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PII: S0022-2836(17)30504-1
DOI: doi:[10.1016/j.jmb.2017.10.022](https://doi.org/10.1016/j.jmb.2017.10.022)
Reference: YJMBI 65535

To appear in: *Journal of Molecular Biology*

Received date: 6 September 2017
Revised date: 8 October 2017
Accepted date: 19 October 2017

Please cite this article as: Li, C.-Y., Zhang, D., Chen, X.-L., Wang, P., Shi, W.-L., Li, P.-Y., Zhang, X.-Y., Qin, Q.-L., Todd, J.D. & Zhang, Y.-Z., Mechanistic insights into dimethylsulfoniopropionate lyase DddY, a new member of the cupin superfamily, *Journal of Molecular Biology* (2017), doi:[10.1016/j.jmb.2017.10.022](https://doi.org/10.1016/j.jmb.2017.10.022)

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**Mechanistic insights into dimethylsulfoniopropionate lyase DddY, a new member
of the cupin superfamily**

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Abstract

The marine osmolyte dimethylsulfoniopropionate (DMSP) is one of Earth's most abundant organosulfur molecules. Bacterial DMSP lyases cleave DMSP, producing acrylate and dimethyl sulfide (DMS), a climate-active gas with roles in global sulfur cycling and atmospheric chemistry. DddY is the only known periplasmic DMSP lyase and is present in β -

γ -, δ - and ϵ -proteobacteria. Unlike other known DMSP lyases, DddY has not been classified into a protein superfamily, and its structure and catalytic mechanism are unknown. Here, we determined the crystal structure of DddY from the γ -proteobacterium *Acinetobacter bereziniae* originally isolated from human clinical specimens. This structure revealed that DddY contains a cap domain and a catalytic domain with a Zn^{2+} bound at its active site. We also observed that the DddY catalytic domain adopts a typical β -barrel fold and contains two conserved cupin motifs. Therefore, we concluded that DddY should belong to the cupin superfamily. Using structural and mutational analyses, we identified key residues involved in Zn^{2+} coordination, DMSP binding and the catalysis of DMSP cleavage, enabling elucidation of the catalytic mechanism, in which the residue Tyr271 of DddY acts as a general base to attack DMSP. Moreover, sequence analysis suggested that this proposed mechanism is common to DddY proteins from β -, γ -, δ - and ϵ -proteobacteria. The DddY structure and proposed catalytic mechanism provide a better understanding of how DMSP is catabolized to generate the important climate-active gas DMS.

Key words

DMSP; DMSP lyase DddY; DMS generation; cupin superfamily; catalytic mechanism

Abbreviations

DMSP, dimethylsulfoniopropionate; DMS, dimethyl sulfide; EDTA, ethylenediaminetetraacetic acid; LB, Lysogeny Broth; WT, wild type; IPTG, isopropyl β -D-1-thiogalactopyranoside; ICP-AES, inductively coupled plasma atomic emission

spectrometry; CD, circular dichroism; PDB, Protein Data Bank.

Introduction

A billion tons of the compatible solute dimethylsulfoniopropionate (DMSP) is produced by marine phytoplankton, macroalgae, corals, some marine α -proteobacteria and a few angiosperms [1-5]. The catabolism of DMSP by microbial communities provides a major source of carbon and sulfur in the marine environment. Although some DMSP is broken down by algae [6, 7], it is believed that bacteria drive the majority of DMSP catabolism through two pathways, the demethylation pathway and the cleavage pathway [8]. This study focuses on the cleavage pathway where DMSP is cleaved by DMSP lyases to produce acrylate (or 3-hydroxypropionate) and ~300 million tons per annum of the climate-active volatile dimethyl sulfide (DMS) [9, 10]. When released into the atmosphere through air-sea flux, DMS can be photochemically oxidized into DMSO or sulfate aerosols, which can scatter solar radiation directly and form cloud condensation nuclei, influencing the global temperature and climate [10, 11]. These sulfurous compounds are returned to land via rain or snow to complete the global sulfur cycle from marine to terra [1, 10]. Furthermore, DMS is also an important infochemical in signaling pathways to wide-ranging organisms [12].

