Structural Basis for Bacterial Quorum Sensing-mediated Oxalogenesis*

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Background: Oxalogenesis in the Burkholderia species is an indispensable event for their survival in the stationary phase. Results: Structural and functional analyses of ObcA, the first enzyme in oxalogenesis, unravel an unprecedented reaction mechanism. Conclusion: ObcA produces a tetrahedral CoA adduct. Significance: This study provides a structural basis for understanding the first step in oxalogenesis.

The Burkholderia species utilize acetyl-CoA and oxaloacetate, substrates for citrate synthase in the TCA cycle, to produce oxalic acid in response to bacterial cell to cell communication, called quorum sensing. Quorum sensing-mediated oxalogenesis via a sequential reaction by ObcA and ObcB counteracts the population-collapsing alkaline pH of the stationary growth phase. Thus, the oxalic acid produced plays an essential role as an excreted public good for survival of the group. Here, we report structural and functional analyses of ObcA, revealing mechanistic features distinct from those of citrate synthase. ObcA exhibits a unique fold, in which a (B/α)8-barrel fold is located in the C-domain with the N-domain inserted into a loop following α1 in the barrel fold. Structural analyses of the complexes with oxaloacetate and with a bisubstrate adduct indicate that each of the oxaloacetate and acetyl-CoA substrates is bound to an independent site near the metal coordination shell in the barrel fold. In catalysis, oxaloacetate serves as a nucleophile by forming an enolate intermediate mediated by Tyr322 as a general base, which then attacks the thioester carbonyl carbon of acetyl-CoA to yield a tetrahedral adduct between the two substrates. Therefore, ObcA catalyzes its reaction by combining the enolase and acetyltransferase superfamilies, but the presence of the metal coordination shell and the absence of general acid(s) produces an unusual tetrahedral CoA adduct as a stable product. These results provide the structural basis for understanding the first step in oxalogenesis and constitute an example of the functional diversity of an enzyme for survival and adaptation in the environment.

Bacterial quorum sensing (QS), a cell to cell communication process in many Proteobacteria, is mediated by small diffusible molecules called autoinducers (1, 2). Once the population of bacteria reaches a threshold density, a bacterium-specific autoinducer binds to a transcription regulator, and the resulting complex controls the gene expression for population-wide characteristics, including bioluminescence, motility, and virulence-related factors (1, 2). Recently, QS has been recognized to provide further benefits at the population level by regulating the production of public goods, the function of which could be beneficial to all members of the group including QS-deficient, exploitative individuals (3–6). The public goods commonly provide fitness benefits, a novel QS concept in bacterial population-wide behaviors.

Oxalic acid was recently identified as an excreted public good for the QS-dependent growth in three related species of the genus Burkholderia (7): the rice pathogen Burkholderia glumae, the animal pathogen Burkholderia mallei, and the non-pathogenic saprophyte Burkholderia thailandensis. In these species, QS-mediated oxalogenesis via the oxalate biosynthetic component is a cellular event indispensable for the survival of bacteria in the stationary phase. Specifically, the acidity of the oxalic acid produced regulates the pH of the environment, avoiding a possible sudden collapse of the bacterial population caused by an alkaline pH in the stationary phase of their growth (7). Oxalic acid is ubiquitously present in humans and plants, as well as many bacteria and fungi. Its physiological roles are diverse, including the formation of calcium oxalate crystals for kidney stones in humans, calcium regulation in plants, and pathogenesis in fungi (8–10). However, a biochemical pathway for endogenous oxalogenesis remains largely uncharacterized, although candidates for oxalate precursors include glyoxylate, glycolate, and glyoxal (8, 9).

In B. glumae, the oxalate biosynthetic component consists of two genes encoding ObcA and ObcB for coordinating the production of oxalic acid, as well as acetoacetate and CoA, by using oxaloacetate and acetyl-CoA as substrates (11). Previous biochemical studies propose that ObcA catalyzes the formation of a C6-CoA adduct between oxaloacetate (four-carbon compound) and acetyl-CoA (two-carbon acetyl moiety); in a subsequent and independent reaction, ObcB utilizes the C6-CoA adduct as a substrate to produce three different products (see Fig. 1a) (12). Substrate-labeling experiments further suggested...
that the C6-CoA adduct produced by ObcA differs regiochemically from the citroyl-CoA intermediate catalyzed by citrate synthase in the TCA cycle (see Fig. 1a) (12, 13), even though these two enzymes utilize identical substrates. Unlike B. glumae, B. thailandensis catalyzes the two-step reaction with a single bifunctional enzyme, Obc1, which consists of an ObcA homolog (54% sequence identity) in its N-terminal domain (see Fig. 2) and a functional ObcB-like enzyme, with no apparent sequence homology to ObcB, in its C-terminal domain (14).

