Crystal Structure and Desulfurization Mechanism of 2’-Hydroxybiphenyl-2-sulfinic Acid Desulfinase*

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The desulfurization of dibenzothiophene in Rhodococcus erythropolis is catalyzed by two monooxygenases, DszA and DszC, and a desulfinase, DszB. In the last step of this pathway, DszB hydrolyzes 2’-hydroxybiphenyl-2-sulfonic acid into 2-hydroxybiphenyl and sulfite. We report on the crystal structures of DszB and an inactive mutant of DszB in complex with substrates at resolutions of 1.8 Å or better. The overall fold of DszB is similar to those of periplasmic substrate-binding proteins. In the substrate complexes, biphenyl rings of substrates are recognized by extensive hydrophobic interactions with the active site residues. Binding of substrates accompanies structural changes of the active site loops and recruits His60 to the active site. The sulfinate group of bound substrates forms hydrogen bonds with side chains of Ser27, His60, Arg70, and Cys27, each of which is shown by site-directed mutagenesis to be essential for the activity. In our proposed reaction mechanism, Cys27 functions as a nucleophile and seems to be activated by the sulfinate group of substrates, whereas His60 and Arg70 orient the syn orbital of sulfinate oxygen to the sulphydryl hydrogen of Cys27 and stabilize the negatively charged reaction intermediate. Cys, His, and Arg residues are conserved in putative proteins homologous to DszB, which are presumed to constitute a new family of desulfinases.

Sulfur oxides released into the atmosphere by combustion of fossil fuel cause serious air pollution, which leads to acid rain and destroys forests and soils. Sulfur oxides also raise public health concerns associated with cardiopulmonary diseases (1). To alleviate these problems, efforts are being made to limit the sulfur content in diesel and gasoline fuel. Inorganic or nonaromatic organic sulfur compounds can easily be removed from fossil fuels, and degradation of DBT by microbial activities has been studied with a keen interest in biodesulfurization (4, 5).

Besides biodesulfurization, knowledge of sulfur metabolism by soil bacteria is particularly interesting because of their competition for sulfur nutrient with plants. Although biochemical studies have shown that DszB is a unique desulfinase, its detailed molecular reaction mechanism remains largely unknown. In an attempt to expand our understanding of bacterial desulfurization in molecular detail, we determined crystal structures of DszB in complex with substrates HBPS and BPS.

EXPERIMENTAL PROCEDURES

Materials—BPS was synthesized as reported previously (12). Sodium salt of HBPS was a gift from Petroleum Energy Center, Shimizu, Japan. Reagents for crystallization buffer were purchased from Hampton Research. Other chemicals used were of the finest grade commercially available.

Crystallization and Diffraction Data Collection—Protein purification and crystallization were performed as described

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The atomic coordinates and structure factors (code 2DE2, 2DE3, and 2DE4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡ The abbreviations used are: DBT, dibenzothiophene; HBP, 2-hydroxybiphenyl; HBPS, 2’-hydroxybiphenyl-2-sulfonic acid; BPS, biphenyl-2-sulfonic acid; PDB, Protein Data Bank; r.m.s.d., root mean square deviation.
previously (11, 13). For phasing, native crystals of DszB were soaked in 10 mM KAu(CN)₂ for 2 days before the diffraction data collection. Single-wavelength anomalous diffraction data set was collected on beamline BL-6A at the Photon Factory (Tsukuba, Japan), at the wavelength of 0.978 Å. Diffraction images were integrated and scaled using MOSFLM and SCALA programs from the CCP4 program suite (14). Native data sets of DszB and substrate complexes were collected on beamlines BL-18B and BL-6A at the Photon Factory, respectively (Table 1).

**Structure Determination, Model Building, and Refinement—**

The x-ray crystal structure of DszB was determined by the single wavelength anomalous dispersion method (Table 1). The single gold site was located, and the program CNS was used to refine the heavy atom parameters (15). After phase improvement by density modification, the single wavelength data produced an interpretable electron density map. The programs ARP/wARP and XtalView were used to build the model (16, 17). The structures of DszB-HBPS and DszB-BPS were solved by the molecular replacement method using the program MolRep (18). Topology files of ligands were prepared using PRODRG server, and models were refined by the program CNS (15, 19) (Table 1). The stereochemistry of the final models was assessed with the program PROCHECK (20). The coordinates and structure factors of DszB, DszB-HBPS, and DszB-BPS structures have been deposited in the Protein Data Bank (PDB) as 2DE2, 2DE3, and 2DE4, respectively. Secondary structures were designated with the program DSSP (21). Structure figures were prepared with the programs Molscript and Raster3D (22, 23).

