Structures of Staphylococcus aureus D-Tagatose-6-phosphate Kinase Implicate Domain Motions in Specificity and Mechanism*

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High resolution structures of Staphylococcus aureus D-tagatose-6-phosphate kinase (LacC) in two crystal forms are herein reported. The structures define LacC in apoform, in binary complexes with ADP or the co-factor analogue AMP-PNP, and in a ternary complex with AMP-PNP and D-tagatose-6-phosphate. The tertiary structure of the LacC monomer, which is closely related to other members of the pfkB subfamily of carbohydrate kinases, is composed of a large αβ core domain and a smaller, largely β “lid.” Four extended polypeptide segments connect these two domains. Dimerization of LacC occurs via interactions between lid domains, which come together to form a β-clasp structure. Residues from both subunits contribute to substrate binding. LacC adopts a closed structure required for phosphoryl transfer only when both substrate and co-factor are bound. A reaction mechanism similar to that used by other phosphoryl transferases is proposed, although unusually, when both substrate and co-factor are bound to the enzyme two Mg2+ ions are observed in the active site. A new motif of amino acid sequence conservation common to the pfkB subfamily of carbohydrate kinases is identified.

In those bacteria that use lactose as a carbohydrate source, the disaccharide is usually either converted to glucose through the Leloir pathway (1) and then metabolized by glycolysis (2) or the disaccharide is usually either converted to glucose through the D-tagatose-6-phosphate pathway (3). Staphylococcus aureus, Staphylococcus epidermidis, and Staphylococcus hominis are the only organisms known to exclusively use enzymes of the D-tagatose-6-phosphate pathway to metabolize lactose and D-galactose (4).

In S. aureus, D-galactose and lactose are imported and metabolized by proteins encoded by the lactose operon, lacABCD, the first step carried out by a D-galactose-6-phosphate isomerase encoded by lacAB (9) converts D-galactose-6-phosphate into D-tagatose-6-phosphate. The second step is an ATP-dependent phosphorylation of D-tagatose-6-phosphate by D-tagatose-6-phosphate kinase (LacC) that yields D-tagatose-1,6-bisphosphate (Fig. 1). A divalent cation is required for this reaction with Mg2+ giving the optimum reaction rate (10). Finally D-tagatose-1,6-bisphosphate aldolase (LacD) cleaves D-tagatose-1,6-bisphosphate to produce glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (5, 11). The pathway is essential for the survival of S. aureus in lactose-rich media such as milk, because if it does not function properly, D-galactose accumulates and bacterial growth is inhibited (12). Interfering with this pathway may therefore be a useful strategy for eradicating infection by S. aureus. Such infections can be important in breast-feeding mothers (13) and are common in livestock where, if not treated, they cause damage to milk-secreting tissues affecting both the quality and quantity of dairy products (14). Structural information concerning the enzymes in the D-tagatose-6-phosphate pathway will play a key role in elucidating a mechanism for its inhibition. Currently, such information is only available for LacD (11).

On the basis of amino acid sequence comparisons, LacC belongs to the pfkB family of carbohydrate kinases, which form a subset of the ribokinase superfamily. Members of this superfamily include ribokinase (RbsK), the minor isoform D-fructose-6-phosphate kinase (PfkB), 1-phosphofructokinase (FruK) and inosine-guanosine kinase (15). A multiple sequence alignment of five members of the ribokinase superfamily including LacC from S. aureus (Fig. 2) shows regions of sequence conservation. Two of these (Motifs I and II) are conserved even in the

The abbreviations used are: LacC, D-tagatose-6-phosphate kinase; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate or adeny1 5′-yl iminodiphosphate; SeMet, selenomethionine; MES, 4-morpholineethanesulfonic acid; PEG, polyethylene glycol.

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sequence of human adenosine kinase (16) and represent the ribokinase superfamily signature. The crystal structure of Escherichia coli ribokinase indicates that these motifs are involved in substrate recognition, catalytic mechanism, and transition state stabilization (17).

