Crystal Structure and Computational Analyses Provide Insights into the Catalytic Mechanism of 2,4-Diacetylphloroglucinol Hydrolase PhlG from *Pseudomonas fluorescens*

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2,4-Diacetylphloroglucinol hydrolase PhlG from *Pseudomonas fluorescens* catalyzes hydrolytic carbon-carbon (C–C) bond cleavage of the antibiotic 2,4-diacetylphloroglucinol to form monoacetylphloroglucinol, a rare class of reactions in chemistry and biochemistry. To investigate the catalytic mechanism of this enzyme, we determined the three-dimensional structure of PhlG at 2.0 Å resolution using x-ray crystallography and MAD methods. The overall structure includes a small N-terminal domain mainly involved in dimerization and a C-terminal domain of Bet v1-like fold, which distinguishes PhlG from the classical α/β-fold hydrolases. A dumbbell-shaped substrate access tunnel was identified to connect a narrow interior amphiphilic pocket to the exterior solvent. The tunnel is likely to undergo a significant conformational change upon substrate binding to the active site. Structural analysis coupled with computational docking studies, site-directed mutagenesis, and enzyme activity analysis revealed that cleavage of the 2,4-diacetylphloroglucinol C–C bond proceeds via nucleophilic attack by a water molecule, which is coordinated by a zinc ion. In addition, residues Tyr121, Tyr229, and Asn132, which are predicted to be hydrogen-bonded to the hydroxyl groups and unhydroylded acetyl group, can finely tune and position the bound substrate in a reactive orientation. Taken together, these results revealed the active sites and zinc-dependent hydrolytic mechanism of PhlG and explained its substrate specificity as well.

The root-colonizing fluorescent *Pseudomonas* species produce a variety of extracellular metabolites with antimicrobial activity and thus are widely utilized as effective biocontrol agents against the plant root diseases caused by soil-borne pathogenic fungi (1, 2). Among these metabolites, the phenolic compound 2,4-diacetylphloroglucinol (DAPG) displays a remarkably broad spectrum of toxic activity toward bacteria, fungi, nematodes, and even plants (3–5). The *Pseudomonas* DAPG biosynthetic cluster includes four genes, termed *phlABCD*, which are transcribed as a single operon (6). Immediately downstream to this operon, the *phlE* gene encodes a putative transmembrane permease that has been implicated in DAPG resistance (7). Adjacent to *phlA*, the genes *phlF* and *phlH* encode two divergently transcribed tetracycline resistance repressor-like regulators involved in DAPG biosynthesis (8–10). In addition to these pathway-specific regulators, a gene located between *phlF* and *phlH*, termed *phlG*, encodes a DAPG hydrolase, which may offer an effective alternative for modulating DAPG levels (11).

The protein encoded by *phlG* was found to catalyze the conversion of DAPG into less toxic monoacetylphloroglucinol (MAPG) by cleaving one of the carbon-carbon (C–C) bonds linking the acetyl groups to the phenolic ring (Fig. 1). This enzyme showed strict substrate specificity as it cannot degrade other compounds of similar structure such as MAPG and triacyltolphloroglucinol (11). The amino acid sequence of PhlG displays 25–37% sequence identity to several hypothetical proteins and the phloretin hydrolase (EC 3.7.1.4) from *Euobacterium ramulus*. Phloretin hydrolase is a known C–C bond hydrolase that specifically converts phloretin to phloroglucinol and 3-(4-hydroxyphenyl) propionic acid (12). However, neither PhlG nor phloretin hydrolase possesses sequence homology to other C–C bond-cleaving hydrolases or motifs typical of the α/β hydrolase family (13), suggesting these two enzymes belong to a distinct hydrolase family.

