The Structure of Sucrose Phosphate Synthase from *Halothermothrix orenii* Reveals Its Mechanism of Action and Binding Mode

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Sucrose phosphate synthase (SPS) catalyzes the transfer of a glycosyl group from an activated donor sugar, such as uridine diphosphate glucose (UDP-Glc), to a saccharide acceptor α-fructose 6-phosphate (F6P), resulting in the formation of UDP and α-sucrose-6′-phosphate (S6P). This is a central regulatory process in the production of sucrose in plants, cyanobacteria, and proteobacteria. Here, we report the crystal structure of SPS from the nonphotosynthetic bacterium *Halothermothrix orenii* and its complexes with the substrate F6P and the product S6P. SPS has two distinct Rossmann-fold domains with a large substrate binding cleft at the interdomain interface. Structures of two complexes show that both the substrate F6P and the product S6P bind to the A-domain of SPS. Based on comparative analysis of the SPS structure with other related enzymes, the donor substrate, nucleotide diphosphate glucose, binds to the B-domain of SPS. Furthermore, we propose a mechanism of catalysis by *H. orenii* SPS. Our findings indicate that SPS from *H. orenii* may represent a valid model for the catalytic domain of plant SPSs and thus may provide useful insight into the reaction mechanism of the plant enzyme.

INTRODUCTION

The enzymes sucrose phosphate synthase (SPS) and sucrose phosphatase (SPP) catalyze the synthesis of sucrose, a process that is believed to be restricted to plants, cyanobacteria (bacterial ancestors of the plant chloroplasts; Cumino et al., 2002), and some proteobacteria (Lunn, 2002). SPS catalyzes the first step in the pathway of sucrose synthesis by transferring a glycosyl group from an activated donor sugar, such as uridine diphosphate glucose (UDP-Glc), to a sugar acceptor α-fructose 6-phosphate (F6P), resulting in the formation of UDP and α-sucrose-6′-phosphate (S6P) (Figure 1). This reversible reaction is followed by an irreversible reaction by SPP, where S6P is dephosphorylated to sucrose, concluding the sucrose biosynthesis pathway.

Expression of SPS, a ubiquitous enzyme in plants and green algae, is regulated by photosynthetic light conditions and osmotic stress (Hite et al., 1993; Lee et al., 2003). The SPSs from the photosynthetic cyanobacteria *Anabaena* sp PCC 7120 and *Synechocystis* sp PCC 6803 (Porchia and Salerno, 1996; Lunn et al., 1999) were characterized, and their respective putative SPS genes have also been identified in several other cyanobacterial species, including *Synechococcus* sp WH 8102 and *Prochlorococcus marinus* (Lunn, 2002). The functional and physiological role of the SPS gene in these photosynthetic prokaryotes, however, is unknown. It has been speculated that, like in plants, SPS may play a role in adaptation to osmotic stress. The presence of SPS in prokaryotes suggests that sucrose synthesis is an ancient trait (Lunn et al., 1999; Cumino et al., 2002).

An open reading frame has been identified as SPS in the course of a random sequence analysis of the *Halothermothrix orenii* genome (Mits and Patel, 2001). *H. orenii* is an anaerobic, thermohalophilic bacterium from the class Clostridia, with optimum growth at 60°C and 10% NaCl (Cayol et al., 1994). Recombinant SPS from *H. orenii* exhibits cross-reactivity with polyclonal antibodies raised against plant SPSs, suggesting antigen conservation among the SPSs of bacteria and plants (Huynh et al., 2005). The identification of a putative SPS gene in a nonphotosynthetic prokaryote provided an opportunity to address questions about the molecular and physiological role of SPS enzymes. Sequence database searches also revealed a large family of sequences similar to *H. orenii* SPS. The top six homologs of *H. orenii* SPS exhibit sequence identities varying from 54% for *Petrotoga mobilis* SJ95 to 33% for *Synechocystis* sp PCC 6803. Similarly, the six closest plant SPS homologs show sequence identities of ~32% (Figure 2A). Further analysis by
CLANS (Frickey and Lupas, 2004) to cluster all sequence homologs into families according to the BLAST sequence similarity P value is shown in Supplemental Figure 1 and Supplemental Data Set 1 online. SPS sequences form a well-defined group, whose nearest neighbors are sucrose synthases (SS; mostly from plants) and bacterial glycogen synthases. Other homologs, including starch synthases from plants (or glycogen synthases from bacteria), are more remotely related. These phylogenetic relationships suggest that the SPS (as well as SS) originated in bacteria and were transferred to plants via the chloroplast endosymbiont. The phylogenetic tree of the SPS family (Figure 2B) shows several well-resolved branches, among which only green plants are monophyletic, while others comprise genes from cyanobacteria and diverse species, suggesting multiple horizontal gene transfers. Thus, SPS from *H. orenii* also appears to have been derived by horizontal gene transfer from cyanobacteria and is a member of a sister group of plant SPS enzymes.

Approximately 180 amino acids at the N-terminal region of plant SPSs are missing in bacterial SPSs (Figure 3). This region contains a phosphorylation site (Ser-162 in maize [*Zea mays*] and Ser-158 in spinach [*Spinacia oleracea*]) that is responsible for light-dark regulation and is essential for the activation of the enzymatic activity in plant SPSs (Huber et al., 1989; Curatti et al., 1998; Lunn, 2002; Lunn and MacRae, 2003; Castleden et al., 2004). According to our bioinformatics analysis performed via the GeneSilico metaserver (Kurowski and Bujnicki, 2003), the N-terminal region of plant SPSs is intrinsically disordered. Nonetheless, it exhibits a potential to form several α-helices, which can fold to form a stable three-dimensional structure under certain specific conditions (e.g., in the presence of a ligand).

