Structural Insights into an Oxalate-producing Serine Hydrolase with an Unusual Oxyanion Hole and Additional Lyase Activity*

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In Burkholderia species, the production of oxalate, an acidic molecule, is a key event for bacterial growth in the stationary phase. Oxalate plays a central role in maintaining environmental pH, which counteracts inevitable population-collapsing alkaline toxicity in amino acid-based culture medium. In the phytopathogen Burkholderia glumae, two enzymes are responsible for oxalate production. First, the enzyme oxalate biosynthetic component A (ObcA) catalyzes the formation of a tetrahedral C6-CoA adduct from the substrates acetyl-CoA and oxaloacetate. Then the ObcB enzyme liberates three products from the C6-CoA adduct: oxalate, acetoacetate, and CoA. Interestingly, these two stepwise reactions are catalyzed by a single bifunctional enzyme, Obc1, from Burkholderia thailandensis and Burkholderia pseudomallei. Obc1 has an ObcA-like N-terminal domain and shows ObcB activity in its C-terminal domain despite no sequence homology with ObcB. We report the crystal structure of Obc1 in its apo and glycerol-bound form at 2.5 Å and 2.8 Å resolution, respectively. The Obc1 N-terminal domain is essentially identical both in structure and function to that of ObcA. Its C-terminal domain has an α/β hydrolase fold that has a catalytic triad for oxalate production and a novel oxanion hole distinct from the canonical HGGG motif in other α/β hydrolases. Functional analyses through mutagenesis studies suggested that His-934 is an additional catalytic acid/base for its lyase activity and liberates two additional products, acetoacetate and CoA. These results provide structural and functional insights into bacterial oxalogenesis and an example of divergent evolution of the α/β hydrolase fold, which has both hydrolase and lyase activity.

Bacteria produce and secrete a wide range of chemicals that can be beneficial to their survival in different environments (1–4). In particular, some molecules often referred to as “public goods” provide a fitness advantage for the whole population, including individuals (i.e. exploiters) that lack the ability to synthesize these molecules. Accordingly, the metabolic costs for producing public goods usually outweigh the benefits, unless the population of bacteria is very large. Therefore, the production of public goods occurs cooperatively in a population-dependent manner by quorum sensing. Bacterial quorum sensing is mediated via diffusible small chemicals, which can sense population density and respond to environmental changes during population growth (5, 6).

In Burkholderia species, quorum sensing-mediated oxalogenesis is an indispensable cellular event for survival during the stationary growth phase (1, 7). Oxalate is a highly acidic molecule that serves as a public good and plays a central role in maintaining pH homeostasis. Without oxalate, the whole population of Burkholderia species would collapse due to ammonia-mediated alkaline toxicity during the stationary growth phase.

In Burkholderia glumae and Burkholderia cepacia, oxalate production is mediated by a two-step enzymatic reaction (Fig. 1A) (8–10). The first enzyme, oxalate biosynthetic component A (ObcA),2 catalyzes the formation of a C6-CoA adduct, using acetyl-CoA and oxaloacetate as substrates (10). A second enzyme, ObcB, is responsible for the production of three different products, namely, oxalate, acetoacetate, and CoA. A tetrahedral C6-CoA adduct was proposed to be a product of ObcA (10), and the C2-C3 and C4-S bonds in the C6-CoA adduct are cleaved by ObcB (Fig. 1A). Interestingly, in Burkholderia thailandensis, Burkholderia pseudomallei, and Burkholderia mallei, the bifunctional enzyme Obc1 catalyzes these two reactions (11). Given that Obc1 functionally complements ObcA and ObcB, it is not surprising that it contains activities of both enzymes. The N-terminal domain of Obc1 exhibits 52% identity with the amino acid sequence of ObcA (10, 11) (Fig. 2); however, the Obc1 C-terminal domain does not show any sequence homology with ObcB even though it exhibits functional identity with ObcB.