To date eight different DMSP lyases (DddD, DddK, DddL, DddP, DddQ, DddW, DddY and Alma1) have been identified in taxonomically diverse bacteria, fungi and phytoplankton [13]. Although these DMSP lyases all cleave DMSP, they possess distinct protein sequences and/or belong to different superfamilies [7, 9, 14-19]. DddD (~93 kDa) is a class III CoA transferase that generates 3-hydroxypropionate and DMS from DMSP [9]. All the other DMSP lyases

cleave DMSP producing acrylate and DMS. DddK, DddL, DddQ and DddW (14-16 kDa) contain a conserved cupin motif, and therefore, belong to the cupin superfamily [14, 17-19], which comprises a functionally diverse group of proteins that usually require a divalent metal co-factor for function [20]. Indeed, the DMSP lyase activity of the DddQ and DddW enzymes have been shown to require a metal co-factor for function [21, 22]. This is very likely the case for all cupin family DMSP lyases. Another metal dependent DMSP lyase is DddP (~50 kDa), a member of the M24 peptidase family, which uses a two-iron core for catalysis [16, 23]. The only known algal DMSP lyase, Alma1 (~39 kDa), is a redox-sensitive enzyme that belongs to the aspartate racemase superfamily [7].

Of the DMSP lyases, DddQ, DddP and DddK have solved crystal structures and their catalytic mechanisms were explained by structural and biochemical analyses [21, 23-25]. In DddQ, a Tyr131 undergoes a conformational change during catalysis, acting as the catalytic base [21]. Similar to DddQ, DddK also needs a tyrosine residue (Tyr64) to initiate the lysis of DMSP [25]. DddP adopts an ion-shift catalytic mechanism for DMSP cleavage [23]. In addition to these structural studies, the catalytic mechanisms of DddD, DddW and Alma1 have also been proposed based on biochemical studies and homologous modelling [7, 22, 26].

Of the DMSP lyases, DddY (~46 kDa) is the most curious. It has no overall similarity to any polypeptide with known function, being classified as a 'Domain of Unknown Function', and it is the only known periplasmic DMSP lyase [15], with all other DMSP lyases being cytoplasmic proteins [7, 10, 15, 19]. Interestingly, DddY was the first DMSP lyase to be characterized [27], but, the corresponding gene was not identified until 2011 [15]. Unlike other bacterial DMSP lyases that occur frequently in α -proteobacteria, mostly in Roseobacters,

DddY is found in strains of β -, γ -, δ - and ϵ -proteobacteria which are spread throughout marine and terrestrial environments [15]. Many of these bacteria containing DddY, for example, *Arcobacter*, *Desulfovibrio* and *Shewanella* spp., are commonly found in microaerobic sediment environments, such as saltmarshes, where DMSP is abundant, and it was proposed that DddY might be very important in these environments [15]. Due to the novelty of its protein sequence and the lack of structural information, very little is known about the molecular mechanism of the unusual DMSP lyase DddY.

Acinetobacter bereziniae, a γ -proteobacterium isolated from human clinical specimens [28], contains a *dddY* gene. In this study, the *A. bereziniae* DddY enzyme was over-expressed in *Escherichia coli*, and characterized. The crystal structures of wild type DddY and of its inactive mutant Y260A complexed with acrylate were solved. Structural assays and sequence analysis indicate that DddY is a cupin-containing DMSP lyase. Based on structural analysis and mutational assays, we propose the molecular mechanism of DMSP cleavage by DddY. The results provide clarity on a previously classified “domain of unknown function” and greatly broaden our knowledge on DMSP cleavage into DMS in organisms from wide-ranging marine, terrestrial and even human associated environments.