Many bacterial and plant homologs also possess an additional C-terminal domain, which is missing from the *H. orenii* SPS (Figure 3). This additional domain possesses sequence similarity to the catalytic domain of SPPs, which catalyzes the final step of sucrose biosynthesis by dephosphorylating S6P to sucrose (Lunn, 2002; Lunn and MacRae, 2003). In plant SPS, the SPP-related C-terminal domain is joined by a linker to the nucleotide diphosphate glucose (NDP-Glc) binding domain. A shorter version of this linker is also present in *H. orenii* and *Synechocystis* sp SPS (Figure 3). It has been proposed that in most cyanobacterial SPS this SPP domain is an inactivated pseudo-enzyme because it lacks conserved Asp residues potentially critical for catalysis (e.g., replaced by Ala-4 and Gln-6 in the enzyme from *Synechocystis* sp 6803), which is further supported by the lack of experimentally detectable SPP activity (Lunn, 2002; Lunn and MacRae, 2003; Fieulaine et al., 2005). However, in some proteobacteria, including *Acidithiobacillus ferroxidans* and *Nitrosomonas europaea*, the SPP-like domain of predicted SPS enzymes contains all of the conserved residues, suggesting that these enzymes are bifunctional with both SPS and SPP activities (Cumino et al., 2002; Lunn, 2002; Lunn and MacRae, 2003). The absence of the active SPP domain from SPS is usually correlated with the presence of a separate SPP-encoding gene (Lunn, 2002). Thus, we predict that if *H. orenii* is able to synthesize sucrose (this capability has yet to be confirmed experimentally), then it must use a separate yet to be discovered SPP enzyme.

Under the classification of glycosyltransferases (GTs) based on sequence similarities and stereochemistries of their substrates and products, SPSs are categorized under the Carbohydrate Active Enzymes (CAZY) database (Coutinho and Henrissat, 1999; http://www.cazy.org/) Family 4, known as retaining GTs (Ullman and Perkins, 1997; MacGregor, 2002). GTs are enzymes involved in the biosynthesis of carbohydrates and glycoconjugates. In general, GT structures are categorized into three folds, dubbed GT-A, GT-B, and GT-C (Gibson et al., 2002; Lunn and MacRae, 2003; Breton et al., 2006; Horcajada et al., 2006). The GT-A fold consists of two dissimilar domains with a nucleotide binding domain that resembles a Rossmann fold and another smaller...
Figure 2. Structural and Sequence Alignment of *H. orenii* SPS with Its Homologs.
acceptor domain (Breton et al., 2006). The GT-B, also known as the glycogen phosphorylase glycosyltransferase superfamily (Wrabl and Grishin, 2001), consists of two distinct Rossmann-fold domains: a sugar acceptor and a sugar donor domain. The GT-C fold is found in integral membrane GTs (Liu and Mushegian, 2003; Breton et al., 2006). SPS is generally categorized under the retaining GT-B family. Although some SPSs have been reported to be metal dependent (Porchia and Salerno, 1996), retaining GT-B members are believed to exhibit a metal ion–independent mechanism (Gibson et al., 2002; Liu and Mushegian, 2003; Buschiazzo et al., 2004; Breton et al., 2006). No metal ions were identified in these structures so far. None of our results support binding of Mg$^{2+}$ under the conditions tested. In addition, it is reported that plant SPSs are specific for UDP-Glc, whereas bacterial SPSs (Synechocystis and Anabaena) are not (Curatti et al., 1998; Lunn et al., 1999; Gibson et al., 2002). The recombinant H. orenii SPS, like the Synechocystis SPS, is able to accept other NDP-Glc, such as ADP-Glc and GDP-Glc (Huynh et al., 2005).

Here, we report the crystal structure of SPS from H. orenii in the apo form as well as complexes with the substrate F6P and the product S6P refined at 1.8-, 2.8-, and 2.4-Å resolutions, respectively. The report on H. orenii SPS provides insight into the structure and function of SPS from cyanobacteria and plants with which it shares a close similarity. Based on comparative analysis of previously published structures of other GT-B enzymes, we propose a mechanism for the transfer of the glycosyl group by SPS from NDP-Glc to F6P, leading to the formation of S6P.

RESULTS

Overall Structure of SPS from H. orenii

The structure of recombinant SPS from H. orenii was solved by the multiwavelength anomalous dispersion method from
was refined at 2.8 Å resolution, to an \( R \)-factor of 0.210 (\( R_{\text{free}} \)).

The SPS molecule consists of two domains (the A-domain: Ile7-Gly229 and Tyr443-Arg462, and the B-domain: Val230-Arg442) that form a deep substrate binding cleft at the interface with a dimension of \( \sim 20 \) Å wide and 30 Å deep. Each domain topology is similar to a Rossmann fold (Figure 4). The A-domain (mostly the N-terminal residues) has a central core \( \beta \)-sheet consisting of eight mostly parallel \( \beta \) strands (\( \beta1 \parallel \beta2 \parallel \beta3 \parallel \beta4 \parallel \beta5 \parallel \beta6 \parallel \beta7 \parallel \beta8 \)) flanked on three sides by seven helices, of which three are one to two turn small helices. The B-domain (mostly comprised of C-terminal residues) has a central parallel \( \beta \)-sheet of six strands (\( \beta9 \parallel \beta10 \parallel \beta11 \parallel \beta12 \parallel \beta13 \parallel \beta14 \)) flanked by nine \( \alpha \)-helices (three of which are one to two turn helices). The A- and the B-domains are connected through the loops Pro228-Val230 and Arg442-Gln446. The latter loop is considered as a part of the kink crossing over the domains and connecting two \( \alpha \)-helices, a general feature for enzymes belonging to the GT-B fold superfamily (Gibson et al., 2002; Breton et al., 2006; Horcajada et al., 2006). The A- and B-domains superimpose with a root mean square deviation (RMSD) of 3.2 Å for 104 C\( \alpha \) atoms and exhibit 11.5% sequence identity.