In contrast to common biochemical hydrolysis of amide and ester bonds, hydrolytic cleavage of C=C bonds is relatively rare in nature (14). To date, only 10 enzymes with this primary function have been described in the KEGG data base (EC 3.7.1.1 to EC 3.7.1.10). Nonetheless, these enzymes are generally found to be involved in the degradation pathway of various hydroxylated aromatic compounds. Recently, the hydrolytic enzymes acting on the C=C bonds in β-diketones were characterized in detail.
and showed surprising structural and mechanistic diversity (14, 15). For instance, fumarylacetoacetate hydrolase, an enzyme that cleaves fumarylacetoacetate to fumarate and acetoacetate, is a metalloenzyme characterized by a novel α/β-fold and uses a His/Asp dyad-activated water molecule as the attacking nucleophile. The bound metal ion, identified to be Ca$^{2+}$, functions both in stabilizing a carbamion leaving group and indirectly positioning the water molecule for nucleophilic attack at a carbonyl group (16). Another β-diketone hydrolase, 2,6-dihydroxy-pseudo-oxynicotine hydrolase, which is involved in the nicotine degradation pathway, belongs to the classical α/β hydrolase family and possesses a catalytic Ser/His/Asp triad for the hydrolytic reaction (17). It was proposed that this diversity possibly reflects the necessity of nature to recruit different protein sequences, structures, and mechanisms to cleave C–C bonds of different susceptibility, for the purpose of detoxification and catabolic processes (15).

In this study, we describe the 2.0-Å resolution crystal structure of PhlG, solved with the MAD method. Moreover, based on structural analysis, computational docking studies, site-directed mutagenesis, and enzyme activity analysis, we propose a catalytic mechanism of the PhlG enzyme and provide a structural interpretation of its strict substrate specificity. To the best of our knowledge, PhlG is the first C–C bond-cleaving hydrolase with a Bet v1-like fold, divergent from other known C–C bond-cleaving enzymes in the classical α/β-fold hydrolase family.

**EXPERIMENTAL PROCEDURES**

Construction, Expression, and Purification of PhlG and Its Mutants—The phlG gene was amplified from purified genomic DNA extracted from *Pseudomonas fluorescens* PT-5 using a Wizard Genomic DNA purification kit (Promega, Madison, WI). Genes encoding PhlG and its mutants were individually cloned into a pET28a-derived vector and overexpressed in *Escherichia coli* Rosetta (DE3) strain (Novagen, Madison, WI) using 2× YT culture medium (5 g of NaCl, 16 g of Bacto-tryptone, and 10 g of yeast extract/liter). A hexahistidine (His$_6$) tag was added to the N terminus of each of the recombinant proteins. The PhlG-expressing cells were grown to an $A_{600}$ of 0.6 at 37 °C. Expression of the recombinant proteins was induced at the exponential phase with 0.2 mM isopropyl-β-d-thiogalactoside, and cell growth was continued for another 20 h at 16 °C before harvesting. Cells were collected by centrifugation at 4,000 × g for 20 min and resuspended in lysis buffer (20 mM Tris-HCl, 100 mM NaCl, pH 8.0). After 3 min of sonication and centrifugation at 12,000 × g for 25 min, the supernatant containing the soluble target protein was collected and loaded onto a nickel-nitrilotriacetic acid column (Qiagen, Valencia, CA) equilibrated with the binding buffer (20 mM Tris-HCl, 100 mM NaCl, pH 8.0). The target protein was eluted with 250 mM imidazole and further loaded onto a Superdex 75 column (GE Healthcare) equilibrated with 20 mM Tris-HCl, 100 mM NaCl, pH 8.0. Fractions containing the target protein were combined and concentrated to 20 mg/ml for crystallization. Samples for enzymatic activity assays were collected at low concentrations (0.5 mg/ml). The purity of protein was evaluated by SDS-PAGE (data not shown), and the protein sample was stored at −80 °C.

The selenium-Met (SeMet)-labeled PhlG protein was expressed in *E. coli* strain B834 (DE3) (Novagen, Madison, WI). A culture of transformed cells was inoculated into LB medium and incubated at 37 °C overnight. The cells were harvested when the $A_{600}$ reached 0.2 and were then washed twice in the M9 medium. The cells were then cultured in SeMet medium (M9 medium with 25 mg/liter l-SeMet and the other essential amino acids at 50 mg/liter) to an $A_{600}$ of 0.6–0.8. The remaining steps in protein purification were the same as those for the native protein.

