tetramers. Second, in mung bean SUS1, Nakai and colleagues (46) noted that the phosphorylation of Ser-11, the equivalent of Ser-13 in AtSus1, increases sucrose affinity, which may enhance UDP-glucose generation. They also found that mutation of Ser-11 to glutamate mimics phosphorylation. Likewise, Sauerzapfe et al. (58) recently demonstrated that mutations at Ser-11 in potato SUS1 actually alter the preference for monosaccharide acceptors in the active site, ~40 Å away.

The structure of AtSus1 provides a model that explains how SUS catalysis may be modulated upon phosphorylation of the CTD or by its interactions with cellular targets. In AtSus1, Ser-13 lies in a disordered region in all but one subunit, subunit H in the AtSus1-UDP-fructose complex (Fig. 2A). Serine 13 in subunit H lies next to helix α8 of the EPBD in subunit G (Fig. 8A). The conserved sequence surrounding Ser-13 is cationic (10RXHSXR/K/ER17), whereas the neighboring helix α8 has both anionic and cationic patches (210LKRAEYLL216). The detailed interactions between the N terminus and helix α8 are unclear, but the phosphorylation of Ser-13 would certainly alter the electrostatic environment in this region. This putative interaction also provides a model explaining how SUS catalysis may be modulated when CTD is phosphorylated at Ser-167 and when the EPBD is thiolated by ENOD40 peptides. As shown in Fig. 8A, the unusually long Nα1 helix (residues 303-326) of the GT-Bc extends out from the active site. The N-terminal end of Nα1 interacts with pyrophosphate and fructose (Fig. 8A), whereas the C-terminal end of Nα1 abuts against helix α10 of the EPBD. Adjacent to α10 is α8, which interacts with the region surrounding Ser-13; nearby is Cys-266, the site of thiolation by ENOD40 peptide A. The juxtaposition of potential regulatory sites around the EPBD suggests that conformational changes in this domain could be readily transmitted into the active site through distortions of the Nα1 helix.

The observation that the CTD can move as a rigid body (Fig. 3C) also presents a possible mode of SUS interaction with actin, and perhaps other cellular targets. Mapping the movement of the CTD onto all the subunits, the overall impact of this conformational change is to move the CTDs closer or farther apart across an ~50-Å wide groove on the face of the AtSus1 tetramer (Fig. 8B), where the α9 helices of two EPBDs form the base of the groove. The overall dimensions and shape of the groove are quite complementary to the size and topography of the F-actin filament (Fig. 8C). The qualitative model for AtSus1 binding to F-actin proposes that the CTD domains would bind within the groove of the F-actin, where the α1 helix of the CTD and helix α9 in the EPBD may directly mediate contact with the actin fiber. Again, the proximity of helix α9 to helix Nα1 creates a pathway for actin binding to modulate the SUS active sites. Hence, a mutagenic analysis of the residues in the EPBD, at the C-terminal end of Nα1, and surrounding Ser-13 may provide
crucial insights into how SUS function and enzymatic activity are regulated.

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