Results and Discussion

Expression and characterization of DddY from *A. bereziniae*

The *dddY* gene of *A. bereziniae* encodes a 401 amino acid polypeptide which is 80% identical to the functional *Alcaligenes faecalis* DddY enzyme characterized [15]. Full length *dddY* of *A. bereziniae* was synthesized, expressed in *E. coli* BL21 (DE3) cells and the recombinant DddY was purified. The pure DddY protein was shown to encode a functional DMSP lyase

generating DMS and acrylate from DMSP. The optimal temperature for DddY enzyme activity was $\sim 60^{\circ}\text{C}$ (Fig. 1A), which was significantly higher than the $37\text{-}40^{\circ}\text{C}$ previously reported for *A. faecalis* DddY, yet the optimal pH values for these DddY enzymes was the same at pH ~ 8.0 (Fig. 1B) [27].

A. bereziniae DddY exhibited a K_m value of ~ 5.0 mM (Fig. 1C), which is similar to that published for *A. faecalis* DddY (1.4 mM) [27], but 12.5-fold higher than that of *Desulfovibrio acrylicus* (0.4 mM) [29]. These relatively high K_m values in the millimolar range are common in many DMSP catabolic enzymes, including the DMSP lyases DddK, DddP, DddQ, DddW and Alma1 [7, 19, 21-23], and the DMSP demethylase DmdA [30]. These values are far higher than the nanomolar DMSP concentrations in seawater and may indicate that DMSP plays important physiological roles in bacterial cells.

Despite its relatively high K_m , the k_{cat} value of *A. bereziniae* DddY, approximately 8.3×10^3 s^{-1} (Fig. 1C), was the highest of any characterized DMSP lyases. The k_{cat} value of *A. bereziniae* DddY was ~ 100 times higher than those of DddD and DddW [22, 26], and 10 times higher than that of Alma1 [7]. The periplasmic localization being in close contact to environmental DMSP and the high k_{cat} values for DddY are wholly consistent with the enzyme having a key role in the DMSP metabolism either to provide acrylate as a carbon source [27], or as a terminal electron acceptor in anaerobic respiration [15, 29].

Overall structure and classification of DddY

In order to study the catalytic mechanism of DddY, we solved the crystal structure of wild type (WT) DddY to 1.5 \AA by the single-wavelength anomalous dispersion (SAD) method using a selenomethionine derivative (Se derivative) (Table 1). To obtain a structure of DddY

in complex with DMSP, several mutants were constructed according to the structure of WT DddY, and an inactive mutant Y260A was obtained. We then co-crystallized this mutant with DMSP. However, after we solved the structure of Y260A, we found that the electron density of the sulfonium group of DMSP is poor and we can only place an acrylate molecule in the active site. Because DMSP is unstable in alkaline solution, this result suggests that DMSP may be spontaneously decomposed in the crystallization buffer (pH8.5) during crystallization. The structures of DddY and Y260A/acrylate complex are nearly identical, with a root mean square deviation (RMSD) of 0.14 Å. Because the structure of WT DddY had a higher resolution, the structural analyses described below are based on the structure of WT DddY unless otherwise noted.

The crystals of DddY belong to the $P2_12_12_1$ space group, with each asymmetric unit contains one DddY molecule. Gel filtration analysis also indicated that DddY was a monomer in solution. Structural analysis showed that the recombinant DddY does not contain the first 21 N-terminal residues, indicating that these residues may be disordered in the structure. Alternatively, the first 21 N-terminal amino acid residues are most likely to be cleaved off during DddY maturation because they are predicted to be a signal peptide (<http://www.cbs.dtu.dk/services/SignalP/>). Structurally, mature DddY contains two domains, the N-terminal domain (Ala22-Val190) and the C-terminal domain (Ser191-Pro401). The N-terminal domain is mainly composed of α -helices, and surrounds the C-terminal domain like a "cap". Thus, we name the N-terminal domain of DddY the "cap domain" (Fig. 2A). The C-terminal domain mainly consists of eight antiparallel β -strands and adopts a β -barrel fold structure, which contains the catalytic pocket of DddY. We name the C-terminal domain of

DddY the "catalytic domain" (Fig. 2A). To investigate the function of the cap domain of DddY, we constructed a truncated mutant DddY Δ 190 that only contained the catalytic domain. The removal of the DddY cap domain caused severe aggregation of the catalytic domain in solution, indicating that the cap domain is essential for DddY. Structural analysis indicates that there are kinds of interactions existing between the cap domain and the catalytic domain, including hydrogen bonds, salt bridges, and hydrophobic interactions. The result of truncation mutation suggests that either the cap domain of DddY is essential for the correct folding of the catalytic domain or the exposure of the interaction surface of the catalytic domain causes its aggregation.