Crystalization, Data Collection, and Processing—Crystals of native PhlG were grown at 16 °C using the hanging drop vapor-diffusion technique, with the initial condition of mixing 1 μl of 20 mg/ml protein sample with an equal volume of the reservoir solution (20% polyethylene glycol 4,000, 17% isopropyl alcohol, 0.1 M sodium citrate, pH 5.6). Typically, crystals appeared in 1 or 2 days and reached the maximum size in 1 week. The SeMet derivative crystals were grown under the same conditions. The crystals were transferred to cryoprotectant (reservoir solution supplemented with 25% glycerol) and flash-frozen with liquid nitrogen. Multiwavelength anomalous dispersion data for a SeMet derivative crystal was collected at radiation wavelengths of 0.9794, 0.9799, and 0.9252 Å at Beijing Synchrotron Radiation Facility, Institute of High Energy Physics, Chinese Academy of Sciences, using beamline 3W1A at 100 K with a MAR 165-mm CCD (MARResearch, Germany). All diffraction data were indexed, integrated, and scaled with HKL2000 (18).

Structure Determination and Refinement—The crystal structure of PhlG was determined using the MAD phasing method from a SeMet-substituted protein crystal to a maximum resolution of 2.0 Å. The SHELX program suite (19) was used to locate the heavy atoms and 22 selenium atoms were identified. The phase was calculated and further improved with the program SOLVE/RESOLVE (20). Electron density maps calculated from solvent-flattened experimental phases showed clear features of secondary structural elements, allowing automatic model building of most residues with the ARP/wARP program (21). The initial model was refined by using the maximum likelihood method implemented in REFMAC5 as part of the CCP4i program suite (22) and rebuilt interactively by using the $σ_A$-weighted electron density maps with coefficients $2F_o - F_c$ and $F_o - F_c$ in the program COOT (23). Refinement converged to an R-factor of 19.1% and R-free of 21.9% at the resolution of 2.0 Å. The final model was evaluated with the programs MOLPROBITY (24) and PROCHECK (25). The final coordinates and structure factors were deposited in the Protein Data Bank under the accession code of 3HWP. The data collection and structure refinement statistics are listed in Table 1. Sequence alignment was performed using the programs ClustalW (26) and ESPript (27). Structure comparison was...
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TABLE 1

Data collection, phasing, and refinement

|                           | MAD peak | MAD edge | MAD remote |
|---------------------------|----------|----------|------------|
| Data collection statistics|          |          |            |
| Wavelength                | 0.9794   | 0.9799   | 0.9252     |
| Space group               | C222     | C222     | C222       |
| Unit cell (Å)             | a = 127.71 Å, b = 132.50 Å, c = 95.02 Å, α = β = γ = 90 Å | a = 124.75 Å, b = 132.58 Å, c = 95.03 Å, α = β = γ = 90 Å | a = 124.75 Å, b = 132.58 Å, c = 95.03 Å, α = β = γ = 90 Å |
| Resolution limit          | 50.00 to 2.00 (2.07 to 2.00) | 50.00 to 2.00 (2.07 to 2.00) | 30.00 to 2.00 (2.07 to 2.00) |
| Uniqueness reflections    | 96,750 (6,164) | 95,198 (5,516) | 101,065 (9,051) |
| Completeness              | 95.3% (66.0) | 94.3% (60.7) | 99.1% (93.4) |
| Rmerge$^*$                | 12.7% (39.9) | 11.5% (39.0) | 10.3% (44.7) |
| Rwork$^*$                 | 14.5% (2.6) | 16.0% (2.6) | 18.1% (2.3) |
| Redundancy                | 4.5 | 4.2 | 5 |
| MAD phasing               | Heavy atom sites | 22 | 22 | 22 |
|                           | Mean figure of merit after solve | 0.3 | 0.3 | 0.61 |
|                           | Mean figure of merit after resolve | 0.3 | 0.3 | 0.61 |
| Refinement statistics     |          |          |            |
| Resolution limit          | 26.00 to 2.00 | 19.1% | 21.9% |
| R-factor$^*$              |          |          | 0.008 Å   |
| R-free$^d$                |          |          | 1.051 Å   |
| r.m.s.d. bond length$^*$  |          |          | 22.94 Å² |
| r.m.s.d. bond angles      |          |          | 22.94 Å² |
| Average of R-factors      |          |          | 22.94 Å² |
| Ramachandran plot$^f$     | Most favored | 97.6% | 2.4% | 3HWP |
|                           | Additional allowed | 97.6% | 2.4% | 3HWP |
|                           | PDB entry | 3HWP | 3HWP | 3HWP |