Here, we present structural studies of ObcA from B. glumae, including a ligand-free form, and its complex with oxaloacetate and with a C4-CoA adduct and oxalate, the degradation products from the C6-CoA adduct. Structural analyses along with functional assays show that ObcA catalyzes its reaction in a unique manner relative to citrate synthase and other acetyl-CoA-dependent enzymes, providing structural insights into the first step in oxalogenesis and the mechanistic features of ObcA.

**EXPERIMENTAL PROCEDURES**

**ObcA Purification**—The gene for ObcA from B. glumae (7) was amplified by PCR with sequence-specific primers. The resulting PCR product was subcloned into the Ndel and Xhol restriction sites of a modified pET28b expression vector (Merck) containing a TEV protease cleavage site at the junction between a His6 tag and a multiple cloning site. The resulting PCR product was subcloned into the NdeI and XhoI sites of the pET28b expression vector, which was amplified by PCR with sequence-specific primers. The resulting PCR product was subcloned into the NdeI and XhoI sites of the pET28b expression vector, which was further subjected to methylation of the lysine residues using the dimethylated enzyme was expressed in E. coli BL21 (DE3). Cells were grown in Luria-Bertani medium at 37 °C, and when the optical density of the culture medium reached 0.7 at 600 nm, protein expression was induced by adding 0.5 mM of isopropyl-β-D-thiogalactopyranoside, followed by an additional 12–14 h of growth at 20 °C. Cells were collected and sonicated in Buffer A containing 50 mM Tris (pH 8.0), 100 mM NaCl, and 5% (v/v) glycerol. The N-terminal His-tagged ObcA was purified using an immobilized metal affinity column (GE Healthcare) that had been equilibrated with Buffer A and then eluted with Buffer A containing additional 500 mM imidazole. The N-terminal His-tag of ObcA was subsequently removed by treatment of TEV protease overnight at 22 °C, using a 20:1 molar ratio of ObcA to TEV protease. The resulting ObcA protein was further purified by immobilized metal affinity and size exclusion chromatography using Superdex 200 (GE Healthcare) with Buffer A.

For the structural determination of ligand-free ObcA, the N-terminal His-tagged seleno-l-methionine (SeMet)-substituted enzyme was expressed in E. coli BL383 (DE3; Merck) using minimal medium supplemented with SeMet. The N-terminal His-tagged SeMet-ObcA was purified, and the His tag was removed as described above. The purified SeMet-ObcA was further subjected to methylation of the lysine residues using established protocols (15). In brief, 2–5 mg/ml of SeMet-ObcA was subjected to the methylation reaction using the dimethylamine-borane complex and formaldehyde overnight at 4 °C. After centrifugation, the soluble fraction from the reaction mixture was further purified by size exclusion chromatography using a Superdex 200 column (GE Healthcare) with Buffer A to remove any possible protein aggregates.

**Crystallization and Data Collection**—Crystallization was conducted at 22 °C by a sitting drop vapor diffusion method using the native or the methylated SeMet-substituted ObcA (10–15 mg/ml). The presence of the Co2⁺ ion in the protein solution caused precipitation of ObcA or resulted in crystals diffracting only to a low resolution, although the Co2⁺ ion was the most effective metal ion for the enzyme catalysis (see below). Addition of one or both substrates was essential for the formation of crystals suitable for a high resolution structure, but under our experimental conditions, the binding of the substrate(s) was not observed in the active site; however, a soaking experiment with the substrate(s) exhibited the binding of substrate(s) of interest. Therefore, crystallization for high resolution structure analysis was performed using various combinations of ligand(s) and metal ions.

For the ligand-free ObcA, the methylated form of SeMet-substituted ObcA was initially crystallized with 2 mM acetyl-CoA under a crystallization solution of 0.1 M Tris (pH 8.0), 0.2 M MgCl₂, and 20% PEG 6000. Later, we collected higher resolution data by soaking the crystal into the crystallization solution plus 100 mM CoA. To obtain an oxaloacetate-bound ObcA structure, the native ObcA crystal was soaked in the crystallization solution plus 20 mM oxaloacetate and 2 mM MgCl₂ after growing the crystal from 5 mM oxaloacetate and 1 mM CoA, with mother liquor containing 0.1 M HEPES (pH 7.5), 3% PEG 400, 2 M ammonium sulfate, 0.1 M MgCl₂, and 15% glycerol. The structure of ObcA in complex with the tetrahedral C4-CoA adduct was obtained using native ObcA. Specifically, the native ObcA crystal was produced by co-crystallizing ObcA with 15 mM acetyl-CoA, 20 mM oxaloacetate, and 1 mM CoCl₂, using a crystallization buffer containing 0.2 M sodium/potassium tartrate, 0.1 M sodium citrate (pH 5.6), 2 M ammonium sulfate, and 15% glycerol. Prior to data collection, the crystal was soaked in a solution of 7.5 mM acetyl-CoA, 10 mM oxaloacetate, and 1 mM CoCl₂.

