Structural Rearrangements of Sucrose Phosphorylase from Bifidobacterium adolescentis during Sucrose Conversion*

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The reaction mechanism of sucrose phosphorylase from Bifidobacterium adolescentis (BiSP) was studied by site-directed mutagenesis and x-ray crystallography. An inactive mutant of BiSP (E232Q) was co-crystallized with sucrose. The structure revealed a substrate-binding mode comparable with that seen in other related sucrose-acting enzymes. Wild-type BiSP was also crystallized in the presence of sucrose. In the dimeric structure, a covalent glucosyl intermediate was formed in one molecule of the BiSP dimer, and after hydrolysis of the glucosyl intermediate, a $\beta$-d-glucose product complex was formed in the other molecule. Although the overall structure of the BiSP-glucosyl intermediate complex is similar to that of the BiSP(E232Q)-sucrose complex, the glucose complex discloses major differences in loop conformations. Two loops (residues 336–344 and 132–137) in the proximity of the active site move up to 16 and 4 Å, respectively. On the basis of these findings, we have suggested a reaction cycle that takes into account the large movements in the active-site entrance loops.

Sucrose phosphorylase (EC 2.4.1.7) is an enzyme that reversibly catalyzes the following reaction; sucrose + orthophosphate = $\alpha$-fructose + $\alpha$-d-glucose 1-phosphate (see Scheme 1). This reaction enables the production of the essential glucose moiety from sucrose. Sucrose phosphorylase from Bifidobacterium adolescentis (BiSP)3 has been recently sequenced, cloned, and characterized (1), and the three-dimensional structure has also been determined by crystallographic methods (2). BiSP consists of 504 amino acid residues, with a molecular mass of 56,189 g/mol.

Based on amino acid sequence similarities, BiSP has been placed in the retaining glycoside hydrolase family 13 (3), also called the $\alpha$-amylase family, in which, however, functions more complex than hydrolysis are not unprecedented. In this family, other members show transglycosidase activity, e.g. cyclomaltodextrin glucanotransferase and amyllosucrase. Structurally, the glycose hydrolase family 13 is characterized by having a $\beta(\alpha)_{8}$-barrel comprising the catalytic domain that is referred to as domain A. Apart from the catalytic domain, the enzymes of the family typically contain several other domains. These include the N-terminal domain, a domain formed by the usually long loop 2 in the $\beta(\alpha)_{8}$-barrel (loop B), and the C-terminal domain. The structure of BiSP is composed of the four domains A, B, B', and C (Fig. 1) (2). Domain B contains two short anti-parallel $\beta$-sheets and two short $\alpha$-helices, whereas domain B' is mainly a coil region, but contains one long and one short $\alpha$-helix. The first 56 residues of the C-terminal domain form a single five-stranded anti-parallel $\beta$-sheet with a topology described as 1,1,1,1 in algebraic notation, and this is unique among the glycoside hydrolase family 13 domains.

Gel filtration (1) and dynamic light scattering and crystal packing analysis (2) have suggested that BiSP is a dimer. The majority of the interactions are confined to the two B domains, but interactions between the loop 8 regions of the two A domain barrels are also observed. This results in a large cavity in the dimer, which includes the entrance to the two active sites. In the glycose hydrolase family 13, dimers are also seen for the enzymes cyclomaltodextrinase (4), neopullulanase (5), and maltogenic amylase (6). In contrast to the BiSP dimer, the other dimers are formed primarily by the hydrolase N-terminal domains. To our knowledge, the BiSP structure represents the first assignment of a functional role of a B domain for dimerization in the entire glycose hydrolase family 13.