$^*$ The values in parentheses refer to statistics in the highest bin.
$^*$ Rmerge $= \Sigma_i$$f_{o,i}$$ - f_{c,i}$/$\Sigma_i$$f_{c,i}$, where $f_{o,i}$ and $f_{c,i}$ are the observed and calculated structure-factor amplitudes, respectively.
$^d$ R-free was calculated with 2% of the data excluded from the refinement.

Site-directed Mutagenesis—Site-directed mutagenesis was performed by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the plasmid encoding the wild-type PhlG as the template. The mutant proteins were expressed, purified, and stored in the same manner as the wild-type protein.

Enzymatic Activity Assay—The enzyme kinetic parameters of native PhlG and its mutants were measured as described in Ref. 11 with minor changes. All the assays were performed at 25 °C in 100 mM NaH2PO4/Na2HPO4 buffer, pH 7.1. The substrate DAPG (Toronto Research Chemicals Inc., North York, Canada) was diluted to a series of concentrations from 100 mM stock solution in methanol. The reaction was triggered by adding the purified protein solution, and the reaction was terminated by mixing with an equal volume of methanol/H2O/acetic acid (50:45:5 volume ratio). The mixture was centrifuged at 12,000 × g for 10 min, and the supernatant was subjected to high performance liquid chromatography (HPLC) analysis. DAPG and MAPG were quantified by HPLC analysis. Acetonitrile and water (both containing 0.1% trifluoroacetic acid) served as the mobile phase. Five percent acetonitrile was used to calibrate the Eclipse XDS-C18 column, 4.6 × 150 mm (Agilent). The gradient was formed as follows: from 0 to 40% acetonitrile in 3 min and then from 40 to 100% acetonitrile in 3 min, at a flow rate of 1.5 ml/min. The initial velocities and substrate concentrations were used to linearly fit the Lineweaver-Burk plot to calculate the $K_m$ and $k_{cat}$ values. Three independent kinetic determinations were made to calculate means ± S.D. for the reported $K_m$ and $k_{cat}$ values.
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Tryptic peptides were separated on line by a fused silica capillary column packed with C18 resin (5 μm, 300 Å, Jupiter, Phenomenex, Torrance, CA) using a linear gradient of 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B) (2–25% acetonitrile for 35 min and 25–90% acetonitrile for 15 min). MS-MS spectra were acquired by a multiple reaction monitoring (MRM) method, in which the m/z corresponding to the cysteine-containing tryptic peptides was targeted for MS-MS. A window of ±1.0 m/z was used for precursor isolation.

**Metal Content Determination of PhlG**—To determine the metal content, 400 μl of PhlG wild-type enzyme was transferred to a 50-ml beaker, digested at 90 °C for 2 h with 1 ml of concentrated HNO₃, and 0.1 ml of 30% H₂O₂, and diluted to final volume of 10 ml with deionized water. 400 μl of the buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl) was also treated with the same procedure and used as the control. The metal contents of the samples were analyzed by inductively coupled plasma-mass spectrometry (Thermo Electron Corp.) at the University of Science and Technology of China.