**Site-directed Mutagenesis—** Site-directed mutagenesis study was conducted as described previously (11). pKK223-3 expression vectors (Amersham Biosciences) coding for wild-type DszB or mutants were transformed into *Escherichia coli* BL21 strain harboring pKY206 plasmid (BL21/pKY206), which contains *E. coli* chaperone genes (24). Cultured cells were resuspended in 50 mM potassium phosphate buffer (10% glycerol (v/v) and 1 mM dithiothreitol) and lysed by sonication (Branson Instruments). The cell debris was removed by centrifugation at 12,000 × g for 30 min, and relative protein concentration was verified by SDS-PAGE. Thus prepared soluble fractions of cell crude extracts were used for activity assay. Activity of DszB and its mutants was assayed at 28 °C by measuring the amount of HBP produced from HBPS with high pressure liquid chromatography system. 1 unit of activity was defined as the amount of DszB necessary to produce 1 nmol of HBP or sulfite per min.

**Sequence Alignment—** Multiple alignment of amino acid sequences was carried out using ClustalX program (25). Fig. 5 was prepared using ESPript program (26).

### RESULTS

**The Overall Structure of DszB—** The crystals of DszB are in space group P2₁2₁2₁ with a = 36.7, b = 82.6, and c = 139.6 Å. The asymmetric unit consists of a single molecule of DszB. In addition to 330 water molecules, 7 glycerol molecules and 1 acetate ion were modeled and included in the structure. The structure of DszB is monomeric and oval-shaped with approximate dimensions of 60 × 50 × 40 Å (Fig. 1A). In the final model of DszB, more than 91% of residues are located in the most favored regions in the Ramachandran plot, and only Leu^{226} is located in generously allowed regions. However, this residue is

### TABLE 1

**Selected crystallographic data and statistics**

SAD indicates single wavelength anomalous dispersion.

|                          | DszB                  | Gold derivative | DszB-HBPS | DszB-BPS |
|--------------------------|-----------------------|----------------|-----------|-----------|
| **Data collection**       |                       |                |           |           |
| Space group              | P2₁2₁2₁               | P2₁2₁2₁        | C2        | C2        |
| Wavelength, Å            | 1.000                 | 0.978          | 0.978     | 0.978     |
| Resolution, Å            | 28.8-1.8              | 34.9-2.0       | 54.2-1.6  | 31.0-1.8  |
| No. of unique reflections| 139.971               | 189.431        | 353.812   | 236.875   |
| Completeness, % (last shell) | 99.4 (98.6)      | 98.8 (94.1)    | 100 (99.8) | 99.4 (96.3) |
| R/σ (last shell)         | 6.8 (3.2)             | 11.3 (6.4)     | 7.1 (3.7) | 9.2 (4.8) |
| Rmerge (last shell)^a     | 0.072 (0.214)         | 0.042 (0.106)  | 0.058 (0.199) | 0.054 (0.138) |
| **SAD phasing**          |                       |                |           |           |
| Resolution, Å            |                       | 34.9-2.2       |           |           |
| Rmerge, σ               |                       | 0.847          |           |           |
| Phasing power, e         |                       | 0.962          |           |           |
| Figure of merit          |                       | 0.216          |           |           |
| **Refinement**           |                       |                |           |           |
| Resolution               | 28.8-1.8              |                 | 54.2-1.6  | 31.0-1.8  |
| R-factor (Rmerge)        | 0.188 (0.201)         | 0.180 (0.196)  | 0.169 (0.198) |           |
| Average B-factor, protein, Å² | 16.535               | 13.463         | 13.452   |           |
| Average B-factor, water, Å² | 31.507              | 23.572         | 26.770   |           |
| Average B-factor, ligands, Å² | 32.595            | 12.388         | 11.609   |           |
| r.m.s.d. bonds, Å        | 0.005                 | 0.005          | 0.004    |           |
| r.m.s.d. angles, °        | 1.694                 | 1.207          | 1.208    |           |

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^a Rmerge = Σh iΣl(lkl) - (lkl))/Σh iΣl(lkl), where lkl is the i-th intensity measurement of reflection lkl, including symmetry-related reflections, and (lkl) is its average.

^b Rmerge is as defined in CNS.

^c Phasing power is as defined in CNS.

^d Average B-factors of a glycerol molecule, HBPS, and BPS are bound to the active site, respectively.
well defined in its electron density. The 18 N-terminal residues were not observed in the electron density map. We deleted these residues to find out whether they have relevance to function, and we found no difference in activity or stability (data not shown). DszB is composed of two α/β domains, with highly curved 5-stranded β-sheets being located between α-helix bundles. We designate the two domains as domain A (residues 19–97 and 251–365) and domain B (residues 98–250) (Fig. 1, A and B).