The proposed reaction mechanism of the glycose hydrolase family 13 is a double displacement reaction (7) involving a covalent enzyme-glucosyl intermediate (Scheme 1). For sucrose phosphorylase from Pseudomonas saccharophila, the existence of the intermediate was indicated already in 1947 by Doudoroff et al. (8), and this was experimentally confirmed by Voet and Abeles (9). The reaction is initiated by simultaneous protonation of the ether linkage oxygen atom by the proton donor (identified as Glu$^{32}$ for BiSP) and nucleophilic attack by Asp$^{192}$ on the anomeric carbon of the glucosyl moiety. This leads to the covalently linked enzyme-substrate intermediate and the release of fructose. The intermediate can then react with phosphate (HPO$_4^{2-}$ or H$_2$PO$_4^{-}$), and glucose 1-phosphate is finally released. Reactions with other nucleophiles such as
In this study, we present the structures of wild-type BiSP reacted with sucrose and the BiSP(E232Q) mutant co-crystallized with sucrose. The structures represent the first intact complexes of a sucrose phosphorylase and provide significant new information on the catalytic mechanism. This study will lead to a number of biochemical investigations that will further enhance our understanding of the basis for substrate specificity of enzymes involved in starch and sucrose metabolism.

MATERIALS AND METHODS

Chemicals and Enzymes—Chemicals and primers were obtained from Sigma unless stated otherwise. Recombinant BiSP was isolated and purified as described (1).

Construction of BiSP Mutant—A QuikChange site-directed mutagenesis kit (Stratagene) was used to make point mutations in the gene (AF543301) according to the instructions of the manufacturer. The following primers were used for the E232Q modification (with the introduced mutations in italics and underlined): E232Qreverse, 5’-GTAGTAGGACTTACCTGATGAGGATTTCCAGACC-3’; and E232Qforward, 5’-GGTCTGGAAATCCTCATCCAAGTGCACTCCTAC-3’. The wild-type BiSP gene (1) was used as a template. After mutagenesis, plasmid DNA from two blue colonies was isolated using a Qiagen plasmid purification kit. To verify that the desired mutation was introduced, the DNA nucleotide sequences of the plasmids were determined using a DYEnamic™ ET terminator cycle sequencing kit (Amersham Biosciences) and an automated ABI PRISM 3100 analyzer (Applied Biosystems).

Isolation of Mutant Sucrose Phosphorylase—Cells from an Escherichia coli culture (1 liter, LB medium containing 50 mg/liter ampicillin, 37 °C) containing the mutant BiSP gene were grown overnight. The mutant sucrose phosphorylase present in the cells was isolated as described for the recombinant sucrose phosphorylase (1). Fractions containing high amounts of mutant BiSP were pooled. The presence of mutant BiSP was determined by SDS-PAGE using the Pharmacia PhastSystem according to the instructions of the supplier. Coomassie Brilliant Blue staining was used for detection of proteins on PhastGel 10–15% gradient gels (Amersham Biosciences). Protein concentration was determined by the method of Bradford (11) using bovine serum albumin as the standard.

Sucrose Phosphorylase Activity—Sucrose phosphorylase activity was measured in 20 mM potassium phosphate buffer (pH 6) containing 1 g/liter sucrose and incubated for 1 h at 37 °C. The concentrations of wild-type and mutant BiSP were 2.8 and 34.0 μg/ml, respectively. The reaction was stopped by raising the temperature to 100 °C for 10 min. After centrifugation for 10 min at 10,000 × g, the supernatant was analyzed by
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high performance anion exchange chromatography as described (1).

Crystalization and Data Collection of the BiSP(E232Q) Mutant in Complex with Sucrose—Crystals of the E232Q mutant were produced as described for native BiSP crystals (2). Hanging drops were prepared by mixing 2.5 μl of protein solution (0.9 mg/ml in 10 mM Tris-HCl (pH 7.1)) with 2.5 μl of precipitant solution (30% (w/v) polyethylene glycol 4000, 0.1 M Bicine (pH 8.6), and 50 mM sodium acetate) and equilibrated with 500 μl of precipitant solution at 20 °C. These crystals were soaked with a solution mimicking the crystalization conditions but supplemented with 20 μM sucrose for 1 h. Small crystals could also be obtained by co-crystallization with sucrose, but these were too small for data collection. The soaked crystals were mounted in a thin fiber loop and flash-cooled in liquid nitrogen. Data collection was performed at beamline BW7B (λ = 0.843 Å) at the European Molecular Biology Laboratory (EMBL) Synchrotron Radiation Facility (Hamburg, Germany) at 120 K using a crystal-to-detector distance of 325 mm (300-mm scan mode of a MAR 345 image plate) and an oscillation range of 0.5°. The collected reflection data were processed with MOSFLM (12) and Scala (13). The crystals belong to the orthorhombic space group P212121, with two molecules in the asymmetric unit. A summary of the data collection statistics is provided in Table 1.