Many Burkholderia species are involved in plant or human pathogenesis. For example, B. glumae causes bacterial panicle blight in rice (12), whereas B. cepacia is an opportunistic pathogen in immunocompromised individuals, including those with cystic fibrosis and chronic granulomatous disease (13). B. pseudomallei causes melioidosis, a lethal infection that leads to the formation of abscesses in internal organs (14). Given that the Burkholderia species mentioned above possess two monofunctional enzymes, ObcA and ObcB, or a bi-functional Obc1 for oxalogenesis (Fig. 2), understanding the molecular basis of

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2 The abbreviations used are: Obc, oxalate biosynthetic component; CC, Pearson correlation coefficient.
these enzymes should provide the groundwork for the development of novel agents for disease control.

We report the crystal structure of Obc1 in its apo form and glycerol-bound form. Using structural and functional analyses, we investigated how Obc1 catalyzes the production of three different products. Together with our previous study on ObcA (10), these results provide structural insights into bacterial oxalogenesis.

**Results**

**Overall Structure of ApoObc1**—The bifunctional enzyme Obc1 has two structurally and functionally distinct domains (Fig. 1B): an N-domain consisting of an ObcA-like N-terminal region (residues Val-2 to Ser-528) and a C-domain consisting of an ObcB activity-exhibiting C-terminal region (residues Arg-529 to Gln-1106). These two domains are arranged in an elon-
gated manner (i.e. 110 Å long and 56 Å wide); therefore, there are no extensive interactions between the two domains. The active sites of the two domains are separated by 47 Å and oriented in an opposite direction.

Monomeric Obc1 was identified in an asymmetric unit, and no obvious oligomeric structure was identified by crystallographic symmetry, consistent with chromatographic analysis that Obc1 is monomeric. The N-domain of Obc1 exhibits 52% sequence identity with ObcA (Fig. 2), and therefore, its structure is essentially identical to that of ObcA (PDB codes 4NNA, 4NNB, and 4NNC) (10). Specifically, the (β/α)6-barrel fold of the Obc1 N-domain is superimposable with ObcA (Fig. 1C), within a root mean square deviation of 1.0 Å for 482 Ca atoms and a Z-score of 51.2 using the structure-similarity search program DALI (15). Structural analysis revealed the presence of a metal binding site and the relative locations of residues in the active site. Given that detailed structural and functional features of ObcA have been reported (10), we decided to focus on C-domain features.

**C-domain of ApoObc1**—The Obc1 C-domain (residues Arg-529 to Gln-1106) extends from the bottom of the N-domain (β/α)6-barrel fold and forms an independent domain that does not interact extensively with the N-domain. The core structural region adopts an α/β hydrolase fold in which the central β-sheet is packed with flanking α-helices on both sides (Fig. 1B). The C-domain has structural features common to canonical α/β hydrolases (16). From the topological view, the C-domain consists of two structural subdomains (Fig. 1B). The first region includes Ser-740 to Gln-1106 and forms an α/β hydrolase fold (Fig. 1D). The second subdomain (Arg-529 to Ala-739) located over a concave region formed by an α/β hydrolase fold, resulting in a crevice between the two regions. Residues Arg-529 to Ala-739 (hereafter, we call this region the cap domain) predominantly form an α-helical fold, with two layers of anti-parallel helices. Specifically, just after the N-domain, two long adjacent α-helices, α19 and α20/21, are oriented in an anti-parallel fashion and form a surface layer of cap domain. Underneath this surface layer, other anti-parallel helices, α24 and α25, are located in a perpendicular orientation relative to the surface layer helices. In addition to these elements, helices α22, α23, and η3 form a four-helix bundle-like fold with the C-terminal region of α21. Due to a kink in the middle of the surface layer α-helices, the cap domain exhibits a convex surface and a concave interior. This cap domain is oriented such that a concave region sits on the edge of the β-sheet in the α/β hydrolase fold, resulting in the formation of a crevice on one side of the β-sheet that serves as the active site of the C-domain (see below).

Following the cap domain α25, a long loop connects a β-strand (i.e. β25) to form an α/β hydrolase fold including Ser-740 to Gln-1106 (Figs. 1D and 2). In the α/β hydrolase fold, nine β-strands (β26, β25, β28, β27, β29, β30, β31, β33, β32 in order) constitute a central β-sheet, in which β32 is the innermost strand near the N-domain and β26 is located farther from the N-domain and forms the surface of Obc1. In the middle of the central β-sheet, six strands (β28, β27, β29, β30, β31, and β33) are in a parallel orientation, with each C-terminal end pointing to the concave interior of the cap domain. At the ends, the other three β-strands are in an anti-parallel orientation (Fig. 1D). Helices α26, α29, α30, and α31 are located on the backside of the β-sheet where the cap domain surface helices α19 and α20/21 are stretched out; this leaves this side of the central β-sheet inaccessible from the solvent. In particular, helix α28 on this side is located under the surface layer of the cap domain. The remaining helices are positioned at the other side of the central β-sheet in an arrangement that generates a crevice on the C-terminal end of the central β-sheet.