We present the structure of LacC in two crystal forms. To solve the structure, it was necessary to incorporate two additional selenomethionine (SeMet) residues by site-directed mutagenesis. Fortuitously, the crystals present a number of different states of the enzyme, and the structures provide details of apoLacC, binary complexes with ADP, or the co-factor analogue adenosine 5'-[(β,γ-imido) triphosphate (AMP-PNP), selected because it is a relatively stable triphosphate), and a ternary complex with AMP-PNP and d-tagatose-6-phosphate. The structures illustrate aspects of co-factor and substrate recognition and suggest a mechanism for phosphoryl transfer dependent on conformational changes. We also identify a new region of amino acid sequence conservation specific to the pfkB subfamily of carbohydrate kinases.

**EXPERIMENTAL PROCEDURES**

**Cloning of LacC from S. aureus**—The gene encoding tagatose-6-phosphate kinase (lacC) from S. aureus (GenBank™ accession number X14827) was amplified by PCR using the specific primers 5'-CAT ATG ATT TTG ACT TTG ACA TTA AAC C-3' (forward) and 5'-GGA TCC TTA CAC CTC TAA AAC TTC-3' (reverse). The forward primer carries an NdeI restriction site and the reverse primer a BamHI restriction site. The resulting PCR product was cloned into a pET15b expression vector (Novagen) and the resulting plasmid heat shock-transformed into E. coli strain BL21 (DE3) (Stratagene).

For the L124M,L125M double mutant of S. aureus LacC, PCR primers containing the mutations (forward primer, 5'-TTT ATT AAA CAT TTT GAA CAA ATG ATG GAA AAA GTT GAA GCA GTT GCT-3'; reverse primer, 5'-AGC AAC TGC TTC AAC TTT TTC CAT CAT TTG AAA ATG TTT AAT AAA-3' (mutations shown in bold)) were prepared using a QuikChange® site-directed mutagenesis kit (Stratagene), the PCR product cloned into a pET15b expression vector, and the resulting plasmid purified and sequenced to check the presence of the desired mutation. The plasmid was then heat shock-transformed into E. coli strain BL21 (DE3).

Recombinant LacC was produced following similar protocols to those employed for 4-diphosphocytidyl-2C-methyl-erythritol kinase (18). After cultivation, cells were harvested and resuspended in 50 mM Tris-HCl, pH 7.7, containing 50 mM NaCl (buffer A). Cell lysis was performed with a cell disruptor at 4 °C, and the cell suspension was centrifuged at 20,000 × g for 30 min. The filtered supernatant was applied to a Hitrap metal-chelating column (Amersham Biosciences). The pure protein, eluted in buffer A and 150 mM imidazole, was dialyzed overnight at 4 °C against buffer A and then concentrated to 34 mg/ml.

An aliquot of the pure apoform of the protein was injected onto a high resolution Superdex 200 HR 10/30 size exclusion column (Amersham Biosciences). The elution profile (data not shown) was compared with that of various protein size markers (obtained from Sigma Aldrich) and indicated that LacC forms a dimer in solution.

To prepare the selenomethionyl derivatives of LacC and the L124M,L125M double mutant, BL21 (DE3) cells, heat shock-transformed with the pET15b/lacC S. aureus, were grown in 3 ml of LB supplemented with 100 μg/ml ampicillin and grown at 37 °C for 3 h. The culture was harvested and cells resuspended in 25 ml of M9 medium supplemented with 100 μg/ml ampicillin and allowed to grow for another 15 h at 37 °C. These cells were used to inoculate 1 liter of M9 medium. When the absorbance at 600 nm reached 0.56 at 37 °C, solid amino acid supplements were added to the culture as follows: 100 mg/liter L-lysine, L-phenylalanine, L-threonine, 50 mg/liter L-isoleucine, L-leucine, L-valine and 60 mg/liter SeMet. Following a delay of 15 min to allow inhibition of methionine synthesis, gene expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside and the culture left overnight. The same purification protocol described for the native enzyme was then followed. The final concentration of the stock solution in buffer A was 17.0 mg/ml.