Crystalization and Data Collection of Wild-type BiSP Reacted with Sucrose—Crystals of wild-type BiSP reacted with sucrose were obtained by a combination of the hanging-drop vapor-diffusion technique and streak seeding. Hanging drops were prepared by mixing 2.5 μl vapor-diffusion technique and streak seeding. Hanging drops were prepared by mixing 2.5 μl of protein solution (30% (w/v) polyethylene glycol 4000, 0.1 M Bicine (pH 8.6), and 50 mM sodium acetate) with 2.5 μl of precipitant solution (0.9 mg/ml in 10 mM Tris-HCl (pH 7.1)) and equilibrated with 500 μl of precipitant solution at 20 °C. These crystals were soaked with a solution mimicking the crystalization conditions but supplemented with 20 μM sucrose for 1 h. Small crystals could also be obtained by co-crystallization with sucrose, but these were too small for data collection. The soaked crystals were mounted in a thin fiber loop and flash-cooled in liquid nitrogen. Data collection was performed at beamline BW7B (λ = 0.843 Å) at the European Molecular Biology Laboratory (EMBL) Synchrotron Radiation Facility (Hamburg, Germany) at 120 K using a crystal-to-detector distance of 325 mm (300-mm scan mode of a MAR 345 image plate) and an oscillation range of 0.5°. The collected reflection data were processed with MOSFLM (12) and Scala (13). The crystals belong to the orthorhombic space group P212121, with two molecules in the asymmetric unit. A summary of the data collection statistics is provided in Table 1.

Structure Determination and Refinements—In both structures, the resulting electron density maps were easily traced using the program ARP/wARP (15). The models were refined with CNS (16) with the MLF target function using a bulk solvent model and anisotropic B-factor correction. Refinement steps were accepted if they produced a lowering of $R_{free}$. Water molecules were picked among spherical peaks of 1.2σ in the $2F_o - F_c$ maps and were analyzed for hydrogen-bonding interactions with the protein or other water molecules. The $B$-values were refined for every atom, but restrained to the values of neighboring atoms. The data and refinement statistics are listed in Table 2.

### Table 1

| Wild-type BiSP-glucose | BiSP(E232Q)-sucrose |
|------------------------|---------------------|
| Space group            | P2 2 1 2            |
| Unit cell (Å³)         | 75.7 x 103.1 x 150.7 |
| Resolution range (Å)   | 20-2.0 (2.07-2.0)     |
| Total no. of reflections measured | 337,497 |
| No. of unique reflections | 80,388               |
| Mosaicity (%)          | 0.30%               |
| Completeness (%)       | 99.6 (96.9)          |
| $R_{free}$ (%)         | 8.2 (43.3)           |

### Table 2

| Wild-type BiSP-glucose | BiSP(E232Q)-sucrose |
|------------------------|---------------------|
| Resolution (Å)         | 20.0-2.0            |
| No. of protein atoms   | 7930                |
| No. of solvent waters  | 1006                |
| No. of hetero-atoms    | 23                  |
| $R_{cryst}$ (%)        | 19.8                |
| $R_{free}$ (%)         | 23.7                |
| R.m.s. deviation       | 0.012               |
| Bonds (Å)              | 2.3°                |
| Angles                 | 3.7°                |
| Average B-value (Å²)   | Protein 26          |
|                        | Solvent 35          |
|                        | Hetero-atom 8        |

$^a$ Data were processed with Denzo and Scalepack (14).
$^b$ Data were processed with MOSFLM (12) and Scala (13).
$^c$ $R_{cryst} = \Sigma_h \Gamma \left( \frac{|F_{calc}| - |F_{obs}|}{|F_{calc}|} \right)$, where $F_{calc}$ is the intensity of an individual measurement of the reflection with Miller indices $h_k, l$, and $F_{obs}$ is the mean intensity of that reflection.