**Active Site of Obc1 C-domain**—The crevice on the Obc1 C-domain suggested the location for putative ObcB activity in the bifunctional enzyme Obc1. Moreover, a DALI-based structure similarity search indicated the presence of a catalytic triad (Ser-His-Asp) in the Obc1 crevice. Specifically, the search revealed that the most similar α/β hydrolase was pancreatic lipase-related protein 2 (PDB code 2PVS) (17); however, this protein only had a Z-score of 9.3 and 8% sequence identity. Due to low similarity in structure and sequence, these two α/β hydrolases exhibited limited structural conservation in the positions of the central β-strands, with large differences in the location of the flanking α-helices. Despite these differences, structural superposition of the Obc1 C-domain with pancreatic lipase-related protein 2 showed conservation of the catalytic triad (Ser-His-Asp) in both position and relative orientation. In Obc1, the putative catalytic triad consists of Ser-935, Asp-997, and His-1069 located in a loop region between β29 and α30, β31 and α32, and β33 and α36, respectively (Figs. 1D and 3A). Not surprisingly, these residues are clustered in a crevice in the C-domain, and their relative locations are conserved in other α/β hydrolases, consistent with members of this superfamily (18).

The Obc1 C-domain displays unique features in its active site, in addition to many structural features conserved in the α/β hydrolase family of proteins (16). First, a GXXG sequence motif (X: any residue) containing a nucleophilic serine residue was not found; instead, an AHSSG (S at position 935) sequence motif was observed in the Obc1 C-domain. Second, an HGGG motif, a tetrapeptide conserved in many α/β hydrolases for an oxyanion hole, was not found. In general, the HGGG motif is located in a part of the loop region that is near a nucleophilic serine residue. In this motif, the main chain nitrogen atoms from glycine stabilize the binding of a tetrahedral reaction intermediate by forming a hydrogen bond with negatively charged oxygen atoms in the intermediate. In this study structural superposition indicated that a proposed oxyanion hole conserved in the α/β hydrolase fold corresponded to a loop region including Ser-785–Thr-786–Pro-787 that connects β27 and α27 in the immediate vicinity of Ser-935 (Fig. 3A). In other α/β hydrolases, the HGGG motif has been found to be oriented in such a manner that the side chain of histidine points to the inner side of the loop, and the main chain nitrogen atoms of glycine are exposed to nucleophilic serine. However, the side chain hydroxyl group of Thr-786 in the Obc1 C-domain protrudes from the corresponding loop and is located 2.5 Å from Ser-936, a residue next to nucleophilic Ser-935, and 2.5 Å from a water molecule that also forms a hydrogen bond with Ser-935, a putative catalytic nucleophile (Fig. 3A). Given this structural feature and the presence of proline at amino acid number 787,
the main chain nitrogen atoms in the loop region are unavailable for hydrogen bonding, suggesting that this loop may not serve as an oxyanion hole in Obc1. Instead, positively charged Arg-856 and Arg-999 are in the vicinity of nucleophilic Ser-935. In particular, Arg-999 appears to be very dynamic based on its disordered electron density.
In our experimental conditions we were unable to detect the binding of a C6-CoA adduct in the Obc1 C-domain. Instead, we characterized glycerol bound to the Obc1 C-domain. The resulting electron density map suggested that the glycerol binding site is located at the proposed crevice surrounded by an α/β hydrolase fold and a cap domain (Fig. 3B). Specifically, glycerol was found at the entrance of the crevice, near the catalytic triad. In fact, glycerol is 3.4–4.0 Å from the side chains of His-934 and Ser-935. Based on the current binding environment, it is chemically sensible in glycerol that the C2 carbon and hydroxyl group on pro-S C1 is located near the side chain nitrogen of His-934 and that the hydroxyl groups at pro-R C3 positions are located near the main chain carbonyl oxygen of His-1069 (Fig. 3C).