The DddY structure was used as a probe against the Dali server [31] to identify any potential structural homologues. Interestingly, the structures with the highest Z-scores to DddY were DddQ enzymes, which are cytoplasmic DMSP lyases that belong to the cupin superfamily. Cupin proteins have a common β -barrel fold and a very diverse suite of biological functions [32]. Sequence alignment of DddY to other cupin-containing DMSP lyases (DddQ, DddL, DddW and DddK) showed that they all contain the two conserved cupin motifs: motif 1, G(X)₅HXH(X)_{3,4}E(X)₆G and motif 2, G(X)₅PXG(X)₂H(X)₃N [32] (Fig. 2B). Although both DddY and DddQ have the typical β -barrel fold of cupins, and contain the conserved cupin motifs, the overall structures of DddY and DddQ exhibit very significant differences (Fig. 2C). DddY (~380 residues) is much larger than DddQ (~190 residues) and their amino acid sequence similarity is very low (< 10%). Furthermore, DddQ contains the β -barrel fold in its structure, but lacks the DddY cap domain [21]. Thus, DddY represents a new member of the cupin superfamily and, more specifically, the cupin DMSP lyase enzymes.

In addition, phylogenetic analysis indicated that DddY formed a separate clade from DddL, DddQ, DddW and DddK (Fig. 3), suggesting the divergent evolution of DddY from other cupin DMSP lyases.

Analysis of the metal ion in DddY

The electron density map of DddY indicates that there are an acetate molecule and a metal ion in the catalytic domain of DddY in close proximity to each other. The acetate was likely from the crystallization buffer for DddY, which contained 0.2 M ammonium acetate. Comparison of the DddY and Y260A/acrylate complex structures showed that the acetate molecule is located in the same position as acrylate. The presence of a metal ion in close proximity to the acrylate, acetate and, very likely, the DMSP substrate, was not surprising because all cupin DMSP lyases studied at the biochemical level have utilized divalent metals to drive the lysis of DMSP generating acrylate, DMS and a proton [21, 22]. Inductively coupled plasma atomic emission spectrometry (ICP-AES) measurements suggested that the metal ion is a Zn^{2+} , occupying ~68% of the DddY molecules. Element iron was also detected and estimated to occupy ~18% of the DddY molecules. Cd, Co, Cr, Cu, Mn or Ni was not detectable. Among metal-containing DMSP lyases, the occupancy of metal ions can hardly reach 100%, with iron in DddP of ~65% [23], zinc in DddQ of ~42% [21] and iron in DddW of ~20% [22]. It is reported that DddW can accommodate multiple metal ions to catalyze DMSP cleavage [22]. We tried to investigate whether DddY can accommodate different metal ions. However, after incubation with 10 mM metal chelator ethylenediaminetetraacetic acid (EDTA) for 1 hour, DddY still retained ~70% residual activity, showing that the Zn^{2+} in DddY is hard to be chelated by EDTA. Structural analysis of DddY provides a potential explanation for

ineffectiveness of the EDTA chelation. The acetate molecule in DddY is hard to be observed in the electrostatic surface representation (Fig. 4A). The cross-sectional view of DddY reveals that there is a cavity for substrate entry, which is also the most probable path for EDTA to access Zn^{2+} (Fig. 4B). In this cavity, residues Glu227, Glu248 and the hydroxyl group of Tyr225 consist an acidic zone (Fig. 4B), which would likely exclude the acidic EDTA from getting into the cavity.