**Crystallization and Data Collection**—The wild-type protein gave crystals that diffracted only to low resolution (~3.3 Å). We proceeded to make selenomethionyl-LacC for structural analyses and discovered that, for reasons not understood, the SeMet protein produced much more ordered crystals, therefore, we worked only with them. Block-shaped monoclinic crystals (crystal form I) of SeMet-LacC preincubated with 5 mM ATP
were obtained from hanging drops consisting of 1.0 μl of the protein stock solution (8.5 mg/ml) and 1.0 μl of a reservoir solution comprising 13% PEG 20000 and 0.1 M MES, pH 6.8, diffused against 0.5 ml of the reservoir solution to which 0.2 μl of 20% w/v benzamidine hydrochloride was added. Cryoprotection was achieved by soaking crystals in the crystallization mother liquid supplemented with 15% 2-methyl-2,4-pentanediol and data collected on ID14-EH2 of the European Synchrotron Radiation Facility (Table 1). Diffraction images were integrated with MOSFLM software and intensities scaled and merged using SCALA software. These were then converted to structure factors and anomalous differences derived using the program TRUNCATE.

Data collected from crystal form I did not allow de novo structure solution. To achieve this, we produced crystals of the SeMet derivative of the L124M,L125M double mutant to enhance the anomalous dispersion effects due to selenium incorporation. Prior to crystallization, this protein was incubated with 5 mM substrate (d-tagatose-6-phosphate) and 5 mM AMP-PNP. Diffraction quality orthorhombic crystals (crystal form II) grew in the optimized crystallization condition 0.2 M magnesium acetate, 0.1 M sodium cacodylate, pH 6.5, 10% PEG 8000, 15% PEG 550, 10 mM ATP. Cryoprotection was achieved by soaking crystals in 0.2 M magnesium acetate tetrahydrate, 0.1 M sodium cacodylate, pH 6.5, 10% PEG 8000 supplemented with 10% glycerol, and 10% PEG 550. Diffraction data, measured on European Synchrotron Radiation Facility beam-line ID14-EH2, were integrated and scaled using XDS (19) and intensities merged using SCALA software packages. Intensities were then converted to structure factors and anomalous differences derived using the program TRUNCATE. See Table 1 for a summary of statistics.

**Crystal Form II**—The structure of crystal form II was solved using the single wavelength anomalous dispersion technique. The programs XPREP (20) and SHELXD (21) were used to determine a substructure comprising 16 selenium sites from a reservoir unit of each form contains four subunits organized as a pair of tetramers in the asymmetric unit. The solvent content and Matthews co-efficient are 50% and 2.5 Å³/Da, respectively. SHELXE (22) was used to determine the correct enantiomorph of the substructure, and the program SHARP (23) was used to calculate phase probability distributions. This resulted in experimental phases with an average figure of merit of 0.245 to d_{min} = 2.1 Å resolution.

The experimental phases were improved using the program DM by density modification using a combination of non-crystallographic symmetry averaging, histogram matching and solvent flattening. Initial non-crystallographic symmetry operators were obtained using the program PROFESS. The resulting phases coupled with the observed structure factors were then input into ARP/wARP (24), which placed 1144 glycine, valine, and serine residues in the asymmetric unit. The GUISIDE module of CCP4i (25) then docked the correct side chains to these residues. Visual inspection of the electron density using O (26) identified model fragments belonging to each of the four monomers. Rounds of refinement using REFMAC5 interspersed with map inspections in O allowed a model of all four monomers in the asymmetric unit to be constructed. The careful placement of solvent molecules and active site ligands concluded the analysis with the refinement process being monitored using R_{work} and R_{free} (27). It was clear early in the map interpretation that there was significant deviation from the expected non-crystallographic symmetry, and therefore all subunits were treated independently. The subunits are labeled A–D.

**Crystal Form I**—Subunit A from crystal form II, stripped of ligands and solvent molecules, provided the search model for molecular replacement calculations to solve the structure of crystal form I. Molecular replacement was carried out using the program Phaser. The asymmetric unit of form I also presents four subunits, again labeled A–D, and each was positioned and then refined as a rigid body using REFMAC5 with data between 20.0 and 2.0 Å. The resulting electron density map provided a basis for automatic model building (ARP/wARP), and a model consisting of 992 glycine residues was built. Similar protocols to those applied in the analysis of form II concluded the structure determination.