**Functional Analysis of Obc1**—There is no direct structural evidence for the existence of a true substrate binding site, and functional roles of residues near the proposed active site have been elusive. Therefore, we selected 19 residues in the C-domain for site-directed mutagenesis to explore their functional roles. These residues form the putative catalytic triad (Ser-935, Asp-997, and His-1069), possible oxyanion binding sites (Ser-935, Asp-997, and His-1069), and putative oxyanion hole-forming residues (Arg-856 and Arg-999; green circle). In particular, His-934, a proposed catalytic residue for lyase activity, is marked with a gray star. This figure was prepared using ESPript (38).
785, Thr-786, Arg-856, Ser-936, and Arg-999), and the putative binding site for a C6-CoA adduct along the crevice and on the surface of the cap domain (Arg-601, Ser-609, Phe-610, Tyr-701, His-934, Asp-1061, Asp-1066, Asp-1067, Ser-1070, Arg-1072, and Arg-1073).

Two different assays were performed using various Obc1 mutants. Previously, we constructed Obc1* that has three mutations (H227A, Y326A, E350A) in the N-domain to eliminate any possible ObcA activity (10). All Obc1 mutants used for functional analysis in this study were engineered to have a mutation at a designated residue in the C-domain as well as three mutations in Obc1*. One assay was used to detect the formation of product CoA, and another assay was used to measure oxalate production. Preliminary experiments indicated that the maximal initial velocity of Obc1 could be achieved in the presence of ObcA, and under these conditions there were no differences in the measured velocity between wild-type Obc1 and Obc1* (Fig. 4A). Under our kinetic assay conditions, we also found that a C6-CoA adduct produced from ObcA was stable and could not be converted into CoA in the absence of Obc1 or Obc1* (Fig. 4A), validating that the formation of CoA from the adduct is enzyme-dependent (Fig. 1A).

Among the Obc1 mutants, the activities of three mutants (S785A, D1066A, R1072A) were essentially identical to that of Obc1*; therefore, these residues were not included in this figure. Putative oxyanion-binding site mutants provided interesting information.
TABLE 1
Kinetic parameters of Obc1* and its mutants
Numbers in parentheses refer to standard error (n = 3). Specifically, three independent purifications and measurements of Obc1* or mutant were performed.

| Enzyme     | $k_{cat}$ | $K_{m}$ | $k_{cat}/K_{m}$ |
|------------|-----------|---------|-----------------|
| Obc1*      | 13.07 (0.17) | 67.0 (8.5) | 1.95 $\times 10^{-1}$ |
| R856K      | 0.51 (0.03)  | 44.2 (3.8)  | 1.16 $\times 10^{-2}$ |
| R999K      | 2.05 (0.14)  | 67.1 (9.7)  | 3.06 $\times 10^{-2}$ |
| H934A      | 0.95 (0.10)  | 74.7 (9.2)  | 1.27 $\times 10^{-2}$ |

Discussion
Structure determination of Obc1, as well as in vitro functional analyses, unraveled details about Obc1. Specifically, we found that the Obc1 C-domain exhibits ObcB-like function. It is unusual that functionally identical proteins have a completely different amino acid sequence and number of residues (i.e., ~600 residues for the Obc1 C-domain versus 176 residues for ObcB). In addition, sequence analyses of ObcB using BetaWrap (19) and Phyre2 (20) suggested that ObcB has a $\beta$-helix structure, in which parallel $\beta$-strands are associated in a helical pattern. Therefore, the Obc1 C-domain and ObcB are dissimilar in all aspects of protein features including sequence, length, and structure. However, a lack of structural information on ObcB precludes further discussion about these unusual discrepancies.

Recent structural studies also revealed another example of a bifunctional enzyme. In Arabidopsis thaliana and the parasitic nematode Haemonchus contortus (21, 22), there is a phospho-base methylation pathway. In A. thaliana, a bifunctional enzyme with N- and C-terminal domains catalyzes the successive methylation of phosphoethanolamine to form phosphocholine. Interestingly, these reactions are catalyzed by two monofunctional enzymes in H. contortus, with each enzyme exhibiting identical catalytic activity to that of a corresponding domain of the A. thaliana bifunctional enzyme.