**Table 1. Data Collection and Refinement Statistics**

| Data Set                  | Peak | Infection | Remote | F6P Complex | S6P Complex | High Resolution |
|---------------------------|------|-----------|--------|-------------|-------------|-----------------|
| Data collection           |      |           |        |             |             |                 |
| Resolution range (Å)      | 50.0–2.0 (2.1–2.0) | 50.0–2.0 (2.1–2.0) | 50.0–2.0 (2.1–2.0) | 50.0–2.7 (2.8–2.7) | 50.0–2.3 (2.4–2.3) | 50.0–1.8 (1.9–1.8) |
| Wavelength (Å)            | 0.978 | 0.974     | 0.960  | 1.5418      | 1.5418      | 0.9788          |
| Observed reflections > 1  | 211355 | 220102 | 243363 | 55492 | 92403 | 266501 |
| Unique reflections        | 37156 | 34739 | 37435 | 13734 | 25868 | 48898 |
| Completeness (%)          | 99.3 | 99.8 | 99.9 | 99.0 | 99.8 | 95.7 |
| Overall (I/σ(I))          | 17.9 (1.8) | 15.0 (3.4) | 14.7 (2.8) | 13.4 (3.1) | 15.5 (2.0) | 12.1 (1.8) |
| \( R_{\text{free}} \) (%) | 5.6 (18.8) | 6.2 (27.0) | 5.9 (32.2) | 7.9 (32.2) | 5.0 (33.5) | 6.0 (29.0) |
| Refinement and quality \( ^b \) |       |           |        |             |             |                 |
| Resolution range (Å)      | 20.0–2.8 | 20.0–2.4 | 20.0–1.8 |           |             |                 |
| \( R_{\text{work}} \) (no. of reflections) \( ^c \) | 0.210 (11318) | 0.210 (17949) | 0.226 (36349) |           |             |                 |
| \( R_{\text{free}} \) (no. of reflections) \( ^d \) | 0.267 (1282) | 0.268 (1998) | 0.252 (4056) |           |             |                 |
| RMSD bond lengths (Å)     | 0.005 | 0.009 | 0.010 |           |             |                 |
| RMSD bond angles (°)      | 1.0 | 1.3 | 1.4 |           |             |                 |
| Average B-factors (Å\(^d\)) | 42.1 | 40.4 | 32.6 |           |             |                 |
| Main chain                | 44.3 | 46.5 | 35.5 |           |             |                 |
| Side chain                |       |           |        |             |             |                 |
| Ramachandran plot         |       |           |        |             |             |                 |
| Most favored regions (%)  | 86.7 | 87.2 | 89.7 |           |             |                 |
| Additional allowed regions (%) | 12.3 | 11.8 | 9.2 |           |             |                 |
| Generously allowed regions (%) | 0.5 | 0.5 | 0.5 |           |             |                 |
| Disallowed regions (%)    | 0.5 | 0.5 | 0.5 |           |             |                 |

\( \text{Peak} = |I_{\text{calc}}| - |I_{\text{obs}}|/|I_{\text{calc}}| \) where \( I_{\text{calc}} \) is the intensity of the \( i \)th measurement, and \( |I_{\text{obs}}| \) is the mean intensity for that reflection.

\( ^c \) For all models, reflections with \( I < 2 \sigma(I) \) were used in the refinement.

\( ^d \) For all models, reflections with \( I < 2 \sigma(I) \) were used in the refinement.

\( ^e \) Individual B-factor refinement was performed.

\( ^f \) Residues in the disallowed regions are well defined in the electron density map.

**Structural Comparisons with Other Proteins**

We compared the structure of *H. orenii* SPS with other protein structures in the PDB database using the program DALI (Holm and Sander, 1993). Significant structural similarities were found with *Agrobacterium tumefaciens* glycogen synthase, *Escherichia coli* OtsA, and *Oryctolagus cuniculus* glycogen phosphorylase, all of which belong to the GT-B family and possess catalytic mechanisms of retaining GTs. The closest structural similarity is...
observed between SPS and A. tumefaciens glycogen synthase complexed with ADP (PDB code 1RZU) from CAZy Family 5, yielding an RMSD of 4.4 Å for 365 Cα atoms, with ~11% sequence identity. This is followed by E. coli OtsA complexed with G6P-UDP (OtsA; PDB code 1GZ5) from CAZy Family 20 (RMSD = 4.4 Å for 269 Cα atoms; ~13% sequence identity) and European rabbit (O. cuniculus) glycogen phosphorylase complexed with glycopyranose spirohydantoin (PDB code 1A8I) from CAZy Family 35 (RMSD = 4.2 Å for 311 Cα atoms; ~7% sequence identity). However, the superimposition of individual domains of SPS and its homologs exhibit a good fit. The A-domain of the SPS superimposes on the corresponding domains of glycogen synthase, OtsA, and glycogen phosphorylase with an RMSD of 2.6 Å for 197 Cα atoms, 3.2 Å for 191 Cα atoms, and 2.9 Å for 184 Cα atoms, respectively. Similarly, the B-domain of SPS superimposes on the corresponding domain of these same homologs with an RMSD of 2.8 Å for 178 Cα atoms, 3.2 Å for 188 Cα atoms, and 3.1 Å for 187 Cα atoms, respectively. Thus, the comparison of the full-length SPS with its structural homologs shows variations in the relative disposition of A- and B-domains of these enzymes (Figure 5A). This type of flexibility in two-domain enzymes is not unusual and has been reported for several two-domain enzymes (MacGregor, 2002; Buschiazzo et al., 2004; Breton et al., 2006; Horcajada et al., 2006). Furthermore, these structural comparisons suggest a possibility of different conformations of GT-B structures. Structures of SPS (or its two complexes) and the glycogen synthase-ADP complex may represent an open conformation (Buschiazzo et al., 2004), whereas the trehalose 6-phosphate synthase-G6P-UDP complex may represent a closed conformation (Gibson et al., 2002; Buschiazzo et al., 2004). GT-B undergoes a small twist between the two domains during transformation from an open to the closed conformation (Buschiazzo et al., 2004), along with the closing of the substrate binding cleft. For instance, in the open GT-B conformation (e.g., H. orenii SPS), the entrance of the substrate binding cleft is >20 Å, whereas in the closed GT-Bs (OtsA) it is ~6 Å. To illustrate this, a closed model of SPS was generated by independently superimposing the A- and B-domains of SPS on the respective domains of the closed OtsA-UDP-G6P complex structure. A figure was prepared by superimposing the B-domain of this closed SPS-UDP model on that of the open SPS-F6P complex (Figure 5B). By comparing the open SPS-F6P complex with the closed SPS-UDP model, the conformational change upon domain movement is shown.