Summaries of the composition and geometry of the final models for both crystal forms along with refinement statistics are presented in Table 1. The stereochemistry of the final models was assessed using PROCHECK. Ramachandran plots (not shown) indicate excellent main chain geometry. However, in all subunits, Asn-233 has $\Phi$ and $\Psi$ angles, which place it in a disallowed region. This residue is well defined by the electron density, and the strained conformation is stabilized by a hydrogen bond between the main chain amide and the carbonyl of Glu-217. The programs DM, MOSFLM, PHASER, PROFESS, PROCHECK, REFMAC5, SCALA, TRUNCATE and GUISIDE are part of the CCP4 software package (28).

**RESULTS AND DISCUSSION**

**Crystallographic Analyses**—To solve the structure of LacC, we produced the SeMet variant of an L124M,L125M double mutant. This sample (crystal form II) provided an anomalous signal large enough to be exploited for structure determination. The choice of residues to mutate was guided by the knowledge that mutation of Leu to Met has little effect on protein structure (29), coupled with secondary structure predictions for LacC (data not shown) that indicated the residues concerned are located in an $\alpha$-helix. Our crystal structure analyses proved this prediction to be correct.

Subsequently, molecular replacement methods allowed us to determine the structure of crystal form I. Both crystal forms have been refined to high resolution (Table 1). The asymmetric unit of each form contains four subunits organized as a pair of homodimers.

**A New Motif for the pfkB Subfamily**—Among the sequences aligned in Fig. 2, there is clearly a previously unreported third region of amino acid conservation common to fructose phosphate kinases and LacC. This conserved region, Motif III, is common to the pfkB subfamily of carbohydrate kinases but not to members of the ribokinase superfamily of proteins in general and has the sequence Ser-Gly-Ser-Leu-Pro-Xaa-Gly. Motif III may thus be assigned as being the characteristic signature of the pfkB family carbohydrate kinases, and as will be described, this motif is specifically involved in the binding of the tagatose substrate.
The Overall Structure of LacC—The primary structure of S. aureus LacC consists of 310 amino acids. The tertiary structure of a LacC monomer is composed of two domains (Fig. 3a). A large α/β core domain comprises residues 1–7, 37–83, and 110–310 and presents a Rossmann-type fold in which the central β-sheet has been extended. Additional α-helices abut this central β-sheet along its entire length. A smaller lid domain protrudes from the α/β domain. It is constructed from residues 10–33 and 89–104 and comprises five β-strands, four of which are organized in a mixed β-sheet with strand order β2-β4-β7-β8. The fifth strand in this domain (β3) is part of the turn linking β2 and β4. Four polypeptide segments comprising residues 7–9, 34–37, 83–88, and 105–110 connect the two domains. The lack of secondary structure for these connecting elements is suggestive of a potential flexibility in the relative orientation of the two domains.

The LacC monomer structure is closely related to other members of the ribokinase superfamily. The common structural feature in this superfamily is the large α/β domain, whereas the lid domain is less well conserved. Thus ribokinase (17), adenosine kinase (30), and glucokinase (31) have lid domains, but 4-amino-5-hydromethyl-2-methylpyrimidine phosphate kinase (32) and 4-methyl-5-β-hydroxy-ethylthiazole kinase (33) lack this feature. When both core and lid domains are present, their folds are conserved, but their relative orientations may differ. A superposition of the LacC monomers in the crystal forms described here (data not shown) confirms this conformational flexibility with subunits being found in either open (monomers A1, B1, C1, A16, B16, D16), semi-closed (D1), or fully closed (C111) conformations (the subscript denotes crystal form).

In common with other members of the ribokinase superfamily, LacC forms a dimer similar to that seen for E. coli ribokinase (Fig. 3b) (17). Two monomers associate via interactions between their lid domains, which come together to form a β-barrel structure known as a β-clasp. The two β-sheets of the lid domains pack face to face and are rotated ~90° with respect to each other. Each face of the β-barrel is made up of a five-stranded β-sheet; four strands from one monomer, the fifth from the second. β-clasp structures display closed and splayed corners. In the LacC dimers, the distances between the diagonally opposite closed and splayed corners are ~17 and 25 Å, respectively. Monomer-monomer interactions within the interior of the barrel are plentiful and are both hydrophobic and polar in nature.