**Analysis of Substrate Entrance**—The on-line version of CAVER was used to explore the putative substrate access tunnel of PhlG. The position of the bound metal ion in the interior pocket was specified to identify tunnels directly connecting the active site to the surface. The tunnel profile, which is the average tunnel cross-section radius along the length, was calculated from the detected accessible path.

**RESULTS**

**Overall Structure**—The PhlG crystal contains two identical subunits, termed A and B, in an asymmetric unit. Subunits A and B are structurally similar, with an overall root mean square deviation (r.m.s.d.) of ~0.5 Å over the 287 Ca atoms. The two subunits associate to form a tight dimer related by a noncrystallographic 2-fold axis (Fig. 2A), which was confirmed in solution by the gel filtration method (data not shown). The dimerization is mainly formed by the small N-terminal domain (Arg²–Gly⁸²), which is composed of a two-stranded anti-parallel β-sheet, three α-helices (α1–α3), and three 3₁₀ helices (η1–η3). Nineteen pairs of hydrogen bonds were involved in the dimerization, most of which come from residues of the N-terminal domain such as Thr¹⁰, Tyr¹¹, Phe¹², Lys¹⁸, Glu³⁹, Arg⁴⁸, Ile⁶⁰, Gln⁵¹, Gly⁵³, and Tyr⁸³. In contrast, only three residues from the C-terminal helix α₁₀, Glu²⁶¹, Tyr²⁶⁶, and Glu²⁶⁶, contribute to hydrogen bonding of the dimer interface. The total buried area of the dimer interface is up to ~2500 Å², indicating a stable dimeric PhlG structure (Fig. 2A).

Besides the small N-terminal domain involved in dimerization, PhlG possesses a large C-terminal catalytic domain (Tyr⁸³–Thr²⁹¹) showing the so-called helix-grip fold (34), which is characterized by a partly opened β-barrel (β3–81) wrapped around a long C-terminal helix α₁⁰ (Fig. 3A). This partly opened β-barrel is sealed by helices α₄ and α₅ on one end and helix α₉ on the other end, leading to the formation of a deep narrow pocket in the center of the protein. Structure-based alignment using the DALI server revealed that the C-terminal catalytic domain closely resembles the tetracenomycin aromatase/cyclase (PDB code 2REZ, r.m.s.d. 2.7 Å), cytokinin-specific binding protein (PDB code 3COV, r.m.s.d. 2.9 Å), and cholesterol-regulated start protein 4 (PDB code 1JSS, r.m.s.d. 3.4 Å), despite their low sequence homology (less than 9% of sequence identity). According to the structural classification of proteins (SCOP) data base, all these structures belong to the Bet v1-like superfamily (SCOP: d.129.3), all members of which share the prototypic structure of a seven-stranded β-barrel (topology 1765432) wrapped around a long C-terminal α-helix with the secondary structural arrangement of β-αββ-αββ-α (35). Conforming to the prototype of Bet v1-like fold, the PhlG C-terminal catalytic domain contains an additional β-strand (β3) adding to the β-barrel on the N-terminal β-strand (topology 12876543) and several internal insertions of α-helices (helices α₆ and α₇ between strand β₅ and β₆, α₈ between β₇ and β₈, and α₉ between β₉ and α₁₀) (Fig. 3B). Because no significant sequence homology is detected between PhlG and known members of Bet v1-like superfamily, PhlG defines a new sequence family of the Bet v1-like superfamily.

Additionally, based on structural and sequence similarities, the Bet v1-like superfamily is further classified into 13 families, including three families of enzymes (polyketide cyclases, ring hydroxylase α-chain, and homotrimeric ring hydroxylase), three families of lipid transfer/ligand-binding proteins (plant pathogen-related proteins Bet v1, phosphatidylinositol transfer proteins, and steroidogenic acute regulatory protein-related lipid transfer domains), and seven families of unclear biochemical profile (35). Because no members of these families are reported to exhibit hydrolytic activity, PhlG is therefore the first discovered hydrolase whose catalytic domain belongs to the Bet v1-like fold.