X-ray diffraction data were collected at 100 K with a 1° oscillation angle on Beamline BL-1A at the Photon Factory (Japan) and 5C at the Pohang Accelerator Laboratory (Korea). Glycerol (15–20%) was used as the cryo-protectant in these experiments. Initially, multiwavelength anomalous dispersion data were collected at 2.8 Å using a crystal of the methylated SeMet-substituted ObcA. Subsequently, higher resolution single-wavelength data became available for the 2.1 Å resolution ligand-free ObcA, the 2.0-Å resolution ObcA in complex with oxaloacetate, and the 2.28 Å resolution ObcA in complex with the C4-CoA adduct. The collected data were processed using the HKL2000 (16), and all crystals had a space group of P4₃2₁2, with one monomer in the asymmetric unit (Table 1), consistent with a size exclusion chromatographic study demonstrating ObcA to be a monomeric protein.

**Structure Determination and Refinement**—The structure of the ligand-free ObcA was determined using the programs SOLVE (17) and RESOLVE (18), with multiwavelength anomalous dispersion data at 2.8 Å resolution. The model was built and refined using the programs COOT (19) and PHENIX (20). After several cycles of manual inspection and refinement, residues ranging from Thr² to Ile⁶₂⁵ were located, except for the highly disordered region of Asp⁷¹—Ala⁹¹ and Arg²⁹⁶—Trp³⁰⁰. The model was then refined against the 2.1 Å resolution data available. During assignment of water molecules in the struct-
ture, we noticed the presence of a metal ion, based on strong density from the $F_o - F_c$ electron density map, as well as its geometry to the nearby residues and other water molecules. We assigned the metal ion as a Mg$^{2+}$ ion because the crystallization solution contained 0.2 M MgCl$_2$. The structure for a ligand-free adduct and oxalate was determined. In particular, the oxalate was bound to the metal-binding site. Similarly, the structure for the tetrahedral C4-CoA adduct showed that oxaloacetate was bound to the metal-binding site. We observed the presence of a metal ion, based on strong density from the $F_o - F_c$ electron density map, as well as its geometry to the nearby residues and other water molecules. We assigned the metal ion as a Mg$^{2+}$ ion because the crystallization solution contained 0.2 M MgCl$_2$. The structure for a ligand-free adduct and oxalate was determined. In particular, the oxalate was bound to the metal-binding site. Similarly, the structure for the tetrahedral C4-CoA adduct showed that oxaloacetate was bound to the metal-binding site.

**Purification of Obc1* and Various ObcA Mutants**—For the assay, the WT and mutant ObcA enzymes were expressed as described above. We also used a mutant enzyme of a bifunctional Obc1 from *B. thailandensis* (hereafter indicated as Obc1*) to replace the function of ObcB in the reaction because of an issue with solubility associated with *B. glumae* ObcB. Genes for the mutant ObcA and Obc1* were constructed by site-directed mutagenesis using mutagenic primers. The N-terminal His-tagged enzyme was purified by immobilized metal affinity chromatography following a desalting step using a column of HiPrep 26/10 (GE Healthcare) and a buffer solution containing 50 mM Tris (pH 8.0), 100 mM NaCl, and 5% (v/v) glycerol for various ObcA mutants or 50 mM HEPES (pH 7.0), 300 mM NaCl, and 5% (v/v) glycerol for Obc1*. The enzymes were used without removing the His tag. For measurement of the metal-dependent activity, purified WT ObcA and Obc1* enzymes were dialyzed against Buffer A plus an additional 10 mM EDTA and subsequently against an EDTA-free Buffer A. The absence of metal ions, including Co$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, or Mg$^{2+}$, in ObcA was further validated by inductively coupled plasma atomic emission spectroscopy analysis.

**Activity Assay of ObcA**—An enzyme activity assay was performed using two different methods, each measuring a different product. First, a steady-state kinetic analysis for ObcA was carried out by monitoring the time-dependent production of free CoA using 2,6-dichlorophenolindophenol, a dye that reacts with the sulfhydryl group of CoA to cause a linear decrease in absorbance at 600 nm (21). In a second assay, total oxalate produced was measured using an oxalate kit (Trinity Biotech).