A comparison of the three-dimensional structures of SPS, OtsA, and glycogen synthases (Figure 5A) indicates that the catalytic domains of these enzymes are evolutionarily related. This is further supported by the presence of several invariant residues at the substrate binding sites (Figure 2A). This structural similarity exists despite their low sequence identities, suggesting that the structure is often more conserved than the primary sequence. However, sequence identities among all SPSs (including plant SPSs) are much higher than sequence identities of these three structural homologs (i.e., SPS, OtsA, and glycogen synthases) (Figure 2A). These observed similarities of sequences and overall structures suggest a common structural and mechanistic framework for all SPS enzymes. Therefore, the structure of H. orenii SPS is a valid model for the catalytic domain of plant SPSs, providing valuable insight into the reaction mechanism of the plant enzyme.

The SPS-F6P Complex

Prior to the crystallization of the SPS-F6P (enzyme-substrate) complex, the formation of the complex was verified by isothermal titration calorimetry (ITC) experiments. The molar ratio between SPS and F6P was determined to be 0.966 (~1), suggesting a 1:1 complex (see Supplemental Figures 2A and 2B online). To obtain this complex, we soaked the apo-SPS crystals in a solution containing the substrate F6P and collected a complete x-ray diffraction data set. The difference electron density map clearly showed a substrate molecule bound to one of the two domains of SPS (Figure 6A). F6P binds in a deep depression in the A-domain, in the interdomain interface cleft (Figures 7A and 7B). The substrate is located between two helices of the A-domain, such that α4 is close to the phosphate group and α1 is close to the sugar side of F6P. Side chains lining the substrate binding pockets are from Gln-16, Gly-33, Gln-35, Lys-96, Tyr-128, Ser-152, Lys-157, and Arg-180 (Figure 6A; see Supplemental Figure 3 online). These residues are conserved among the bacterial and plant SPSs (Figure 2A). In addition, the structure and sequence

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**Figure 4. Ribbon Diagram Showing the Structure of SPS.**

A-domain (residues 7 to 229; 443 to 462) is depicted in blue and the B-domain (residues 230 to 442) in red. The bound substrate molecule D-fructose-6-phosphate (F6P) is depicted as a ball-and-stick representation. The N and C termini are labeled. This figure was prepared using the programs MOLSCRIPT (Kraulis, 1991) and Raster3D (Merritt and Bacon, 1997).
analyses reveal that the binding residues of SPS to the fructose moiety of F6P (Gly-33, Gln-35, Lys-96, and Tyr-128) and to the diphosphate group of UDP-Glc (Arg-270, Lys-275, Glu-369, and Phe-367) are conserved in plant and bacteria SS. A total of nine hydrogen bonds and several hydrophobic interactions were formed between F6P and the SPS molecule. Of these, four strong hydrogen bonding contacts (<3 Å) were found between the phosphate group of F6P and highly conserved residues of SPS, namely, Tyr-128, Ser-152, Lys-157, and Arg-180. In the substrate binding cleft adjacent to the F6P binding pocket there are several well-ordered water molecules, which can be replaced by the incoming second substrate NDP-Glc.

The binding of F6P does not cause any major conformational changes in the SPS structure. Furthermore, the superimposition of SPS apo- and F6P complex structures (RMSD of 0.266 Å for 455 Cα atoms) reveals no domain movement. Only the key side chains, Gln-35, Lys-157, and Arg-180, show a small movement toward F6P. It must be emphasized that the substrate was soaked into the crystal; therefore, no major structural rearrangement of SPS was anticipated.

The SPS-S6P Complex

Similar to the SPS-F6P complex, apo-SPS crystals were soaked in a solution containing S6P (product), and a complete x-ray diffraction data set was collected. The electron density map clearly showed the presence of one S6P molecule bound at the A-domain in the domain interface cleft (Figure 6B). The location of the product molecule, S6P, is in the same region as F6P of the F6P-SPS complex, between the two helices of the A-domain, such that α4 is close to the phosphate group and α1 is close to the sugar side of S6P. Similar to F6P, the S6P binds in a deep depression in the A-domain, at the domain interface cleft (Figure 7C). The overall hydrogen bonding contacts of F6P and S6P complexes are the same except for His-151 (see Supplemental

Figure 5. Open and Closed Forms of Retaining GT-B Enzymes.

(A) Ribbon diagrams showing three complex structures side by side: I, H. arenii SPS-F6P complex (open conformation); II, A. tumefaciens glycogen synthase-ADP complex (open conformation); III, E. coli OtsA-G6P-UDP complex (closed conformation). The ball-and-stick representation shows the bound F6P, ADP, and G6P-UDP, respectively.