**Active Site, Substrate Docking Simulation, and Mutational Analysis**—The most important characteristic of Bet v1-like proteins is the presence of an interior hydrophobic/amphipathic pocket accessible to the exterior. As mentioned above, this pocket is also present in the C-terminal domain of PhlG, and is ~20 Å deep and 480 Å³ large. The fine distribution of hydrophobic and polar residues in the pocket creates an ideal environment for accommodating both hydrophobic and polar surfaces of the DAPG molecule. Interestingly, a metal ion was identified inside the pocket, coordinated by His¹²⁹(β5), Glu¹⁶⁰(β6), His¹⁷⁰(α10), Glu²⁷⁴(α10), and a water molecule (Wat1) with pentagonal bipyramidal coordination geometry. It may play an important structural role in stabilizing the PhlG catalytic domain by holding the strands β₅ and β₆ and the helix α₁₀ together. By having a high occupancy as indicated by the strong electron density peak, this metal ion is present in the structure despite the presence of metal ion chelators (0.1 M sodium citrate) in the crystallization buffer. It interacts with the N-terminus of His¹²⁹ (2.15 Å) and His¹⁷⁰ (2.16 Å), the O atoms of Glu¹⁶⁰ (2.18 Å) and Glu²⁷⁴ (2.13 Å), and the water molecule Wat1 (2.31 Å) with pentagonal bipyramidal configuration, where the two Glu residues occupy the axial positions (Fig. 4A). Because the electron density in this metal-binding site is so strong that even Ca²⁺ cannot be well fitted into electron density.
assuming the occupancy equals 1, we speculate the bound metal ions should have more electrons than Ca$^{2+}$ and are most likely to be transition metal ions, as is also supported by the anomalous difference signal at the wavelength of 0.9250 Å (Fig. 4A). By using the inductively coupled plasma-mass spectrometry method, we further determined that the molar ratio of Zn$^{2+}$...
to the protein is 1.15:1, which strongly suggests that the Zn²⁺ ion is the cognate metal ion of PhIG.

Besides a structural role, this metal ion may also be essential for catalytic activity because most hydrolytic enzymes such as metalloprotease and phosphatase utilize zinc ions to either stabilize the transition intermediate or activate a water molecule as a nucleophile for catalysis (36–38). To confirm the catalytic role of the zinc ion, three metal ligand residues, namely H270A, E274A, and E160A, were constructed, and enzyme kinetics were measured using an HPLC-based assay (Table 2). Because H270A has no detectable activities, further kinetic characterization of this enzyme was not attempted. All these mutants showed at least by 10⁻⁵-fold reduced k_cat values compared with the k_cat value of the wild-type enzyme, indicating that the

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**FIGURE 3. Structure and topology of PhIG monomer.** A, schematic representation of PhIG monomer, colored and labeled according to secondary structural elements. The metal ion is denoted as a gray sphere. B, topology diagram of the C-terminal Bet v 1-like domain of PhIG. Compared with the prototypic Bet v 1-like fold, the PhIG C-terminal domain has an additional β-strand (β3) in the β-barrel and several insertions of helices (helices a6, α7, a8, and a9).