To monitor the production of free CoA, enzyme assays were performed at 30°C using a UV-visible spectrophotometer (Jasco). The reaction mixture included 50 mM Tris buffer (pH 8.0), 100 mM NaCl, 100 μM 2,6-dichlorophenolindophenol, 100 μM EDTA, and subsequently against an EDTA-free Buffer A. The absence of metal ions, including Co$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, or Mg$^{2+}$, in ObcA was further validated by inductively coupled plasma atomic emission spectroscopy analysis.

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**Structure and Function of ObcA**

The overall reaction of oxalogenesis in *B. glumae* and the structure of ObcA. **a**, in ObcA-dependent catalysis, acetyl-CoA (black) and oxaloacetate (red) are proposed to be converted into a C6-CoA adduct (12). The oxal group of the adduct is colored in blue. Subsequently, ObcB produces CoA, acetoacetate, and oxalic acid. **b**, the overall structure of ObcA in a ligand-free form is displayed, indicating an N-domain (blue) and a C-terminal (β/α)8-barrel fold (magenta). Additional structural elements from the C-domain (green), β1 (yellow), and a metal ion (green sphere) are also indicated. **c**, the topology of ObcA is shown with color coding identical to that described in b. The C terminus of βH11 to the N terminus of αN3 is highly disordered and not modeled. Those termini are marked with black dots. Secondary structure elements are shown in Fig. 2.

**RESULTS**

**Structure of ObcA in the Ligand-free Form**—ObcA (residues Thr2–Ile325) consists of two structurally distinct domains: a small N-domain and a large C-domain (Figs. 1b and 2). The overall structure comprises a (β/α)8-barrel fold for the C-domain, with the N-domain (Pro15–Tyr198) inserted into a loop between β1 and α1 of the barrel fold (Fig. 1c). This topology represents a unique structural architecture. Homologous structure searches using DALI (22) indicated that despite the abundance of (β/α)8-barrel folds in the Protein Data Bank, no other structures resemble the overall features of ObcA. In particular, DALI search using the N-domain indicates that the most homologous structure found exhibits a Z-score of only 1.3. The N-terminal 14 residues form the β1 in the barrel fold. Other insertions occur in the loop regions of the large C-domain, specifically in the loops following β2, β6, and β8 (Figs. 1c and 2). First, two long antiparallel β-strands (β2 and β6) following β2 protrude vertically from the C-domain and belong structurally to the N-domain (hereafter, subscripts used in this manuscript represent additional structural elements present in the corresponding region of the canonical (β/α)8-barrel fold, and the N-domain structural elements are indicated by “N”). The sec-

μM CoCl2, and 400–800 μM acetyl-CoA. The mixture was incubated for 20 min at 30 °C, followed by the addition of 50–500 nM WT ObcA or its mutant and 800 nM Obc1*, after which the mixture was incubated for an additional 4 min. Enzyme reaction was initiated by adding 1–10 mM oxaloacetate to the resulting reaction mixture, and the initial velocity was determined by measuring the linear decrease in absorbance at 600 nm from the time range of 60–105 s; nonlinear decreases in absorbance were observed in the first 60 s. The 800 nM Obc1* was confirmed as the saturating concentration for the coupled reaction of a steady-state kinetic assay for ObcA. Free CoA concentration produced per minute was calculated from the standard curve. Specifically, the standard curve was measured after 5 min. Total oxalate produced was calculated from the standard curve. Specifically, the standard curve as a function of oxalate concentration was obtained in the absence of two enzymes and two substrates.

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ond insertion consists of two β-strands and one α-helix inserted into the loop connecting β6 and α6. The resulting antiparallel β-strands (β61 and β62) protrude horizontally from the barrel, and the α61, is packed in an antiparallel orientation to the N-terminal region of α6. Additional alterations are seen in a loop following β8, in which two antiparallel β-strands (β81 and β82) project horizontally from the barrel. In the successive α8, a loop runs through the bottom of the barrel, and the C-terminal α9 turns nearly 90° relative to the preceding loop, thus sealing off any possible opening in the bottom of the barrel fold. In addition to these obvious modifications, an additional helix α51 exists between α5 and β6.

The small N-domain is located along the C-terminal ends of the central β-strands in the barrel fold. It does not share any structural similarity with the known folds and consists of two segments (Fig. 1b). In one segment, two antiparallel β-strands (β21 and β22) from the C-domain and three twisted β-strands (β31, β32, and β33) are arranged perpendicularly along the long axis of the strands, with two helices (α51 and α52) filling the open space between them. In the second segment, a layer of helices and β-strands is packed on the other side of the three twisted β-strands from the first segment, forming a funnel-shaped fold. The potential small opening in the funnel-like structure is located toward the C-domain but completely occluded by two β-strands (β81 and β82) from the C-domain barrel fold, whereas the larger opening at the opposite end comprises a highly disordered region.