(B) Superimposed, stereodiagram of the open SPS-F6P complex (yellow) and the closed SPS-UDP model (blue). The ball-and-stick representation shows the bound F6P and UDP at the substrate binding cleft observed in the open and closed SPS. The superimposition was performed with DALI (Holm and Sander, 1993) and O programs (Jones et al., 1991). These figures were prepared using the program PyMOL (DeLano, 2002).
In the SPS-F6P complex, His-151 has no interaction with the F6P molecule due to the absence of the glycosyl group. Here, in the SPS-S6P complex, His-151 forms a strong hydrogen bond (<3 Å) with the O atom of the transferred glycosyl group (Figure 6B). A total of 13 hydrogen bonding contacts and several hydrophobic interactions are formed between S6P and SPS molecules. Noteworthy, similar to the SPS-F6P complex, four strong hydrogen bonding contacts (<3 Å) are found between the phosphate group of S6P and the highly conserved residues (Tyr-128, Ser-152, Lys-157, and Arg-180). The binding of S6P does not cause any major conformational changes in the SPS structure. The superimposition of apo-SPS and S6P complex structures (RMSD of 0.266 Å for 455 Cα atoms) reveals no significant differences. Key side chains, such as Gln-35, Lys-157, and Arg-180, interacting with S6P show a small movement (<1 Å) toward S6P.

**Putative ADP / UDP Binding Pocket**

It was reported that plant SPSs are specific for UDP-Glc, whereas bacterial SPSs (*Synechocystis* and *Anabaena*) are not (Curatti et al., 1998; Lunn et al., 1999; Gibson et al., 2002). Recombinant *H. orenii* SPS, like the *Synechocystis* SPS, is able to accept other NDP-Glc, such as ADP-Glc and GDP-Glc (Huynh et al., 2005). Although we did not obtain the position of a second NDP-Glc substrate in the SPS structure through crystallization, the binding site of ADP-Glc and UDP-Glc can be predicted by a comparison with the structure of the glycogen synthase-ADP complex (Buschiazzo et al., 2004) and the OtsA-UDP complex (Gibson et al., 2002), respectively (Figure 5A). The overall architecture of the nucleotide binding site is very similar in SPS, glycogen synthase, and OtsA. In the open form of SPS, ADP/UDP is predicted to bind to a pocket on the B-domain of the interdomain cleft adjacent to the A-domain F6P binding pocket. This binding pocket is lined by Ser268-Arg270, Thr299-Ile303, Pro370-Ser381, and Pro341-Tyr352.

To provide independent support for the predicted NDP-Glc binding site in *H. orenii* SPS, we performed computational docking of NDP molecules with the FlexX algorithm (Kramer et al., 1999). The result gave 10 docked models of UDP to *H. orenii* SPS (Figure 8A). A similar result was obtained in the course of ADP docking to SPS with a similar orientation of the ligand (Figure 8B). Furthermore, to validate the proposed NDP-Glc binding site of SPS, we superimposed B-domains of SPS-UDP/ADP docked models on OtsA-UDP (see Supplemental Figure 4A).
It clearly shows the agreement between the NDP-Glc predicted binding pocket and key conserved residues of SPS, OtsA, and glycogen synthase. Arg-270, Lys-275, Glu-369, and Glu-377 are key residues and highly conserved among the plant SPS homologs (Figure 2A). The NDP-Glc binding pocket remains the same for both the open form (SPS, glycogen synthase) and closed form (OtsA) of these GTs. By analogy, we propose that the NDP-Glc will occupy the same binding pocket in the closed form of SPS. It should be noted that for the binding of NDP-Glc, the side chains of Arg-270 and Arg-301 may have a different orientation. Docked ADP and UDP at the SPS NDP-Glc binding site form several hydrogen bonds with the SPS molecule (Figures 8A and 8B). It is known that bacterial SPSs exhibit little specificity against NDP-Glc, whereas plants’ SPSs are more specific to UDP-Glc than other NDP-Glc (Jones et al., 1991; Curatti et al., 1998; Gibson et al., 2002). Most of the residues interacting with the diphosphate group and the ribose moiety of NDP-Glc are well conserved in both plants and bacteria, whereas residues interacting with the base moiety of the NDP-Glc are less conserved among bacteria than plant SPSs. The above considerations may indicate why plant SPS are specific for UDP-Glc, while bacterial SPSs do not discriminate among NDP-Glc (Figure 8A; see Supplemental Figure 4A online). It is worth mentioning here that, unlike plant SPS, both plant and bacterial SS show similarities to bacterial SPS, using NDP-Glc as the glycosyl donor (Porchia et al., 1999). Based on the docked models (Figures 8A and 8B) and sequence analysis, three nucleotide binding residues of H. orenii SPS (Thr-299, Leu-300, and Leu-342; Figure 2A) were identified. By contrast, the corresponding positions in plant SPS are substituted by conserved large side chain residues, Ile, Met, and His (Figure 2A). These variations also suggest a possible basis for the more diverse binding modes of bacterial SS, plant SS, and bacterial SPS and the stringent binding mode of plant SPS to UDP-Glc.

Cid et al. (2000) proposed the presence of an E-X7-E motif at the C terminus of GTs, where the flanking Glu residues play a catalytic role in the reaction (Cid et al., 2000). This motif is also found in H. orenii SPS, and residues Glu-377 and Glu-369 (Figure 2A) are known as the motif positions 1 and 2, respectively (Cid et al., 2000; Wrabl and Grishin, 2001; Gibson et al., 2002; Liu and Mushegian, 2003). In SPS-UDP docked models (Figure 8A), we observed that the carboxylate group of Glu-369 interacts with the distal phosphate group of a few UDP docked models, while that of Glu-377 interacts with the ribose moiety in all 10 docked models of UDP. Since all NDP-Glc share the same ribose and diphosphate backbone, we speculate that these conserved Glu residues almost certainly play an equivalent role in binding to other NDP-Glc donors.

**DISCUSSION**

The successful crystallization of the H. orenii enzyme provided an insight into the structure of SPS of other organisms. Based on our structural and bioinformatics analysis of the NDP-Glc binding pocket, in particular the detected similarity to retaining GTs of known structure, we propose a possible mechanism of SPS action.

The inverting GT-A, in the presence of a DXD motif, adopts a divalent metal ion–dependent catalytic mechanism, whereas the retaining GT-B, in the absence of such a motif, exhibits the metal ion–independent mechanism (Gibson et al., 2002; Liu and Mushegian, 2003; Buschiazzo et al., 2004; Breton et al., 2006). Although the mechanism of retaining GTs is not well understood,
Figure 9. Mechanism of Action.