The BiSP(E232Q)-Sucrose Structure—Two BiSP molecules (A and B) resulting in a total of 1008 amino acid residues, two sucrose molecules, and 999 water molecules were included in the final model. In both chains, Cys356 has been oxidized (probably by the synchrotron radiation) to a sulfone, and residue 232 has been modeled as Gln according to the introduced mutation. The Ramachandran plot as calculated by the program PROCHECK (17) shows 88.4% of the residues in the most favorable regions, 11.1% in the additional allowed regions, 0.4% (Asp946 in both molecules, Asp447 in molecule A, and Phe156 in molecule B) in the generously allowed regions, and no residues in the disallowed regions of the plot.

The Wild-type BiSP-Glucose Structure—Two BiSP molecules (A and B) resulting in a total of 1008 amino acid residues, two sucrose molecules (one of which is covalently attached to Asp192 of chain A), and 1006 water molecules were included in the final model. The Ramachandran plot as calculated by the program PROCHECK (17) shows 90.9% of the residues in the most favorable regions, 8.7% in the addi-
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RESULTS AND DISCUSSION

Sucrose phosphorylases are retaining enzymes, and the crystal structure of wild-type BiSP indicates that Glu\textsuperscript{232} and Asp\textsuperscript{192} are the catalytic residues acting as acid/base catalyst and nucleophile, respectively (2). To obtain detailed information on the binding site of the substrate, mutants that capture sucrose in the active site can be created. In this study, the BiSP gene was subjected to site-directed mutagenesis to replace Glu\textsuperscript{232} with Gln to inactivate the enzyme. The products formed by sucrose phosphorylase were measured by high-performance anion exchange chromatography. In comparison with wild-type BiSP, the BiSP mutant showed no activity toward sucrose, not even at a >10-fold higher protein concentration (data not shown).

The BiSP-Substrate Complex—To obtain the sucrose complex, crystals of the inactive BiSP(E232Q) mutant were soaked in sucrose. Both monomers (A and B) have identical overall conformations (root mean square [r.m.s.] deviation of 0.3 Å on 504 C-\(\alpha\) atoms and an estimated coordinate error of the structure of 0.3 Å) that are highly similar to those in the wild-type BiSP-Tris complex (r.m.s. deviation of 0.3 Å on 504 C-\(\alpha\) atoms of molecule A) (2). The sucrose molecule is bound within the active-site pocket (Fig. 2A) at approximately the same position as the Tris molecule in the previously determined structure (2). The interactions of BiSP(E232Q) and sucrose are shown in Fig. 2B and Table 3. The binding of sucrose is very similar to that observed in the mutant E328Q amylosucrase-sucrose complex (Fig. 2C) (18). The hydroxyl groups of the glucosyl moieties (\(^{1}C\), conformation) have conserved hydrogen-bonding partners. Also, the water molecule that mediates the contact between Asp\textsuperscript{50} (Asp\textsuperscript{144} in amylosucrase) and the glucosyl O-4 is conserved. However, there are differences in the binding of the fructosyl moieties. O-3 is within hydrogen-bonding distance of His\textsuperscript{345}, whereas O-3 interacts with a water molecule in amylosucrase. Furthermore, BiSP(E232Q)-sucrose has one hydrogen bond with a water molecule and one with Asp\textsuperscript{345}; instead, a water-mediated hydrogen bond with O-4' is formed in the E328Q amylosucrase-sucrose complex. The E328Q amylosucrase-sucrose O-6' interactions with Asp\textsuperscript{394} and Arg\textsuperscript{446} are replaced in the BiSP(E232Q)-sucrose complex by hydrogen bonds with Gln\textsuperscript{345} only. Unlike in E328Q amylosucrase and other related enzymes, the sucrose molecule in BiSP(E232Q) is packed tightly within the active-site pocket.