The Active Site of LacC—Crystal form I was obtained by co-crystallization with ATP. However, only two molecules of ADP, bound to monomers B1 and D1, were identified. Monomers A1 and C1 represent the structure of the apoform of the enzyme. That ADP, and not ATP, is found bound to monomers B1 and D1
should not be surprising. In the crystal structure of \textit{E. coli} ribokinase (17), ADP is found in the co-factor binding site even though the protein was incubated with AMP-PNP (often used because of its stability) before crystallization. In that case, the authors speculated that AMP-PNP had hydrolyzed before binding to the protein. Given the much greater propensity of ATP to hydrolyze, it is likely that the same situation has occurred here.

The ADP binding site is on the surface of the domain constructed using residues from 11, the C terminus of 12 and the turn linking it to 13, the long loop linking 14 to the N terminus of the latter, and the C terminus of 9. Aliphatic side chains form a binding pocket in which the adenine base is held in an anti-conformation stabilized only by hydrophobic interactions. The ribose O-2* interacts with the main chain carbonyl and ND2 of Asn-278. A well ordered water molecule mediates hydrogen bonds between the ribose O-3* and the amide and carbonyl groups of Gly-224 and Gly-227. The ribose O-3* atom also accepts a hydrogen bond from Asn-278 ND2. The -phosphate group interacts with Ser-222 OG and via a water molecule with Lys-38 NZ. The O-3A link between - and -phosphate groups accepts a hydrogen bond from the main chain amide of Gly-253. The -phosphate forms hydrogen bonds with the carbonyl group of Pro-249 (we assume that a phosphate O is protonated), the amide group of Gly-253, and (via water molecules) the amide groups of Gly-251 and Asp-254.

Crystal form II, from which the first experimental phases were actually determined, was obtained from protein incubated with 5 mM substrate (D-tagatose-6-phosphate) and a co-factor analogue (AMP-PNP). Here, positive difference electron den-

FIGURE 2. \textbf{Amino acid sequence comparisons.} A multiple sequence alignment of 1-phosphofructokinase from \textit{E. coli} (K1PF), d-fructose-6-phosphate kinase from \textit{E. coli} (K6PF2), LacC from \textit{S. aureus} (LACC), ribokinase from \textit{Lactococcus lactis} (RBSK), and human adenosine kinase (ADK). Residues blocked in red are identical in the sequences aligned, whereas yellow blocks indicate regions of sequence homology. Motifs I and II previously identified for the ribokinase superfamily are indicated as is Motif III common to only the pfkB family of carbohydrate kinases (see “Results and Discussion”). This sequence alignment was made with the T-Coffee program (47).
sity \((mF_o - DF_o, \alpha_{calc})\) was observed in the active sites of all four monomers of the asymmetric unit. In the active sites of subunits A\textsubscript{II}, B\textsubscript{II}, and D\textsubscript{II}, this was assigned as AMP-PNP with a hydrated \(\text{Mg}^{2+}\) ion binding between the \(\alpha\) and \(\beta\)-phosphates.

In monomer C\textsubscript{II}, two different moieties were bound in the active site. One is clearly tagatose-6-phosphate (Fig. 4a, right). The OMIT \((mF_o - DF_o, \alpha_{calc})\) difference density (Fig. 4a, left) indicated that an initial model for the second moiety should be AMP-PNP with two hydrated cations bound to its phosphate tail. However, such a model resulted in significant negative difference density centered on the \(\beta\)-phosphate group of the AMP-PNP, an indication that this model was not correct. Clean difference density maps were eventually obtained in the active site of subunit C\textsubscript{II} by modeling AMP-PNP and ADP, each with half-occupancy and two fully occupied hydrated \(\text{Mg}^{2+}\) ions (see “Results and Discussion”). Mg\(^{2+}\) ions are shown as orange and green spheres and water molecules as light blue spheres. C, N, O, P positions are black, blue, red, and purple, respectively. Shown on the right side are tagatose-6-phosphate and its associated density. The atomic positions C, O, and P are orange, red, and purple, respectively. b, the C\textsubscript{II} subunit is shown as in Fig. 3a with the addition of a semitransparent van der Waals surface (gray). The ligands are depicted as sticks, AMP-PNP is shown in black and \(\delta\)-tagatose-6-phosphate in orange.