(A) Superimposition of the catalytic regions of the open SPS-F6P complex (cyan) and the closed SPS-S6P-UDP model (magenta). SPS residues proposed to bind to the glycosyl group of S6P (or UDP-Glc) and its substrate binding residues from the A-domain are shown. An arrow illustrates the movement of the binding residues from the A-domain upon domain closure. Substrate binding residues from the B-domain shown on this figure are underlined. Carbon atoms of F6P and UDP are shown in green and gray, respectively. The carbon atoms of S6P are also shown in green with its glycosyl group shown in yellow. The rest of the atoms are blue (N), red (O), and orange (P). The superimposition was performed with DALI (Holm and Sander, 1993) and O programs (Jones et al., 1991). This figure was prepared using the program PyMOL (DeLano, 2002).

(B) Schematic diagram of the reaction between F6P and UDP-Glc in the binding cleft of SPS. The A- and B-domain binding residues and two substrates are labeled in blue, red, and black, respectively. The hypothetical hydrogen bond between O2 of the F6P and the C1 of UDP-Glc is shown as a dotted line. Both the orientation of O2 of F6P and the C1 of UDP-Glc are labeled accordingly. The red arrow indicates the transfer of the glycosyl group (shaded) from UDP-Glc (sugar donor) to F6P (sugar acceptor).
Gibson et al. (2002) proposed a putative transition state for the transfer of a glycosyl group by OtsA, which is metal ion independent (Gibson et al., 2002). To verify that H. orenii SPS binds to a divalent metal ion in the presence and absence of substrates, we made several attempts using ITC and cocRYSTALLIZATION/soaking experiments to trap the Mg2+ ion. None of the results supported the binding of Mg2+ under the conditions tested, and in the crystal structure no electron density corresponding to a divalent metal ion was observed near the diphosphate groups of the docked NDPs.

Figure 9A shows the superimposed model of catalytic open SPS-F6P and closed SPS-S6P-UDP. The closed model of SPS was generated by independently superimposing the A- and B-domains of SPS on the respective domains of the closed OtsA-UDP-G6P complex structure. Gly-33, Gly-34, and Gln-35 of domain A of SPS are highly conserved among SPS homologs (Figure 2A). These three residues also correspond to the conserved Gly-Gly-Leu motif of OtsA (Gibson et al., 2002). In the open SPS-F6P and SPS-S6P complex crystal structures, F6P (or S6P) forms hydrogen bonds with the main chain amide of Gly-33 and Gln-35 (see Supplemental Figure 3 online). In the closed SPS model (Figure 9A), the main chain amide of Gly-34 is found to interact with the diphosphate group of UDP at the B-domain, while Gly-33 and Gln-35 maintain the interactions with F6P (or S6P). In the case of closed OtsA structure, UDP at the B-domain interacts with the main-chain amides of the two corresponding Gly residues of the Gly-Gly-Leu motif at the A-domain (Gibson et al., 2002). Although the role of the SPS Gly-33 is different from its corresponding Gly in OtsA, both the second Gly of the Gly-Gly-Leu motif in SPS and OtsA binds to the diphosphate group of UDP at their respective B-domain. Hence, based on the closed model of SPS and the OtsA complex structure, Gly-34 of SPS may play a crucial role in providing a linkage between NDP-Glc and the A-domain and may also be involved in domain closure upon substrate binding.

The position of the glycosyl group of S6P in the closed SPS model is believed to be the catalytic reaction center of SPS. The closed SPS model revealed several interactions between the glycosyl group of S6P and SPS residues (Figure 9A): Conserved residues Glu-369, Phe-371, Gly-372, Leu-373 (B-domain), and His-151 (A-domain) of the closed SPS model were found to interact with the glycosyl group of S6P. Likewise, the corresponding residues of OtsA also interacted with the glycosyl group of UDP-Glc in the closed OtsA-UDP-Glc complex structure (PDB code 1UQU; Gibson et al., 2004). UDP-Glc in this complex structure was constrained to adopt a folded shape by the interactions from the binding residues from OtsA (Gibson et al., 2004; Breton et al., 2006). Interestingly, the same conformation of a glycosyl group was also observed in UDP-Glc-OtsA complex structure.

In the structure of the SPS-F6P complex, atom O2 of F6P was found to have a strong hydrogen bond (<3.0 Å) with one water molecule. By comparison with the structure of OtsA, this water molecule may get replaced by the phosphate of the incoming donor molecule (Breton et al., 2006). In addition, it is possible that as the two domains close upon binding of a second substrate NDP-Glc, a hydrogen bond is established between the O2 atom of F6P and the diphosphate group of NDP-Glc, which is observed in the closed SPS model (Figure 9A). This hydrogen bond lowers the energy barrier, facilitates the formation of a late oxonium ion–like transition state, as a result of a nucleophilic attack by the deprotonated atom O2 of F6P at the weakened, anomeric C1 of NDP-Glc, leading to the cleavage of NDP-Glc (Gibson et al., 2002; Breton et al., 2006).

In the SPS-F6P and the SPS-S6P complexes, highly conserved His-151 from the A-domain of SPS is found to be the only residue that binds to the glycosyl group of the S6P product and has no interaction with F6P (see Supplemental Figure 3 online). Previously, Gibson et al. (2002) proposed a possible interaction between the corresponding His-154 and the glycosyl group of UDP-Glc (substrate) in the OtsA-G6P-UDP complex, which was later confirmed in the OtsA-UDP-Glc complex (Gibson et al., 2004). According to the closed SPS model, while the conserved Gly-34 from the A-domain interacts with the diphosphate group of UDP at the B-domain, His-151 remains the only residue from the A-domain to interact with the glycosyl moiety of S6P (or UDP-Glc). Similar to Gly-34, His-151 may provide a linkage between NDP-Glc and the A-domain of SPS and possibly be involved in domain closure upon substrate binding. Most importantly, we propose an active role for the conserved His-151 during the transfer of the glycosyl group from NDP-Glc to the B-domain to F6P on the A-domain, resulting in the formation of S6P and followed by its release from this domain.