The BiSP-Glucosyl Intermediate Complex—Crystals of the wild-type BiSP complex were obtained by co-crystallization...
with sucrose. The asymmetric unit of the crystals comprises two molecules forming the same dimer interface as observed in the Tris complex. Here, however, the two molecules adopt different conformations. The conformation of molecule A is very similar to that in the BiSP(E232Q)-sucrose complex (r.m.s. deviation of 0.3 Å on 504 C-\(^\alpha\) atoms), whereas the conformation of molecule B shows larger deviations (1.6 Å to molecules A of the Tris and E232Q complexes on all 504 C-\(^\alpha\) atoms). Both enzyme molecules contain glucose within the active site, but only one glucose is covalently bound to the nucleophile, i.e. forming the catalytic covalent intermediate (molecule A) (Fig. 3A) The other glucose is noncovalently bound (molecule B) (Fig. 3B). The interactions of the glucosyl intermediate are shown in Fig. 3C and Table 3. Because the anomic carbon of sucrose changes configuration from \(\alpha\) to \(\beta\) upon formation of the covalent intermediate, we observed the \(\beta\)-anomer bound as expected.

To date, two covalent intermediate structures have been solved for enzymes of glycoside hydrolase family 13, \(\text{viz.}\) cyclodextrin glucosyltransferase (19) and amylosucrase (20). The most relevant structure for comparison with the covalent BiSP intermediate is that of amylosucrase, because amylosucrase and BiSP share sucrose as the primary substrate; furthermore, hydrolysis is only a minor side reaction for both enzymes. The glucosyl moiety in BiSP is in a slightly twisted 1,4\(\beta\) conformation compared with 4\(\beta\) observed in amylosucrase. However, O-6, O-2, O-3, and O-4 occupy nearly the same positions in the two structures. O-6 is shifted by \(\sim 1\) Å, but forms a hydrogen bond with a conserved His residue in both structures.

The active-site architecture of the covalent intermediate appears to be very similar to that of the sucrose-bound form. The glucosyl moiety occupies the same position in the covalent intermediate as in the BiSP(E232Q)-sucrose complex, and all residues in close vicinity of the glucosyl moieties are located at very similar positions, \(\text{i.e.}\) no structural rearrangements accompany the formation of a covalent intermediate in BiSP. The flipping of the glucosyl ring from 4\(\beta\) in sucrose to 1,4\(\beta\) in the intermediate does, however, have consequences in terms of binding. C-1 moves by 1.2 Å toward Phe\(^{33}\), and this movement adds to the van der Waals interactions. A similar situation was observed upon formation of the covalent intermediate in amylosucrase, where the glucosyl moiety was translated toward Tyr\(^{147}\) with a shift of 1.5 Å (20). This suggests that favorable van der Waals interactions contribute to stabilize the covalent intermediates in BiSP as well as in amylosucrase.

The BiSP-Product Complex after Hydrolysis—As the enzyme goes from the covalent intermediate-bound state to the product-bound state, a number of changes to the active-site topology occur. The glucosyl-binding site remains unaltered. Hence, the active-site interactions with the glucose are very similar to those seen with the glucosyl part of sucrose in the BiSP(E232Q)-sucrose complex (Fig. 3D). The noncovalently bound glucose (in molecule B) adopts the \(\beta\)-configuration with a 4\(\beta\) conformation.

The most striking differences between the glucose product-bound form (after hydrolysis of the covalently bound intermediate) and the substrate- and intermediate-bound forms are the conformations of the loops between residues 336 and 344 (loop A) and residues 130 and 140 (loop B), which differ by up to 16 and 4 Å, respectively (Fig. 4A). Excluding these regions, the r.m.s. deviation is 0.3 Å (on 484 C-\(^\alpha\) atoms) compared with all other BiSP monomers. The rigid-body movement of loop B can be ascribed to major changes in backbone torsion angles of residues 133 and 137. Both loops A and B are in close proximity in the BiSP(E232Q)-sucrose and BiSP-glucosyl intermediate structures. Therefore, it is conceivable that the conformational changes observed in the wild-type BiSP-glucose structure are not independent. As a consequence of these differences in loop conformations, the architecture of the active site changes. Asp\(^{342}\) of loop A moves out of the binding site and becomes solvent-exposed. Tyr\(^{344}\) replaces Asp\(^{342}\), whereas Arg\(^{135}\) of loop B moves into the active site, with the guanidino group replacing the side chain of Leu\(^{341}\). Because of the movement of Asp\(^{342}\) out of and the movement of Arg\(^{135}\) and Tyr\(^{344}\) into the active site, a net change in charge of +2 is seen.