Based on coordination geometry, the cation bridging the \(\alpha\)- and \(\beta\)-phosphates of AMP-PNP is assigned as \(\text{Mg}^{2+}\). The nature of the cation bridging the \(\beta\)- and \(\gamma\)-phosphates of the AMP-PNP moiety in the active site on molecule C of crystal form II is less obvious. Given its square pyramidal coordination, it could be assigned as either \(\text{Na}^+\) or \(\text{K}^+\) with the ion–oxygen

FIGURE 4. Electron density maps for ligands and location of the active site. a, the \(mF_o - DF_o, \alpha_{calc}\) OMIT density contoured at 2.5\(\sigma\) in the active site of monomer C\textsubscript{II}. That shown on the left side represents AMP-PNP and ADP, each with half-occupancy and two fully occupied hydrated \(\text{Mg}^{2+}\) ions (see “Results and Discussion”). Mg\(^{2+}\) ions are shown as orange and green spheres and water molecules as light blue spheres. C, N, O, P positions are black, blue, red, and purple, respectively. Shown on the right side are tagatose-6-phosphate and its associated density. The atomic positions C, O, and P are orange, red, and purple, respectively. b, the C\textsubscript{II} subunit is shown as in Fig. 3a with the addition of a semitransparent van der Waals surface (gray). The ligands are depicted as sticks, AMP-PNP is shown in black and \(\delta\)-tagatose-6-phosphate in orange.
atom distances observed (2.0–2.3 Å) favoring assignment as Na⁺ (34). However, Mg²⁺ can also exhibit square pyramidal coordination with Mg²⁺–oxygen distances similar to those observed here (35). Given the assignment as Mg²⁺ of a five-coordinate cation bridging the β- and γ-phosphates of the AMP-PNP moiety in active site B of this crystal form of LacC this cation was also assigned as Mg²⁺. The temperature factors of the cations (and their associated water molecules) suggest that they are fully occupied. Thus, when both substrate and ATP are bound in the active site of LacC, hydrated Mg²⁺ ions bridge both the α- and β-phosphate groups and the β- and γ-phosphate groups of the co-factor. When ADP is bound in the presence of substrate, a hydrated Mg²⁺ ion bridges the α- and β-phosphate groups, whereas a second forms a bridge between its terminal β-phosphate and the co-factor binding site residues of LacC.

As described for ADP binding, the co-factor binding sites are located on the surface of the core domain (Fig. 4b), and interactions between the enzyme and the adenine base are conserved. However, interactions between the sugar/phosphate tail of the AMP-PNP/ATP and the protein are different from those seen for ADP binding to LacC (not shown).

When substrate is absent (monomers AIII, BIII, DIII), the ribose O-2° of AMP-PNP interacts with the protein in the same way as when ADP is bound. However, the AMP-PNP ribose O-3° now accepts a hydrogen bond directly from Asn-278 ND2 and the previously observed solvent-mediated hydrogen bonds between the ribose O-3° and the protein are replaced by a direct interaction of this group with the carbonyl oxygen of Gly-227. This difference is due to a change in the position of the loop involving residues Ser-222 to Gly-227 when AMP-PNP rather than ADP is bound, is shifted toward the co-factor by ~1.6 Å. The α-phosphate accepts hydrogen bonds from the amide of Gly-224 and the hydroxyl of Ser-222. When substrate is not present, the α-phosphate is also involved in water-mediated hydrogen bonds with the carbonyl group of Pro-249 and the linking N-3B atom. The β-phosphate accepts hydrogen bonds from ND2 of Asn-185 and NZ of Lys-183. The γ-phosphate is held in place tightly, with O-1G accepting a hydrogen bond from the Gly-251 amide, whereas O-2G accepts two hydrogen bonds donated by the amide groups of Gly-253 and Asp-254 and, in addition, interacts with a water molecule, which coordinates the Mg²⁺ ion. This Mg²⁺ is coordinated by β- and γ-phosphate groups of the nucleotide via two non-bridging oxygen atoms. The coordination sphere of the cation is completed by three water molecules in the active site of monomer D and four water molecules in those of monomers A and B. These water molecules form hydrogen bonds with a number of amino acid side chains including those of Lys-38 and Asp-254.