METHODS

Cloning, Expression, and Purification

Primers containing BamHI and KpnI restriction sites at the 5’ and 3’ ends, respectively, were used in PCR to amplify the spsA gene (Mijts and Patel, 2001). The PCR product was digested by these restriction enzymes, followed by its ligation with the pTrcHisA expression vector (Invitrogen) encoding an N-terminal, noncleavable His6 tag (Mijts and Patel, 2001). The plasmid was transformed into BL21 (DE3) and grown in 1 liter of Luria-Bertani broth with 0.1 mM ampicillin at 37°C until it reached an optical density (OD600) of ~0.6 to 0.7. The culture was cooled to 25°C and induced with 1 mM isopropyl-β-D-galactoside overnight. The Halothermorthrix orenii SPS has 499 amino acid residues with a molecular mass of 56.815 kDa. The recombinant H. orenii SPS, consisting of a hexahistidine tag and a linker, is expressed as a 61.1-kD protein. The cells were harvested by centrifugation (9000g, 30 min. 4°C) and resuspended in 30 mL of 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 10 mM imidazole and one tablet of EDTA-free Complete Protease Inhibitor Cocktail (Roche Diagnostics). Selenomethionine-substituted SPS was expressed using Met auxotroph Escherichia coli DL41 in LeMaster medium supplemented with 25 mg/L selenomethionine (SeMet). The cells were lysed by sonication, followed by centrifugation at 11,000 rpm (Eppendorf 5804R) for 30 min. Cell lysate was centrifuged at 11,000 rpm (Eppendorf 5804R) for 30 min. Cell lysate was centrifuged at 11,000 rpm (Eppendorf 5804R) for 30 min. Cell lysate was centrifuged at 11,000 rpm (Eppendorf 5804R) for 30 min. Cell lysate was centrifuged at 11,000 rpm (Eppendorf 5804R) for 30 min. Cell lysate was centrifuged at 11,000 rpm (Eppendorf 5804R) for 30 min.
Matrix-Assisted Laser-Desorption Ionization Time of Flight Analysis

The native and SeMet-substituted SPS was further analyzed for the incorporation of selenium on a Voyager STR matrix-assisted laser-desorption ionization time of flight mass spectrometer (Applied Biosystems) by comparing the experimentally measured molecular mass of the native SPS with that of the SeMet protein, and this confirmed the proper incorporation of selenium.

Dynamic Light Scattering

Dynamic light scattering measurements were performed at room temperature by a DynaPro DLS instrument (Protein Solutions). The homogeneity of native SPS and SeMet-SPS was monitored during the various stages of concentration steps to avoid aggregation prior to crystallization. The percentage of polydispersity was below 14.1% for all protein samples at various concentrations.

ITC

ITC experiments were performed by a VP-ITC calorimeter (Microcal) using 0.01 to 0.02 mM of the SPS in the sample cell and 0.1 to 0.2 mM of F6P in the injector. Sixty injections of volume 5 μL each were used. The heat of dilution for each ligand was measured in a separate titration experiment by titrating ligand into buffer. Consecutive injections were separated by at least 4 min to allow the peak to return to the baseline. The ITC data were analyzed using a single site fitting model using Origin 7.0 software (OriginLab).

Crystallization

Initial crystallization conditions were screened at 25°C. Further optimization with extensive additive screens (Hampton Research crystallization screens and by the micro batch under-oil technique using JB crystallization screens (Jena Biosciences). Initially, apo- and SeMet SPS crystals were plate like and were obtained after 2 d directly from JB3 screen C2. Further optimization with extensive additive screens (Hampton Research) for best diffraction quality crystals was obtained by the hanging-drop vapor diffusion method using a 1-mL reservoir solution containing 20% polyethylene glycol 4000, 0.6 M NaCl, and 0.1 M Na MES, pH 6.5, with a drop size 1 μL of the reservoir solution with 1 μL of protein. Crystals had approximate dimensions of 0.45 × 0.3 × 0.2 mm. They diffracted up to 1.8 Å and belonged to space group C2 with a = 154.23, b = 48.50, c = 75.05 Å, and β = 100.92°.

Data Collection, Structure Solution, and Refinement

Crystals were directly taken from the drop and flash cooled in a N2 cold stream at 100K. The apo-SPS crystals were diffcracted up to 2.4 Å resolution using an R-axis IV++ image plate detector mounted on a RU-H3RHB rotating anode generator (Rigaku), Synchrotron data for the SeMet-substituted SPS protein was collected at beamlines X29 (National Synchrotron Light Source, Brookhaven National Laboratory). Complete mult-wavelength anomalous dispersion data sets were collected at three wavelengths (Table 1) using a Quantum 4-CCD detector (Area Detector Systems) to 2.0 Å resolution. Data were processed and scaled using the program HKL2000 (Otwinowski and Minor, 1997).

Structure Solution and Refinement

Out of the seven expected selenium sites in the asymmetric unit, five were located by the program SOLVE (Terwilliger and Berendzen, 1999). The N-terminal, as well as the C-terminal Met, was disordered. Initial phases were further developed by RESOLVE (Terwilliger, 2000), and the overall figure of merit was improved to 0.73, which made it possible to build automatically ~70% of the molecule. The remaining parts of the model were built manually using the program O (Jones et al., 1991). Alternating cycles of model building and refinement using the program CNS (Bruguier et al., 1998) resulted in the final model refined to 1.8 Å resolution with an R-factor of 0.226 (Rfree = 0.252). Reflections I>2I was used in the refinement. The final model consists of 455 residues (Ile7-Arg462) and 287 water molecules. The N-terminal His tag with the linker residues and the C-terminal 32 amino acids were not visible in the electron density map. PROCHECK (Laskowski et al., 1993) analysis shows two residues in the disallowed regions of the Ramachandran plot.