Based on the structural and biochemical data presented here, the following reaction mechanism for Obc1 is proposed (Fig. 5). With high identity both in sequence and structure to ObcA, the Obc1 N-domain should catalyze its first reaction by following the previously reported mechanism for ObcA (10). In the Obc1 N-domain, two different substrates, acetyl-CoA and oxaloacetate, are converted into a C6-CoA adduct (Fig. 1A). Tyr-326, a proposed key catalytic base, and other active site residues including metal-coordinating residues are conserved in the Obc1 N-domain (Fig. 2). Given that the C6-CoA adduct from the first reaction is not chemically labile and the liberation of CoA occurs in Obc1*-dependent catalysis (Fig. 4A), the second step should occur in the C-domain and involve the release of three products, namely, oxalate, acetoacetate, and CoA. This second enzyme reaction requires cleavage of two different bonds, the C2-C3 bond and the C4-S bond in the C6-CoA adduct (Fig. 1A). From a chemical structure perspective, C2-C3 bond cleavage accounts for oxalo production, and C4-S bond cleavage is responsible for the release of acetoacetate and CoA.

It is highly evident that within the C-domain crevice, the catalytic triad is responsible for C2-C3 bond cleavage in a manner identical to other serine hydrolases that cleave C-C bonds between trigonal and tetrahedral carbon atoms (18, 23). Specifically, given that C2 in the C6-CoA adduct is the only sp$^2$ carbon, Ser-935, upon activation by His-1069, performs nucleophilic attack on the C2 carbonyl carbon, forming a tetrahedral reaction intermediate (Fig. 5, steps I and II). The negative charge on the enolate oxygen atom of the tetrahedral intermediate could be stabilized by the presence of Arg-856 or Arg-999 (Fig. 4B and Table 1). Subsequent collapse of the tetrahedral
intermediate by His-1069 as a proton donor cleaves the C2-C3 bond and leaves an acyl moiety covalently attached to Obc1 (Fig. 5, step III). The resulting acyl-Obc1 complex is subsequently hydrolyzed by a water molecule, leading to the release of one molecule of oxalic acid (Fig. 5, steps IV and V). The two other products, acetoacetate and CoA, should be liberated from the remaining CoA adduct (Fig. 5, steps II and III).

Our analyses suggest a catalytic role for His-934 in the cleavage reaction of the C4-S bond, leading to the production of acetoacetate and CoA. First, the presence of a hydrogen-bonding network supports that His-934 acts as a catalytic base during an initial stage of catalysis and later as a catalytic acid (Fig. 5, steps II and III). Specifically, the ND1 atom (nitrogen near C/H9251) in the His-934 side chain likely forms a hydrogen bond with the main chain carbonyl oxygen of Phe-974 (Fig. 3, B and C), allowing the NE2 atom (nitrogen far from C/H9251) to exist in a deprotonated state for a catalytic base. Furthermore, the binding mode of glycerol could provide a clue to that of a C6 moiety in the C6-CoA adduct (Fig. 5, steps II and III). If we assume that the C6-CoA adduct, whose structure is based on a C4-CoA adduct previously identified (10), is bound to the active site of the Obc1 C-domain, then the C2 atom of the adduct should be placed toward the catalytic Ser-935 residue (Fig. 5, step I). This placement suggests that the pro-S C1 and C2 atoms in glycerol correspond to the C4 and S atoms in the C6-CoA adduct, respectively. Under these structural circumstances, His-934 is proximal to the hydroxyl group at C4 in the C6-CoA adduct. In fact, His-934 is within 3.6 Å from the hydroxyl group at the pro-S C1 and C2 carbons of glycerol (Fig. 3, B and C). Moreover, our kinetic analysis indicated a significant role for His-934 in catalysis, in particular $k_{cat}$ not $K_m$ (Table 1). Taken together, His-934 could accept a proton from the C4 hydroxyl group of the C6-CoA adduct and then donate a proton back to the sulfur atom of an intermediate (Fig. 5, steps II and III), cleaving the C4-S bond and producing acetoacetate and CoA.