**FIGURE 4. Active site, substrate docking model, and mutational analysis of PhIG.** A, anomalous difference Fourier map confirms the zinc ion. The anomalous difference map density of the metal is comparable with that of selenium atoms of SeMet residues (Mse, SeMet). The anomalous difference Fourier map calculated with data collected at the wavelength of 0.9250 Å is shown in pink contoured at 5σ. At this wavelength, zinc has a modest anomalous signal (f = 2.24), whereas magnesium (f = 0.06) and calcium (f = 0.52) do not. The 2F_o – F_c-weighted map is shown in gray and contoured at 1.5σ. B, model of DAPG intermediate bound to the active site of PhIG. The docked DAPG intermediate is shown in ball and stick format, and the residues within 4 Å of DAPG are shown in stick format. The distance between the hydroxyl group of the acetyl group and the zinc ion is 2.2 Å. The water molecule coordinated to the zinc ion may serve as the attacking nucleophile because its distance to the carbon atom of the carbonyl group is 2.3 Å. Hydrogen bonds between the active site and docked DAPG are also shown in black dashes and the distances are labeled.
Putative Substrate Access Tunnel and Its Implication in Glutathionylation-induced Inhibition of PhIG Activity—Because the catalytic zinc ion is buried more than 15 Å from the protein surface, there should be a direct path connecting the catalytic site with the exterior solvent. A putative substrate tunnel starting from the bound zinc ion to the exposed protein surface was identified by using the on-line version of the CAVER program. The tunnel is dumbbell-shaped, with a small opening formed between helix α4 and the loop linking strand β5 and helix α6 (Fig. 5A). This small opening is only about 2 Å wide, which is not wide enough for the substrate DAPG to pass through, suggesting that the residues around this opening should undergo conformational changes to allow the substrate access to the active site during catalysis. The residues participating in guarding this opening include His114 and Pro115 from helix α4 and Asn115, Pro133, and Cys134 from the loop linking strand β5 and helix α6 (Fig. 5B). In the CRET SMART domain, incorporation of the ligand ceramide is also limited by a small opening, which is guarded by two helices (one corresponds to the helix α4 in PhIG) and a loop (39). In the phosphatidylinositol transfer proteins, there is an additional helix (corresponding to the helix α4 in PhIG) adjacent to the C-terminal helix, which can undergo large conformational changes between a closed, ligand-bound structure and an open, membrane-bound structure, thus enabling the binding and release of large phospholipid molecules (40). All these examples suggest that members of the Bet v1-like superfamily regulate the entry/release of ligand/substrate by controlling an opening of limited size to the interior pocket.

Of the four cysteine residues in one PhIG monomer, Cys134 and Cys157 are buried and far away from the active site. The other two cysteines, namely Cys134 and Cys157, are around the opening of the calculated tunnel. Thus, to confirm the significance of the tunnel opening calculated by CAVER, we attempted to modify Cys134 and Cys157 with excessive GSSG to sterically block the opening of this substrate access tunnel. Both of these two residues were detected to form mixed disulfide bonds with GSH using liquid chromatography-mass spectrometry (data not shown). As shown in Fig. 3C, the GSSG-modified PhIG showed significantly abolished activity, which is in agreement with our inference from the calculated substrate access tunnel. However, we do not have available structural evidence at present to rule out the possibility that the conformation of the finely arranged active site was not also altered upon the GSSG treatment.
DISCUSSION

PhlG Defines a Novel Family of C–C Bond-cleaving Hydrolase—
We showed that the overall structure of a C–C bond hydrolase, PhlG, uses the Bet v1-like fold as the catalytic domain, which is distinct from the classical H9251/H9252-fold hydrolases. To the best of our knowledge, this is the first identified hydrolytic enzyme with the Bet v1-like fold.

The penta-coordinated catalytic zinc ion bound in the pocket of PhlG is a unique feature among the proteins with the Bet v1-like fold and other C–C bond hydrolases. For those Bet v1-like fold proteins such as plant PR-10, human START5, and tetracenomycin aromatase/cyclase, although one or more metal-binding sites are located in the conserved internal pocket, none of these bound metal ions play catalytic roles or have the similar coordination geometry to the zinc ion found in PhlG. Rather, the reported C–C bond hydrolases either hexa-coordinated Ca2+ to stabilize the reaction intermediates or use the strictly conserved triad (His, Asp, and Ser) of α/β fold for catalysis. Using the protein metal site data base MESPEUS, we searched for the hydrolases containing catalytic metal ions with similar coordination residues and geometry as the zinc ion of PhlG. The phosphodiesterase was identified to coordinate a Zn2+ with two histidines, two aspartates, and a solvent molecule in pentagonal bipyramidal configuration, quite similar to PhlG (36, 41). Because it has been widely accepted that phosphodiesterases as well as other Zn2+-dependent hydrolases, such as alkaline phosphatase (42) and carboxypeptidase A (43), possess a zinc-bound water or hydroxyl ion to execute the nucleophilic attack, we hypothesized that this metal ion bound in PhlG may play an essential role in catalysis and consequently performed the computational docking studies.