toward Understanding the Reaction Mechanism—The study has provided structural information on a substrate (sucrose)-bound form of the enzyme, the enzyme-glucosyl intermediate, and a product (glucose)-bound form. So far, the structures of the apoenzyme and \(\alpha\)-d-glucose 1-phosphate product-bound form remain to be determined.

The first step in the proposed reaction cycle (Scheme 2) is binding of sucrose to the active site of the enzyme. Upon formation of the covalent intermediate, the fructose group is cleaved off and is required to move away from the covalent intermediate C-1 atom by \(\sim 3\) Å. This is compensated, to some extent, by the 1.2-Å movement caused by the 4\(\beta\)-to-1,4\(\beta\) conformational change, but it still requires loop A to be pushed \(\sim 2\) Å away from the active-site residues. In the covalent interme-

### TABLE 3

| Ligand  | Interatomic distance (Å) | BiSP(E232Q)-sucrose molecule A | Wild-type BiSP-glucose Glucosyl intermediate | Wild-type BiSP-glucose \(\beta\)-D-Glucose |
|---------|--------------------------|---------------------------------|---------------------------------------------|----------------------------------------|
| Glucosyl |                          |                                 |                                              |                                        |
| O-2     | 3.0                      | 3.0                             | 2.9                                         |                                        |
| Arg\(^{190}\) N-\(\eta_2\) | 3.3                      | 2.9                             |                                              |                                        |
| Gln/Glu\(^{327}\) N-\(\varepsilon_2/O-82\) | 3.1                      | 3.1                             | 2.9                                         |                                        |
| Asp\(^{290}\) O-52 | 2.6                      | 2.7                             | 2.7                                         |                                        |
| O-3     |                          |                                 |                                              |                                        |
| His\(^{289}\) N-\(\varepsilon_2\) | 2.9                      | 3.1                             | 2.9                                         |                                        |
| Asp\(^{290}\) O-62 | 2.7                      | 2.8                             | 2.8                                         |                                        |
| O-4     |                          |                                 |                                              |                                        |
| Asp\(^{50}\) O-62 | 2.8                      | 3.0                             | 2.8                                         |                                        |
| Arg\(^{299}\) N-\(\eta_1\) | 2.6                      | 2.7                             | 2.7                                         |                                        |
| O-6     |                          |                                 |                                              |                                        |
| His\(^{288}\) N-\(\varepsilon_2\) | 2.9                      | 3.0                             | 2.9                                         |                                        |
| Asp\(^{192}\) O-62 | 2.9                      | 2.7                             | 2.8                                         |                                        |
| Fructosyl |                          |                                 |                                              |                                        |
| O-1', Gln\(^{232}\) O-\(\varepsilon_1\) | 2.6                      |                                 |                                              |                                        |
| O-3', His\(^{244}\) N-\(\varepsilon_2\) | 3.5                      |                                 |                                              |                                        |
| O-4', Asp\(^{342}\) O-62 | 2.8                      |                                 |                                              |                                        |
| O-5', Gln\(^{340}\) O-\(\varepsilon_1\) | 2.8                      |                                 |                                              |                                        |
backbone atoms of Pro$^{134}$ and Leu$^{343}$. Upon hydrolysis of the enzyme-glucosyl intermediate and formation of the glucose product-bound form, this interaction is lost because of large movements of loop A. Loop A now adopts an entirely different conformation that brings Tyr$^{344}$ into the fructose-binding site. The large conformational change in loop A possibly causes loop B to move farther in toward the active site.