We searched for proteins with similar structures using the DALI server (27) and found matches, including ovotransferrin (PDB code 1NNT, Z score = 8.7, root mean square deviation (r.m.s.d.) = 4.3 Å) and substrate-binding component from a bacterial ABC transporter of sulfate (PDB code 1SBP, Z score = 7.3, r.m.s.d. = 3.8 Å) (Fig. 1C). These results show that DszB belongs to the phosphate-binding protein family as defined in the SCOP protein fold data base (28). Two enzymes, porphobilinogen deaminase (29) and thiaminase-I (30), also belong to this family. Additionally, amino acid homology search on the NCBI protein sequence data base (www.ncbi.nlm.nih.gov/) showed that DszB has significant amino acid sequence similarity to the putative substrate-binding proteins. Specifically, the C-terminal half of DszB shares a 26% amino acid identity with AtsR, the substrate-binding protein component for the sulfate ester transport system of Pseudomonas putida S-313 (31). Based on three-dimensional structure similarity and amino acid homology, we propose that DszB has been evolved from substrate-binding proteins.

The active site of DszB, in which the critical residue Cys\(^{27}\) is located, is in the cavity between the two domains and includes a glycerol molecule (Fig. 1D). Adding glycerol in the buffers (10% (w/v)) during the purification steps was essential in stabilizing the protein (11). The binding of a glycerol molecule in the active site may be responsible for stabilizing the protein. The domain B part of the cavity is mainly hydrophobic, whereas that of domain A contains Cys\(^{27}\), whose sulfhydryl group is free of interactions with nearby residues. In the course of searching for a potential general base, we found the carboxylate group of Glu\(^{192}\) to be adjacent to the residue, separated by about 4 Å (Fig. 1D), and we mutated it to Gln to examine its function. However, the mutation had no effect on the desulfination activity (data not shown).

*Structures of DszB in Complex with Substrates—To investigate the substrate-binding mode of DszB, an inactive mutant
Crystal Structures of DszB

(DszB-C27S) was crystallized in complex with substrates HBPS and BPS, as attempts to soak substrates into crystals were unsuccessful (13). Complex crystals could be obtained by incubating the protein (~10 mg/ml) in a buffer containing 20 mM Tris, pH 8.0, 10% (v/v) glycerol, and 20 mM HBPS or BPS prior to the crystallization setup. The structures of HBPS and BPS complexes were determined at resolutions of 1.6 and 1.8 Å, respectively, by the molecular replacement method. Crystals of DszB in complex with HBPS with a rainbow gradient coloring scheme from the N terminus (blue) to the C terminus (red). HBPS is depicted as a space-filling model. C, superposition of the native and HBPS-bound structures of DszB illustrates structural change near the active site by the induced fit. Regions with significant structural changes are indicated by the colors cyan (native structure) and red (structure in complex with HBPS). HBPS is depicted as a space-filling model. The movement of His60 induced by the binding of HBPS is indicated with an arrow. D, the conformational change of residues 24–28 by the binding of HBPS, by which Cys27 (Ser27 in the complex structure) is exposed to the substrate. Carbon atoms of residues are colored in dark gray (native structure) or light gray (complex structure). Hydrogen bonds are indicated with dashed lines. Ribbon model is from the complex structure and colored as in B.

The overall structure is similar to that of the native protein, and we can superimpose the structure of DszB with that of DszB-HBPS or DszB-BPS with an approximate r.m.s.d. of 0.5 Å (Fig. 2B) except for residues 55–62 and 187–204, which confer the most notable change as a result of substrate binding (Fig. 2C). These residues, which were loops in the native structure, form α-helices to accommodate the biphenyl rings of substrates (Fig. 2C). The structural change introduces His50 into the active site, whose side chain interacts with Ser25 and HBPS, and the conformation of loop β1-αA changes to accommodate this residue, exposing Ser27, which is Cys in the native protein, to the sulfinate group of substrates (Fig. 2D). The glycerol molecule in the active site of the native structure is replaced by phenyl sulfinate moieties of substrates in the complex structures.

In the active site, Pro28, Phe61, Leu152, Trp155, Gly183, Phe203, and Leu226 mainly form hydrophobic interactions with substrates (Fig. 3A). Except for the additional hydroxyl group of HBPS, which is hydrogen-bonded to a water molecule in the active site, HBPS and BPS bind the active site in a similar manner. A sulfinate oxygen interacts with O−y of Ser27, Nε of His50, and N-η2 of Arg70, all at distances of 2.6–2.7 Å. As a result, the sulfinate group, Cys27, His50, and Arg70, forms a tetrahedral hydrogen bond network, with the guanidine moiety of Arg70 forming additional hydrogen bonds with the carbonyl oxygen of Gly73 (Fig. 3B). O−y of Ser27 also lies adjacent to the main chain nitrogen of Gly73 at a distance of ~3.0 Å. The configuration of Gly73 is reminiscent of the oxyanion hole in serine proteases, which is usually observed in various hydrolases and is
known to stabilize the negative charge of the reaction intermediate (32). It is also noteworthy that a water molecule interacts with the other sulfinate oxygen at a distance of 2.8 Å (Fig. 3B).