The Active Site in Ligand-free ObcA—The putative active site of ObcA was indicated by the presence of a metal-binding site in a cavity of the C-terminal barrel fold, consistent with a previous observation that ObcA activity requires a metal ion (12). Our functional analysis also provided evidence for a metal-dependent activity of ObcA, in which the Co2+ ion is the strongest (see below). Given that purified ObcA does not contain metal ions characterized by inductively coupled plasma atomic emission spectroscopy analysis and that the crystallization condition of a ligand-free ObcA requires 200 mM Mg2+, the metal-binding site identified in a ligand-free ObcA is likely occupied by the Mg2+ ion. The active site is ~20 Å from the surface of the enzyme (Fig. 3, a and b). Three residues, including His222 and His224 in β2 and Glu473 in the loop of β8, along with three water molecules, ligate the metal ion, representing an octahedral coordination (Fig. 3c). In particular, His224 and Glu473, as well as two water molecules spanning these residues, form a square coordination for the equatorial plane, whereas His222 in the interior floor of the cavity and a water molecule proximal to the surface of the enzyme represent the axial ligands. Arg279 on the tip of the loop following β3 covers the metal-binding site, but the electron density for the loop, particularly Arg279–Gly284, is relatively disordered, suggesting dynamic features of the loop. Many residues are present in the vicinity of the metal-coordinating ligands. In particular, water molecules present in the shell are within a hydrogen bonding distance from the nearby residues, including Ser275 and Ser308, for one trans to Glu473, and Arg469 for a water molecule across from His224 (Fig. 3d).

Noticeably, a relatively large pocket remained vacant, located adjacent to the metal-binding site and extending from the active site cavity into the enzyme surface along the central β-strands of the barrel fold (Fig. 3, a and b). Most pocket-forming residues are more than 4.5 Å distant from the equatorial water molecule trans to His224, and the wall of the pocket is lined with three ladders of residues: Glu346 and Arg469 constituted the bottom floor; and the second ladder consisted of Asp474, Phe348, Val376, and Leu447, whereas the rim of the pocket on the surface involved Phe316, Val379, and Pro449. Among those residues, the innermost residue Arg469 is within a hydrogen bonding distance from the equatorial water molecule.
Structural and Function of ObcA

Structure of ObcA in Complex with Oxaloacetate—The structure of ObcA in complex with oxaloacetate shows that oxaloacetate is identified in the metal-binding site, with its oxalo group pointing toward Arg279 (Fig. 4a). The binding of oxaloacetate caused no noticeable change in conformation, relative to a ligand-free ObcA, with an root mean square deviation of 0.41 Å for 484 Cα atoms. Specifically, the C4 carboxylate of oxaloacetate replaced the metal-ligating water molecule trans to Glu473 in the ligand-free ObcA, and the O2 atom in the oxalo group occupied the sixth coordination. The two carboxylates of oxaloacetate are stabilized by the nearby residues, including Ser277, Arg279, and Tyr322 for the C1 carboxylate and Ser275 and Ser308 for the C4 carboxylate.

In addition to these nearby active site residues, possibly facilitating the binding of oxaloacetate, Tyr322 was brought into the immediate vicinity of the substrate, and the side chain hydroxyl group was placed ~3.3 Å distant from the C3 carbon of oxaloacetate (Fig. 4, a and b). In particular, its configuration fulfills the stereochemical requirements of an in-line projection to abstract a proton from the C3 methylene group, with an angle of 109° connecting the OH group in Tyr322, and C3 and C4 atoms of oxaloacetate. The binding of oxaloacetate stabilized the loop covering the metal-binding site, presenting a well defined interaction between Arg279 and the C1 carboxylate of oxaloacetate. The pocket adjacent to the metal-coordinating shell exhibited no major changes, with the exception that the side chain of Glu346 was closer to a metal-ligating water molecule across from His224 (Figs. 3d and 4b).