The majority of the hydrogen bonds and metal ion-co-factor interactions are maintained when AMP-PNP/ATP is bound in the presence of substrate (Fig. 5). However, in this case, a major difference is that a second Mg²⁺ ion bridges the α- and β-phosphate groups. Octahedral coordination of this cation is completed by water molecules, which mediate interactions of the α- and β-phosphate groups with the side chain of Gln-99 and the main chain carbonyl of Pro-249. As well as interacting with this Mg²⁺ ion, the β-phosphate O-1B also accepts a hydrogen bond donated by Asn-185 ND2. Lys-183 NZ donates a hydrogen bond to the β-phosphate O-2B, which also interacts with the second Mg²⁺ ion. This ion bridges the β-phosphate O-2B and the γ-phosphate O-2G, and square pyramidal coordination is completed by three water molecules, the positions of which are conserved in all four active sites of crystal form II of LacC. The linking N-3B interacts with the amide group of Gly-253. The γ-phosphate O-1G accepts hydrogen bonds donated by the amide group and the likely protonated OD2 of Asp-254 and participates in a solvent-mediated interaction with the cation bridging the β- and γ-phosphate groups. The γ-phosphate O-3G accepts a hydrogen bond donated by the amide group of Gly-251.
In monomer CII, the D-tagatose-6-phosphate substrate binds to residues from both domains of LacC and to residues in the linker region (Figs. 4b and 5). The substrate O-1 group, the phosphorylation site, forms hydrogen bonds with Asp-254 OD2 and Lys-38 NZ, with the latter also donating a hydrogen bond to the D-tagatose-6-phosphate O-5 hydroxyl and interacting with the substrate phosphate via a water molecule (not shown). The substrate O-2 interacts with Asn-41 ND2, whereas its O-3 and O-4 groups form hydrogen bonds with Asp-12 OD2 and OD1, respectively. As well as the solvent-mediated interaction with Lys-38, the substrate phosphate also forms direct hydrogen bonds with the side chains of Arg-88 and (from the partner subunit) Arg-27, the side chain hydroxyl of Ser-136, and the amide of Gly-135. The dimeric nature of LacC is thus important to construct the active site, in particular to position the basic Arg-27 so that it can participate in substrate binding.

The Catalytic Mechanism of LacC—As described above, monomer CII represents the structure of LacC with both the substrate and co-factor simultaneously bound. The active site comprises residues from the lid domain, the linking fragment, and the \( \alpha/\beta \) domain. Amino acids in all three conserved motifs (Fig. 2) play a role in the binding of substrate and/or co-factor. In particular, and as also seen in the structure of ribokinase (17), the amino acids in Motif I form an \( \alpha \)-helix of which the N terminus and the side chain of Lys-38 point toward the substrate phosphorylation site. From Motif II, the main chain amides of Gly-251 and Gly-253 form hydrogen bonds with the \( \gamma \)-phosphate of the co-factor, and the side chain of Asp-254 points toward the substrate phosphorylation site. Ser-136 from Motif III donates a hydrogen bond to the substrate’s phosphate group. This phosphate is present only in substrates processed by 1-phosphofructokinase, D-fructose-6-phosphate kinase, and D-tagatose-6-phosphate kinase, and this explains the conservation of this serine in these sequences only.

The catalytic center (Fig. 5) is located at the interface between the co-factor and substrate. The \( \gamma \)-phosphate of AMP-PNP is 2.9 Å distant from the substrate phosphorylation site, which is sandwiched between the side chains of Asp-254 and Lys-38. This configuration suggests a catalytic mechanism for LacC in which the side chain of Asp-254 abstracts the proton from the substrate C1 hydroxyl group to generate a nucleophile, which then attacks the \( \gamma \)-phosphate of the co-factor to yield the phosphorylated product (Fig. 6). The optimal positioning of the side chain of Lys-38 likely stabilizes the negative charge of the transition state.