Site-directed Mutagenesis—A site-directed mutagenesis study to address the role of each residue in the active site triad demonstrates that each residue is crucial to the activity of DszB. We found that the H60Q mutation caused an ~17-fold reduction in the specific activity (Table 2). The structural integrity of DszB may have been affected by the R70I or R70K mutations as those expressed mutants mostly existed in the insoluble fraction of cell extracts (data not shown).

The highly conserved $^{70}$RXGG motif forms the structural core of domain A, and thus Arg$^{70}$ may be essential in maintaining the structure of domain A. We could not detect desulfination activity from the crude cell extracts of Arg$^{70}$ mutants (Table 2).

**DISCUSSION**

To our knowledge, the only desulfinase documented so far is aspartate $\beta$-decarboxylase (EC 4.1.1.12), which can accept structurally similar cysteine sulfinites (33, 34). Aspartate $\beta$-decarboxylase is a member of the pyridoxal phosphate-dependent aminotransferase family, and $\beta$-elimination of aspartate or cysteine sulfinate is considered to occur by the formation of a Schiff’s base with the pyridoxal phosphate cofactor, thus stabilizing the transition state (35). However, DszB does not require any form of cofactor for desulfurization (11) and seems to have adopted a distinct mechanism of desulfination (36).

**Proposed Reaction Mechanism of DszB**—In the active site of DszB-C27S in complex with substrates, Ser$^{27}$ (Cys in the native protein) is positioned for nucleophilic attack on the sulfinate sulfur (Fig. 3B), and thus the thiolate ion of Cys$^{27}$ and the sulfinate sulfur form a thiolsulfonate-like intermediate as a plausible first step of the reaction. It has been proposed that the Cys-His dyad in cysteine proteases forms a thiolate-imidazolium pair to activate the catalytic cysteine residue (37), and we find Ser$^{27}$ and His$^{60}$ with a similar configuration in the complex structures. However, the ion pair formation mechanism for DszB is not reasonable because S-$\gamma$ of Cys$^{27}$ and N-$\epsilon$ of His$^{60}$ are separated by a distance of ~17 Å in the absence of a substrate (Fig. ID). Furthermore, the H60Q mutant retains desulfination activity, which suggests His$^{60}$ does not function as a general base and may be required for the proper orientation of the sulfinate group. We could not find any other base in the vicinity of Cys$^{27}$ except for the sulfinate group from the substrate. Hence, we propose the sulfinate group to be the general base.

Sulfinic acids are stronger acids than carboxylic acids and consequently poor bases (38). Nonetheless, optimal positioning and orientation of the catalytic base can increase its basicity by about several orders of magnitude (39). In the case of the sulfinate group, because of the lone pair electron of the sulfur atom, the $\sigma_{n}$ orbital of the sulfinate oxygen and the $\sigma$-C bond are predicted to be on the same side of the SO$_2$.
plane (40). Consequently, we find the sulfinate group of bound substrates in a configuration that directs its syn orbital to the hydroxyl hydrogen of Ser27 (Fig. 3B). Moreover, protonation of the sulfinate group can reduce the negative charge building on the reaction intermediate. Similar substrate-assisted catalysis has been proposed for GT-Pases such as p21Ras and type II restriction endonucleases (41). Arg70 and the main chain nitrogen of Gly73 seem to be required for the stabilization of the negatively charged reaction intermediate. A water molecule coordinated to the sulfinate oxygen could be added in a similar fashion to release a sulfite molecule in the final steps of desulfination. The proposed reaction mechanism of DszB is illustrated in Fig. 4.

DszB Belongs to a New Family of Desulfinases—The structure of DszB defines a new group of hydrolases in which conserved Cys, His, and Arg participate in hydrolysis of the C–S bond in organic sulfinic acids. Based on this definition, homologs of DszB were searched for in NCBI protein sequence data base. Homologs of DszB could be found primarily in genome sequences of soil bacteria, and all include Cys, His, and Arg in their N-terminal regions (Fig. 5). A multiple alignment of the amino acid sequences also shows apparent homology (data not shown), and thus these homologs appear to have similar folds. Interestingly, some of these putative proteins are from protein clusters whose genomic contexts are different from that of dsz operon. As organic sulfur compounds of various structures exist in soil, these uncharacterized DszB homologs might function as desulfinase components of sulfur metabolic pathways yet to be discovered.

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