Structure of ObcA in Complex with the Bisubstrate Adduct—We unexpectedly identified a C4-CoA adduct and oxalate in the active site of ObcA at 2.28 Å resolution (Fig. 5a). The electron density map in the active site indicated that it does not represent an authentic C6-CoA adduct as we had anticipated. Rather, the density was separated into two segments, one for a C4-CoA adduct lacking the oxalo group in the oxaloacetate moiety and a second one for oxalate (Figs. 5b and 6, a and b). Given that all three products, including oxalate, acetoacetate, and CoA, are readily produced only in ObcB-dependent reactions (11), it is highly likely that under our experimental conditions, the oxalo group was spontaneously released from a C6-CoA adduct, forming oxalate, which was then trapped within the active site. Consistent with this crystallographic observation, our activity assay in solution (see Fig. 7a), in addition to previous evidence (11), both indicate that oxalate is very slowly and spontaneously produced in an ObcA-dependent manner. Therefore, we conclude that the C4-CoA adduct and oxalate are degradation products from the C6-CoA adduct, a proposed ObcA product (Fig. 1a).

The structure of ObcA in complex with a C4-CoA adduct and oxalate is almost identical with a ligand-free ObcA, with a root mean square deviation of 0.40 Å for 483 Cα atoms. The resulting C4-CoA adduct and oxalate occupy the metal-coordinating shell and its nearby pocket (Fig. 5c). The phosphopantetheine arm of the adduct is positioned in the pocket along the central β-strands of the C-domain. Specifically, this arm is bound to a concave region between the N- and C-domains composed of three different loops following B1, B7, and B8 in the barrel fold. This region was filled with a string of water molecules in the oxaloacetate-bound ObcA complex. Several positively charged residues, such as Arg127, Arg128, Lys193, and Lys489, are localized around the phosphate group of the arm (Fig. 5d). Noticeably, the side chain of Lys193 underwent large changes that prompted its interaction with the phosphate group, whereas the 3′-phosphoadenosine monophosphate moiety interacted with few residues (Fig. 6d). In contrast, oxalate is located near the C4-CoA adduct (Figs. 5, a–c, and 6, a and b), and its binding site is almost identical to the oxalo group in the oxaloacetate-bound ObcA but with a different orientation (Fig. 6c). As a result, oxalate is at least 2.6 Å away from the C4-CoA adduct. One carboxylate present in oxalate is within hydrogen bonding distance to Ser277 and Tyr322, as we observed in the oxaloacetate-bound ObcA (Fig. 4a), whereas the other carboxylate is nearly in the same posi-
tion with the O2 atom present in oxaloacetate, occupying a position for the axial water molecule in a metal-coordinating shell (Fig. 6).

Notable features exist in the C4-CoA adduct. The carbon atom adjoined to the sulfhydryl group of CoA is in the tetrahedral configuration (Fig. 6b). The resulting tetrahedral C4-CoA adduct therefore consists of acetoacetate and CoA moieties (Fig. 1a), consistent with the observation that acetoacetate and free CoA moieties comprise the two other products in ObcB-dependent reactions. However, the adduct differs in chemical structure from a cognate acetoacetyl-CoA containing a thioester linkage between the sulfhydryl group of CoA and the carboxylate of acetoacetate. The acetoacetate moiety of the C4-CoA adduct occupies two adjacent positions in the equatorial plane of the metal coordination shell (Figs. 5a and 6d). The carboxylate in the adduct replaced the C4 carboxylate position of oxaloacetate, whereas the oxygen atom on the tetrahedral carbon occupied a position for the water molecule trans to His224. The resulting geometry around the metal ion is essentially identical to those of the ligand-free and oxaloacetate-bound ObcA. The binding of the phosphopantetheine arm was further stabilized by interactions with the pocket residues: for example, the Arg469 interaction with the oxygen atom in the tetrahedral carbon of the adduct, the Asp474 and Arg279 interactions with the arm of the adduct, and many other hydrophobic interactions.

FIGURE 4. The active site of ObcA in an oxaloacetate bound form. a, the stereo view of the active site of ObcA in complex with oxaloacetate (yellow) is shown. The dashed line between Tyr322 and the C3 group of oxaloacetate does not represent a hydrogen bond. Oxaloacetate in the right panel of (a) is overlaid with an Fo−Fc map contoured at 2.3 σ. b, the respective interactions are displayed. Dashed lines with an interatomic distance indicate possible hydrogen bonds.