This mechanism is similar to that proposed for other phosphoryl transferases. However, unlike other enzymes that carry out the reaction in an \( \text{Mg}^{2+} \)-dependent manner (36–41), the crystal structures described here indicate that the
active site of LacC contains two, rather than one, Mg$^{2+}$. The cation that bridges the $\beta$- and $\gamma$-phosphates of the co-factor serves to correctly orient both the $\gamma$-phosphate and the side chain of the catalytic Asp-254. This cation may also serve to neutralize any negative charge on the transition state and may favor an associative reaction mechanism by polarizing the co-factor $\gamma$-phosphate thus making it more susceptible to nucleophilic attack (42). The role of the Mg$^{2+}$ ion that bridges the $\alpha$- and $\beta$-phosphates is less clear, although it may enhance the effectiveness of ADP as a leaving group (43).

Conformational Changes in LacC—LacC monomers adopt distinct conformations in which the relative orientations of the lid and core domains vary. Conformational changes upon substrate binding are a well known strategy for nucleotide-dependant transferases to position appropriate amino acids around the side chain of Gln-99 help stabilize binding of a second Mg$^{2+}$ substrate is bound. Only when domain closure is complete can ATP or ATP and substrate revealed that only local structural changes seen in LacC involve a movement of the $\alpha/\beta$ domain toward the lid domain resulting in a closure of the active site. The fully closed conformation seen for the CII monomer of LacC is essential for transfer of the phosphoryl group to the substrate, as only in this configuration are the catalytic partners close to each other and the catalytic Lys-38 and Asp-254 in the correct position to facilitate phosphoryl transfer. Moreover, in LacC, full domain closure is also required to provide a fully formed substrate binding pocket (Fig. 7).

The conservation of the distances (~25 Å) between the splayed corners of the $\beta$-claps formed in the AB and CD dimers in crystal form II suggest that the conformational changes seen in LacC involve a movement of the $\alpha/\beta$ domain toward the lid domain resulting in a closure of the active site. The fully closed conformation seen for the CII monomer of LacC is essential for transfer of the phosphoryl group to the substrate, as only in this configuration are the catalytic partners close to each other and the catalytic Lys-38 and Asp-254 in the correct position to facilitate phosphoryl transfer. Moreover, in LacC, full domain closure is also required to provide a fully formed substrate binding pocket (Fig. 7).

The phenomenon of domain closure has been observed in many multidomain enzymes. In one such enzyme, phosphoglycerate kinase, complete domain closure may only be achieved with binding of both substrate and AMP co-factor (45). Such a situation also seems to be the case for LacC. However, domain closure is not common to all enzymes that possess a lid domain. Thus, the crystal structures of 2-keto-3-deoxygluconate kinase from Thermus thermophilus in complex with ATP or ATP and substrate revealed that only local structural (and not large scale conformational) changes are required to enable catalysis (46).

The conformational changes observed in monomer CII of LacC are also critical in allowing the recruitment of the Mg$^{2+}$ ion that bridges the $\alpha$ and $\beta$-phosphates of the co-factor when substrate is bound. Only when domain closure is complete can the side chain of Gln-99 help stabilize binding of a second Mg$^{2+}$ ion by the ATP co-factor (Figs. 5 and 7b). That the fully closed conformation of LacC is also crucial to allow proper substrate binding suggests that the presence of this second Mg$^{2+}$ ion may be important to phosphoryl transfer as catalyzed by LacC.

CONCLUSIONS

The structure of LacC from S. aureus has been determined in two different crystal forms. The fold is characteristic of the ribokinase superfamily and consists of two domains, a core $\alpha/\beta$ domain, and a lid domain comprising $\beta$-strands that is involved in dimer formation. These two domains are linked by unstructured polypeptides, which confer flexibility in the relative orientation of the two domains. As the dimer interface forms a rigid $\beta$-clasp structure, the conformational changes observed involve a movement of the core domain toward the lid domain. Domain closure has to be complete to bring substrate and co-factor close enough to react, to provide a fully formed substrate binding pocket and to allow the recruitment of a second Mg$^{2+}$ ion that may be important in catalysis.

Our study provides understanding of how LacC recognizes and binds its substrates and allows us to propose a mechanism for the catalysis of phosphoryl transfer. The structural information may, in addition, provide the basis for the design of a specific LacC inhibitor, which might have implications for human and animal health.

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