Phosphorolysis of the glucosyl intermediate is not observed in the wild-type BiSP structure, as phosphate ions were not present during crystallization. To locate possible phosphate-binding site(s) in BiSP, soaking and co-crystallization experiments with phosphate, sulfate, vanadate, and cacodylate were performed. Only co-crystallization of wild-type BiSP with cacodylate resulted in a complex structure to a resolution of 3 Å (data not shown). However, no cacodylate ions were observed within the active site. To investigate the phosphorylase activity of BiSP, the position of the glucose molecule hydrolysis product can be used to dock the glucose 1-phosphate reaction product into the active site (Fig. 4 B). Arg$^{135}$ and Tyr$^{344}$ are positioned nearly optimally for the binding of a phosphate group. By extruding Arg$^{135}$ into the active site, the BiSP enzyme ensures that phosphate is able to compete out water, i.e. prevent hydrolysis via strong electrostatic interaction. Hydrolysis is a significant reaction in other transferases utilizing a covalent intermediate. Notably, Asp$^{342}$ and Tyr$^{344}$ of loop A are completely conserved, and Arg$^{135}$ of loop B is substituted only with lysine among the known sucrose phosphorylases, emphasizing the importance of these residues for sucrose phosphorylase activity.

A question that remains to be answered is why a mixed dimer consisting of the intermediate- and hydrolysis product-bound forms is found upon treatment of wild-type BiSP with sucrose. One possible explanation is that it is a consequence of crystal packing. However, it has been previously indirectly observed
FIGURE 4. Structural changes occurring during the enzyme reaction. A, close-up view of loops A and B of the wild-type BiSP covalent intermediate (molecule A; cyan) superimposed on the glucose product-bound form (molecule B; yellow). The bound glucose of molecule B is shown for clarity. B, close-up view of loops A and B and the noncovalently bound glucose molecule of the wild-type BiSP-glucose complex. Glucose 1-phosphate (yellow) has been modeled based on the position of the glucose interacting with Arg135 and Tyr344. C, proposed intermolecular phosphate-binding site created by two Arg135 residues. The distances indicated by dashed lines are 3.9 Å. The bound sucrose molecules are shown as red van der Waals spheres.
using complementary methods that such mixed dimers also exist in solution (9). Interestingly, although structurally different from BiSP, the crystal structure of maltose phosphorylase (an enzyme that undertakes phosphorolysis of maltose into glucose and glucose 1-phosphate by inversion) (21) also shows a mixed dimer when phosphate is present. In maltose phosphorylase, the phosphate-binding loops rearrange in only one molecule to create a phosphate-binding site near the proposed maltose-binding site. As in BiSP, these phosphate-binding loops are also involved in forming the dimer interface in maltose phosphorylase, and it is possible that both enzymes work in a similar fashion, even though they do not belong to the same family of enzymes.

Overall, essentially the same dimer as identified in the BiSP-Tris structure (2) is found in the our two structures. When analyzing the protein-protein contacts using the PISA server at the EMBL European Bioinformatics Institute, we found that, for the BiSP(E232Q)-sucrose complex, the size of the accessible surface area is 957 Å². For the glucosyl intermediate, the dimer interface is generally maintained, but because of the movements of the loops in the glucose product-bound form of the enzyme, the interactions are slightly different. The size increases to 1052 Å², and the number of hydrogen bonds also increases.

This asymmetry of the dimers may reflect the way that phosphate ions are brought to the active site. Analyses of the dimer interface in the BiSP-sucrose and BiSP-glucosyl intermediate complexes revealed that the guanidino groups of Arg135 are pointing toward the interface and that the distance between them is ~10 Å. Slight movements of these side chains would make the guanidino groups from both monomers create a phosphate-binding site (Fig. 4C). This is in agreement with the observation that cacodylate ions are capable of binding to this site (data not shown). It implies that BiSP has only one surface phosphate-binding site from which phosphate ions are transported to the active site by Arg135. Thus, BiSP might work in such a way that the two monomers are out of phase and require phosphate at different times; whereas one monomer phosphorylates the covalent intermediate and releases glucose 1-phosphate, the other monomer binds sucrose and creates the covalent intermediate.

Acknowledgments—We acknowledge EMBL Hamburg and the staff at beamline BW7B for provision of synchrotron radiation facilities.

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