F6P-SPS and S6P-SPS Complexes

F6P-SPS and S6P-SPS complexes were obtained by soaking crystals of apo-SPS in 20 mM F6P and 20 mM S6P, respectively, for 12 h at 25°C. Complete data sets of both complexes were collected on an R-axis IV++ area detector with an RU300 rotating anode generator as the x-ray source and diffracted to 2.8 and 2.4 Å, respectively. Crystals were cryoprotected as described above. The apo-SPS model used to calculate the difference electron density maps revealed the presence of ligands. Two models were refined with CNS (Bruguier et al., 1998) and combined with manual refitting with the program O, and appropriate entries were made in their respective dictionaries. Both F6P-SPS and S6P-SPS complex models consist of residues from Ile7 to Arg462 with 312 and 294 water molecules, respectively. The simulated annealing Fo-Fc omit map of the ligands is shown in Figures 6A and 6B. Crystallographic statistics are presented in Table 1.

Bioinformatics Analyses

Sequence database searches were performed with PSI-BLAST run iteratively until convergence with e-value threshold of 1e-3 (Altschul et al., 1997). Sequences of SPS homologs were clustered using CLANS using the P value threshold of 1e-10 (Frickey and Lupas, 2004). Genuine members of the SPS family were identified visually as a very tight cluster in the CLANS output. The SPS family sequences were aligned using ClustalX (run with default parameters; e.g., amino acid substitution matrix Gonnet250, slow pairwise alignments, gap opening penalty 10, gap extension penalty 0.1 for pairwise, and 0.2 for multiple alignment), and the resulting alignment was optimized manually. The evolutionary tree was inferred from the alignment using MEGA 3.1 (Kumar et al., 2004). The minimum evolution method was used with the following options: JTT matrix, pairwise deletion of gaps, initial tree calculated with the neighbor joining algorithm, followed by branch swapping with the close-neighbor-interchange algorithm (level = 2). The robustness of nodes in the tree has been assessed with 1000 rounds of the interior branch test and expressed as percentile values. Protein structure prediction for sequence segments present in SPS homologs from other species, but missing from the H. orenii SPS, was performed via the GeneSilico metaserver (Kurowski and Bujnicki, 2003); results were analyzed visually. Docking of flexible ADP and UDP structures to SPS (with the binding pocket defined as composed of residues 268, 269, 275, 299, 342, 345, 348, 367, and 377) was performed using FlexX (Kramer et al., 1999) with the following parameters: standard energy mode, no implicit particle placement, flexible pose optimization, flexible ring fitting, standard torsion angle model, energy threshold: 0 kJ/mol, relative energy threshold: 50 kJ/mol. Twelve hundred docking solutions were generated and ordered according to the FlexX weighted physical affinity score. Ten top-scoring poses were considered for detailed analysis.

Phylogenetic Analysis

Sequence database searches were performed with PSI-BLAST (Altschul et al., 1997). Sequences of SPS homologs were clustered using CLANS (Frickey and Lupas, 2004), and genuine members of the SPS family were
aligned using ClustalX. Phylogenetic analyses were done with MEGA 3.1 (Kumar et al., 2004) using the minimum evolution method, JTT matrix, and pairwise deletion of gaps. Protein structure prediction for sequence segments present in SPS homologs from other species but missing from the _H. orenii_ SPS was performed via the GeneSilico metaserver (Kurowski and Bujnicki, 2003). Docking of flexible ADP and UDP structures to SPS was performed using FlexX (Kramer et al., 1999) with default parameters. Ten top-scoring poses were considered.

**Accession Numbers**

Coordinates and structure factors for the apo-SPS (EC 2.4.1.14), F6P, and S6P complexes have been deposited with Research Collaboratory for Structural Bioinformatics PDB with codes 2R60, 2R66, and 2R68, respectively.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Sequence Similarity between SPS and its Homologs, as Visualized with CLANS.

**Supplemental Figure 2.** ITC Profile of _H. orenii_ SPS and Substrate F6P.

**Supplemental Figure 3.** Superimposition of F6P-SPS and S6P-SPS Complexes.

**Supplemental Figure 4.** Superimposition of One Docked-NDP Ligand and the Actual NDP Ligand.

**Supplemental Figure 5.** Superimposition Diagram of the SPS-Closed Model and Closed OtsA Structure Represented by _Ca_ Chains.

**Supplemental Figure 6.** SDS-PAGE Gel Image of _H. orenii_ SPS Purification.

**Supplemental Data Set 1.** Amino Acid Sequence Alignment of SPS Family in FASTA Format.

**ACKNOWLEDGMENTS**

We thank Anand Saxena (Brookhaven National Laboratory) for assistance in data collection and Tomasz Jarzynka (International Institute of Molecular and Cell Biology) for assistance in docking analyses. Data for this study were measured at beamline X12C of the National Synchrotron Light Source, Brookhaven National Laboratory. J.S. acknowledges full research support from the Academic Research Fund (Grant R154000245112), National University of Singapore. We thank Shashikant Joshi for the useful discussion and for extending the Proteins and Proteomics Center facility. J.M.B. was supported by the European Union 6th Framework Program (Grant LSHG-CT-2003-503238) and the accompanying grant from the Polish Ministry of Science. B.K.P. and F.H. acknowledge the support from the Griffith University Research Grants scheme. C.T.K. is a graduate scholar in receipt of a research scholarship from the National University of Singapore.

Received February 25, 2007; revised February 5, 2008; accepted March 27, 2008; published April 18, 2008.

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*Plant Cell* 2008;20;1059-1072; originally published online April 18, 2008;
DOI 10.1105/tpc.107.051193

This information is current as of April 26, 2019