However, it is unknown whether cleavage of the C2-C3 and C4-S bonds in the C6-CoA adduct are performed in a sequential or concerted manner. In this study we did not detect any mutant enzymes that liberated only one product, suggesting that these two bonds are likely cleaved in a concerted manner (Fig. 4B). Further kinetic experiments are required to resolve this issue.

The crystal structure of Obc1 in its apo form and a glycerol-bound form provides the first structural insights into the second step of oxalogenesis. This study provides insight into an unusual enzymatic feature, the dual catalytic role of the Obc1 C-domain. Specifically, the C-domain has hydrolase activity mediated by a well known catalytic triad and lyase activity.
mediated by a single histidine residue. Given that the overall structural features of the Obc1 C-domain are similar to that of α/β hydrolase containing a catalytic triad, this suggests that the presence of additional lyase activity is an example of divergent evolution. Consistent with this, oxalogenesis in *Burkholderia* species is indispensable for their survival in the stationary phase.

**Experimental Procedures**

**Cloning and Purification of Wild-type Obc1 and Its Mutants**—The gene for Obc1 from *B. thailandensis* (7, 11) was amplified by colony PCR and cloned into a modified pET28b expression vector (Merck) containing a tobacco etch virus protease recognition sequence between the His tag and multiple cloning site. The resulting plasmid containing N-terminal His-tagged Obc1 was transformed into *Escherichia coli* BL21 (DE3) cells (Novagen). After induction of protein expression with 0.5 mM isopropyl β-D-1-thiogalactopyranoside, *E. coli* cells were cultured for an additional 14–16 h at 20 °C. Cells were sonicated in Buffer A containing 50 mM HEPES (pH 7.0), 300 mM NaCl, and 5% (v/v) glycerol. N-terminal His-tagged Obc1 was purified using a HisTrap HP column (GE Healthcare) and eluted with Buffer A plus 250 mM imidazole. The N-terminal His tag was subsequently cleaved by treatment with 2 mM dithiothreitol and tobacco etch virus protease overnight at 22 °C using a 20:1 molar ratio of Obc1 to tobacco etch virus protein. The resulting tag-free Obc1 protein was further purified using a HisTrap HP column and subjected to size-exclusion chromatography using Superdex-200 (GE Healthcare) with Buffer A. The protein concentration was determined using an extinction coefficient of 129,150 M⁻¹ cm⁻¹ at 280 nm and calculated using the ProParam tool in ExPASy (24).

For the activity assay, Obc1* was constructed as described previously (10). Briefly, Obc1* exhibits ObcB activity but essentially lacks ObcA activity due to the introduction of three mutations (H227A, Y326A, E350A) into the active site of the N-terminal domain (Fig. 2). Genes for the various mutants used in the functional assay (Fig. 2) were amplified by colony PCR and used as templates and mutagenic sequences as primers. N-terminal His-tagged Obc1*, Obc1* mutants, and wild-type Obc1 as a template and mutagenic sequences as primers. Figure 2). Genes for the various mutants used in the functional assay (Fig. 2) were amplified by colony PCR and used as templates and mutagenic sequences as primers. N-terminal His-tagged Obc1*, Obc1* mutants, and wild-type Obc1 (10) were purified using a HisTrap HP column and then desalted using a HiPrep 26/10 column (GE Healthcare).

**Crystallization and Data Collection**—Crystallization was conducted at 22 °C using the sitting drop vapor diffusion method. Initial crystals were produced in a buffer containing 0.1 M HEPES (pH 7.0), 1 M sodium citrate tribasic, and 10 mg/ml protein. Later, we found that co-crystallization of Obc1 with 5 mM oxaloacetate and 2 mM acetyl-CoA led to the production of larger crystals with higher diffraction quality. However, the added substrates were not bound to the active site. Under these conditions, we obtained a crystal for the apo form of Obc1.

We also report the structure of Obc1 in complex with glycerol in its active site. Crystallization conditions for this complex were the same as those used for the apo form, except that a pre-grown crystal was soaked in the crystallization mother liquor plus 5 mM acetyl-CoA and 10 mM oxaloacetate. During protein purification, glycerol was a component of Buffer A; however, for unknown reasons, it was only bound to Obc1 when the crystal was soaked in the mother liquor-containing substrates.