FIGURE 5. The binding mode of the C4-CoA adduct and oxalate. a, the stereo view of the active site is shown for ObcA in complex with the bisubstrate C4-CoA adduct (green) and oxalate (yellow), noting a tetrahedral carbon (black asterisk) and Co2+ ion (black sphere). Dashed lines are potential hydrogen bonds within an interatomic distance of 3.5 Å, and the schematic drawing for these interactions is shown in Fig. 6d. b, the model for the C4-CoA adduct, oxalate, and Co2+ is overlaid with an Fo−Fc electron density map at 2.3 σ. c, the binding cavity of the C4-CoA adduct and oxalate is displayed in a surface representation of ObcA, with the metal ion indicated in gray. d, the surface charge distribution of the adduct-binding region is shown in an orientation similar to Fig. 3b. Positive charge distribution and negative charge distribution are indicated in blue and red, respectively. Note that the adenosine 3′,5′-diphosphate moiety is placed in the vicinity of the positive charge.
study is unusual, considering that the tetrahedral CoA is thought to be a reaction intermediate in many CoA-dependent reactions, subsequently collapsing into a free CoA by protonating the leaving CoA thiolate anion (23). In contrast, the presence of the tetrahedral C4-CoA adduct in ObcA is made possible by the fact that no obvious candidates for general acid(s) are positioned around the sulfur atom of the adduct in ObcA. Further inspection of the adduct suggests that the oxygen atom on the tetrahedral carbon is possibly in a hydroxyl form rather than a labile anionic form, providing a structural foundation for the stability of the tetrahedral adduct. Specifically, the nearby Arg469, which is conserved in ObcA-like proteins (Fig. 2), likely protonates the oxygen atom on the tetrahedral carbon (Figs. 5a and 6a). Identification of a bisubstrate adduct in the active site also suggests that the reaction proceeds via a ternary complex mechanism requiring the binding of both substrates prior to catalysis.

Functional Analysis of ObcA—We performed an enzymatic analysis of the WT and mutant ObcA enzymes. In particular, production of a free CoA and oxalate was measured in a coupled reaction with Obc1*. In the presence of the WT ObcA, Obc1*, and the Co2+ ion, the reaction completed in our assay produced oxalate as well as CoA (Fig. 7, a–c). The Obc1* enzyme essentially lacks in its own ObcA activity, but not ObcB function, because of mutations at three residues located within the ObcA-like N-terminal domain. Those mutations are H227A, Y326A, and E350A, which correspond to His224, Tyr322, and Glu346, respectively, of B. glumae ObcA (Fig. 2) and are essential for ObcA function (see below). Measurement of the total oxalate produced, as well as kinetic analysis of CoA production by Obc1*, indicated that Obc1* exhibits less than 0.5% of the WT ObcA activity (Figs. 7, d and e, and 8a), with 176- and 328-fold decreases in the kcat value for acetyl-CoA and oxaloacetate, respectively, relative to that of the WT ObcA (Fig. 8b).

A steady-state kinetic analysis for the production of free CoA and measurement of total oxalate production indicated that residues interacting with the metal ion as well as oxaloacetate or a bisubstrate adduct play an essential role in enzyme activity (Fig. 7d). Greater activity for total oxalate production than CoA production is possibly attributable to the experimental condition of oxalate production in which the measurement was performed after 5 min of reaction time for each mutant ObcA. Mutations of metal-binding residues (Fig. 3a), H222A, H224A, and E473A, significantly reduced ObcA functions; H224A became essentially inactive, although H222A and E473A have some marginal activity, suggesting that His224 is most important in metal binding. Similar results were also observed for oxaloacetate-binding residues (Fig. 4a); mutants, such as S275A, S277A, S308A, R279K, and R279A, also largely defect ObcA activity, except for R279K. Unlike R279A, which exhibits less than 5% of the WT ObcA activity, the R279K mutant maintained a nearly identical kcat value with the WT ObcA, but with a 35-fold increase in its Km value for oxaloacetate and only a 3-fold increase for acetyl-CoA (Figs. 4b and 7e), indicating that the positive charge on Arg279 is crucial for the binding of oxaloacetate. Other mutations also largely affected the Km value for oxaloacetate, consistent with their proposed structural roles.

Taken altogether, we conclude that ObcA catalyzes the formation of a C6-CoA adduct by joining the oxaloacetate C3 carbon to the thioester carbonyl carbon of acetyl-CoA, resulting in a tetrahedral C6-CoA adduct, which in turn serves as a substrate for ObcB in the production of oxalic acid, acetoacetate, and CoA. Identification of a tetrahedral C4-CoA adduct in this structure and function of ObcA.
For example, S277A and S308A exhibited 8- and 27-fold increases, respectively, in the $K_m$ value for oxaloacetate, but with much smaller changes (1–2-fold) in the $K_m$ values for acetyl-CoA.