Structural Basis of Strict Substrate Specificity—The main feature of the Bet v1-like fold is a deep interior pocket formed between a seven- or eight-stranded antiparallel β-sheet and a long C-terminal α-helix, yet the volume of this pocket shows remarkable variability in different members of this superfamily. For instance, a plant pathogenesis-related protein of class 10 (PR-10) (PDB code 2QIM) that binds plant hormones such as zeatin and cytokinin has an internal pocket of 4500 Å3 in volume (44), START5 (the human star-related lipid transfer protein 5 (PDB code 2R55)) that binds cholesterol or other sterols has a pocket of 800 Å3, and tetracenomycin aromatase/cyclase (PDB code 2REZ) that catalyzes the cyclization of polyketide has a pocket of 600 Å3 (45). In contrast, PhlG has a much smaller pocket of less than 500 Å3 in volume, which can barely accommodate a DAPG molecule but not a triacetylphloroglucinol molecule or other larger analogs of DAPG, providing a structural interpretation of the strict substrate specificity of the enzyme.

Based on the results from computational docking, site-directed mutagenesis, and enzymatic assays, we propose that cleavage of the C–C bond in DAPG proceeds via nucleophilic attack at the carbonyl group of one of the two acetyl groups by a water molecule coordinated by a zinc ion. This zinc ion can effectively activate the coordinated water molecule by dramatically lowering its pKa. The two zinc ion ligands Glu160 and Glu270, both of which are hydrogen-bonded to the coordinated water, can be great candidates for conveying the released proton to the keto-O of the departing acetyl group. Aromatic residues such as Phe111, Trp224, Phe173, and Phe267 can be essential in shaping the substrate binding pocket and positioning the phenyl group of DAPG via aromatic π-π stacking interactions and hydrophobic interactions. Residues Tyr121, Tyr229, and Asn132, which are predicted to be hydrogen-bonded to the hydroxyl groups and unhydrolyzed acetyl group, can...
finely tune and position the bound substrate in a reactive orientation. It is noteworthy that Asn\textsuperscript{132} is not preserved in phlorotelin hydrolase (Fig. 2B), which is consistent with the fact the phlorotelin molecule does not have an acetyl group (12). Although multiple sequence alignment of distantly related PhlG homologs reveals that the His\textsuperscript{214} is strictly conserved and the docking simulation also suggests a catalytic/substrate binding role for His\textsuperscript{214}, site mutagenesis analysis indicates that this residue does not play a key role in the catalysis of the enzyme.

**Structural Implication of the Substrate Entry**—The entrance to the active site appears to be rather restricted in the PhlG structure, as indicated by the dumbbell-shaped substrate access tunnel. A small opening to the active site is defined by the finely arranged His\textsuperscript{114} and Pro\textsuperscript{115} on one side and Asn\textsuperscript{132}, Pro\textsuperscript{133}, and Cys\textsuperscript{134} on the other side, separated by distances of \(-2\) Å when hydrogen atoms are added to the protein structure (Fig. 5B). Considering the size of DAPG and MAPG, this opening is not large enough to allow substrate entry and product release. Therefore, conformational changes near the opening must happen in order for the substrate to access the active site. Because proline residues usually have exceptional rigidity compared with other amino acids due to their locked backbone dihedral angle at approximately \(-75^\circ\) (46), it is more likely residues His\textsuperscript{114} and Asn\textsuperscript{132} serve as tunnel gatekeepers that control the substrate entry through conformational changes of their side chains. It is also possible that the substrate can induce conformational changes near the opening of the substrate access tunnel. However, because DAPG and MAPG are molecules of relatively small sizes, we speculate that the side-chain conformational changes of His\textsuperscript{114} and Asn\textsuperscript{132} are sufficient for substrate entry and product release.

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