X-ray diffraction data were collected at 100 K with a 0.5° oscillation angle on beamline 7A at the Pohang Accelerator Laboratory (Korea). Ethylene glycol (20% v/v) was used as the cryo-protectant during data collection, and the collected image files were processed using IMOSFLM (25). The space group of Obc1 crystals is R3₂2₁ with one monomer in the asymmetric unit (Table 2). In particular, a high resolution cut-off of each data set was based on a CC₁/₂ statistical value of ~−0.5. The CC₁/₂ statistical value is superior to the Rmerge or signal-to-noise ratio as a statistical guide for deciding the usefulness of data (26–28). In this study the CC₁/₂ value-based resolution cut-off indeed provided a higher-quality electron density map, compared with the map calculated using data based on the Rmerge value or signal-to-noise ratio.

**Structure Determination and Refinement**—The structure of apoObc1 was determined using the molecular replacement program MrBUMP (29, 30) with an apoObcA structure (10) (Protein Data Bank code 4NNA) using the N-terminal region of Obc1 as a search model. Unlike the N-terminal region, the quality of the electron density map for the remaining C-terminal region was relatively poor. The initial model was manually built and refined using the programs COOT (31), Buccaneer (30, 32), and PHENIX (33). Several cycles of model building and refine-
ment improved the quality of the map, in particular for the C-terminal region. In the final model, 1048 residues were located, except for some highly disordered residues (Table 2). As seen in the structure of ObcA (10), an electron density map for a metal ion was found in the active site of the apoObc1 N-terminal domain even though no metal ion was added during purification or crystallization. Subsequently, the refined structure of apoObc1 was used as a starting model for refining Obc1 in complex with glycerol. In particular, an electron density map corresponding to glycerol was identified in the putative active site of the Obc1 C-domain. Further refinement was carried out using PHENIX.

**Steady-state Kinetic Assay**—Obc1 enzymatic activity was measured in a manner similar to that for ObcA (10). In brief, two different assays were employed, each measuring the production of different products. First, a steady-state kinetic assay was performed in the presence of 2,6-dichlorophenolindophenol, which is reduced by the free sulfhydryl group of CoA, causing a linear decrease in absorbance at 600 nm (34–36). Second, total oxalate production was measured using an oxalate assay kit (Trinity Biotech) following the manufacturer’s protocol. In both assays, the reaction mixture contained Co2+ as the most effective ion for ObcA activity (10).

Using 2,6-dichlorophenolindophenol, a steady-state kinetic assay was performed at 30 °C in a reaction mixture containing 50 mM HEPES (pH 8.0), 100 mM NaCl, 120 μM 2,6-dichlorophenolindophenol, 100 μM CoCl2, and 7.8–500 μM acetyl-CoA and oxaloacetate. Then 250 nM ObcA was added to the reaction mixture and preincubated for 10 min at 30 °C to produce C6-CoA adducts that served as substrates for the Obc1 C-terminal domain. After preincubation, 100–2000 nM Obc1* or its mutant was added to the reaction mixture for adduct cleavage to free CoA, acetocacetate, and oxalate. A decrease in absorbance at 600 nm was measured using a UV-visible spectrophotometer (Jasco), and the initial velocity was determined from 70 to 130 s. The initial velocity was used to determine the CoA production per minute based on a standard reaction curve (10). K_m and V_max values were obtained by fitting initial velocity versus substrate concentration data to the Michaelis-Menten equation using SigmaPlot. In this analysis we assumed that the concentration of the C6-CoA adduct produced was equal to the concentration of the substrates acetyl-CoA and oxaloacetate. Unlike the kinetic assays, to determine the relative activity of the Obc1* mutants, the assays were performed using 500 μM substrate and 100 nM Obc1* or its mutants, and the initial velocity was determined.

Total oxalate production was measured at 30 °C using the reaction mixture for kinetic analysis in the presence of 500 μM acetyl-CoA and 500 μM oxaloacetate but without 2,6-dichlorophenolindophenol. In brief, 500 nM ObcA was added to the reaction mixture and incubated for 5 min. Cleavage of the C6-CoA adduct was initiated and incubated for another 5 min after the addition of 100 nM Obc1*. The resulting reaction mixture was mixed with an oxalate assay reagent, and the absorbance at 590 nm was measured after 5 min. The concentration of oxalate was calculated from a standard curve obtained using oxalic acid.

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