In addition to residues in the metal coordination shell and oxaloacetate binding environment, several residues in the acetyl-CoA-binding pocket (Fig. 5a) such as Glu$^{346}$, Leu$^{447}$, Arg$^{469}$, and Asp$^{474}$ were also characterized as essential elements for activity (Fig. 7d). Mutations on these residues caused their activities less than 3% of the WT ObcA, consistent with their proposed roles in the binding of acetyl-CoA or adduct. For example, Leu$^{447}$ is within distance of the CoA moiety to mediate hydrophobic interactions, and Arg$^{469}$ was suggested in this study to protonate the oxygen atom on the tetrahedral carbon of the adduct, resulting in the hydroxyl group (Fig. 6d). Asp$^{474}$ interacting with Arg$^{469}$ appears to play a role in positioning of the Arg$^{469}$ side chain toward the adduct and regenerating Arg$^{469}$ in a catalytically competent form (see legend of Fig. 9).

Although most residues described above play a role in the binding of substrate or a possible adduct, we conclude that Tyr$^{322}$ is a key catalytic residue in ObcA reaction, based on its regiospecific location to the C3 atom of oxaloacetate (Fig. 4a; see “Discussion”). Mutation of Tyr$^{322}$ with either alanine or phenylalanine also greatly diminished the enzyme activity (4–10% of the WT ObcA activity), suggesting a possible catalytic role of the side chain hydroxyl group in the reaction. In particular, further kinetic analysis of Y322F indicated that catalytic efficiency ($k_{cat}/K_m$) of the mutant is only 0.3–2.0% of the WT ObcA.

**DISCUSSION**

In *Burkholderia*, endogenous oxalogenesis occurs using oxaloacetate and acetyl-CoA, the substrates also utilized by citrate synthase in the TCA cycle. However, ObcA and citrate synthase are structurally unrelated (13). Accordingly, apparent functional discrepancies between the two enzymes originate from
Asp474 could regenerate Arg469; in particular, Asp474 is a solvent-exposed residue, and the nearby water molecule is located in the binding pocket for acetyl-CoA. Note that mutation of either Tyr322, Arg469, or Asp474 greatly affects the activity of ObcA, consistent with this proposal, mutation of Tyr322 greatly diminished the enzyme activity (Fig. 7, d and e). Comparison of the binding mode of oxaloacetate and the bisubstrate adduct in this study indicated that the C3 atom of oxaloacetate is within 2.6 Å from the tetrahedral carbon of the adduct (Fig. 6c), suggesting that a direct nucleophilic attack of the proposed enolate of oxaloacetate occurs on the si-face of the thioester linkage of acetyl-CoA, the trigonal plane facing toward a bound oxaloacetate. Mechanistically, the formation of an enolate intermediate is similar to members of the enolase superfamily, in which a general base abstracts the α-proton of the carboxylate substrate and a metal ion stabilizes the enolate anion intermediate (24).

Apparently, the proposed ObcA-dependent catalysis (Fig. 9) shares some characteristics with other acetyltransferases involved in the acetylation of histone lysine, carnitine, chloramphenicol, and choline (23, 25–28). These acetyltransferases using acetyl-CoA as an acetyl donor commonly produce an acetylated substrate via a proposed tetrahedral intermediate, particularly by employing a general base to deprotonate a substrate, either directly or indirectly via a water-mediated proton wire, and its subsequent attack on the thioester carbonyl carbon of acetyl-CoA. These enzymes containing general acid(s) but lacking metal ions facilitate collapse of the resulting tetrahedral CoA intermediate into an acetylated substrate and CoA. In contrast, the tetrahedral CoA adduct in ObcA is likely stabilized by the bound metal ion and furthermore by the absence of possible general acid(s). Therefore, ObcA exhibits combined features from the enolase and acetyltransferase superfamily, but the presence of the metal coordination shell and the absence of general acid(s) appear to be the key factors for functional diversity. The metal-ligating shell has multiple roles in ObcA-dependent catalysis, not only dictating the binding mode of oxaloacetate to facilitate formation and stabilization of the enolate anion intermediate of oxaloacetate, but also contributing to the polarization and positioning of the thioester group of the incoming acetyl-CoA for a nucleophilic attack. Furthermore, the metal-coordinating shell also serves as a platform for a direct nucleophilic attack, as well as stabilization of the resulting tetrahedral CoA adduct.

In conclusion, we demonstrated that in the Burkholderia species, ObcA utilizes two substrates, oxaloacetate and acetyl-CoA, for the TCA cycle and forms a tetrahedral C6-CoA adduct in an unprecedented manner compared with other acetyl-CoA-dependent reactions. The C6-CoA adduct serves as a substrate for ObcB to produce oxalyl acid, acetoacetate, and CoA. Current genome data indicate that ObcA homologs are predominantly present in Burkholderia species (14). Our results provide
a structural basis for understanding the first step in oxalogenesis using metabolites from the primary metabolic pathway, as well as an example of the functional diversity of an enzyme for survival and adaptation in the growth environment.

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