Key residues in the active site of DddY for DMSP binding and cleavage

In the crystal structure of DddY, residues His265, Glu269, His338 and the acetate molecule participate in coordinating Zn^{2+} (Fig. 5A). As expected, the exact same residues coordinate the acrylate in the Y260A/acrylate complex crystal structure (Fig. 5B). To ascertain which molecule or residue coordinates the metal ion when there is no acetate/acrylate existing in DddY's structure, the crystal structure of the Se derivative of DddY that does not contain an acetate/acrylate molecule was carefully refined. In the Se derivative structure, two adjacent water molecules occupy the position where the carboxyl oxygen atoms of acetate/acrylate are located, and one of the two waters coordinates Zn^{2+} . Despite that the Se derivative does not exist in biological system, it is possible that water molecules participate in coordinating Zn^{2+} in DddY in the absence of ligands. His265, Glu269 and His338 residues comprises a common structural motif, the 2-His-1-carboxylate facial triad, whose function is probably to stabilize metal ions [33-35]. The DMSP lyase DddQ possesses the same motif to coordinate the metal ion [21]. Site-directed mutations of His265, Glu269 or His338 to alanine abolished the activity of DddY (Fig. 6A), indicating the key roles of these residues in DMSP cleavage.

With the positions of acetate/acrylate in DddY and Y260A/acrylate complex matching, we suggest that DMSP would be located in the same position. In this substrate-binding pocket of DddY, the side chains of three aromatic residues (Phe207, Tyr225 and Trp359) form a hydrophobic box (Fig. 5C), which probably accommodates the tertiary sulfonium group of DMSP. Residues His263, Tyr271 and Arg361 also likely participate in orientating DMSP through the formation of hydrogen bonds with the carboxyl group of DMSP (Fig. 5C). Mutations of these residues involved in binding DMSP severely decreased the enzymatic activity of DddY (Fig. 6A), indicating the key roles of these residues in DMSP cleavage.

The cleavage of DMSP into DMS and acrylate is an alkaline-induced β -elimination reaction. There should be a general base in the active site of DddY to initiate DMSP cleavage. Structural analysis suggests that Tyr260 and Tyr271 are the potential candidates to act as the general base to abstract the C_{α} -H proton of DMSP. Tyrosine usually exhibits a high pK_a in solution. To act as a general base, a tyrosine should achieve a deprotonation state. The deprotonation state of Tyr260 could be achieved through an interaction with Tyr208 (Fig. 5C). Tyr271 can form hydrogen bonds with the carboxyl oxygen of acetate and with Tyr223 through a water molecule (Fig. 5C). It has been reported that a hydrogen-bond network of a ketosteroid isomerase facilitates the deprotonation of tyrosine residues [36]. The hydrogen-bond network of DddY could also allow Tyr271 to achieve its deprotonation state. Mutations of Tyr208, Tyr223, Tyr260 or Tyr271 to alanine almost abolished the activity of DddY (Fig. 6A). Sequence alignment demonstrates that Tyr271 is conserved in DMSP lyases, whereas in DddW and DddK, the corresponding residue of Tyr260 is a leucine (Fig. 2B), which cannot be a general base. Moreover, the distance between Tyr271 and acetate (3.1 Å) is

shorter than that between Tyr260 and acetate (3.6 Å) (Fig. 5C). These results suggest that Tyr271 is the most probable catalytic base of DddY.

Circular-dichroism (CD) spectroscopy assays showed that the secondary structures of all the mutants are very similar to that of WT DddY (Fig. 6B). This indicates that the decrease in the enzymatic activities of the mutants is a result of residue replacement rather than structural changes. Altogether, the mutational analyses are consistent with the structural observations.

Mechanism of DMSP cleavage to generate DMS by DddY

Based on the above results, the following catalytic mechanism of DMSP cleavage by DddY is proposed. In the absence of DMSP, residues His265, Glu269, His338 and a water molecule coordinate Zn^{2+} in the active site of DddY (Fig. 7A). After DMSP enters the active site, it displaces the water molecule, and forms a coordination bond with the Zn^{2+} (Fig. 7B). Then, Tyr271 likely attracts the C_{α} -H proton of DMSP, forming a C_{α} carbanion. This C_{α} carbanion would subsequently attack the C_{β} of DMSP, leading to the breaking of the C_{β} -S bond (Fig. 7C). Finally, DMSP would be cleaved to DMS and acrylate, which are released from the active site (Fig. 7D). After this reaction, DddY can reacquire water molecules from the solution, re-construct the hydrogen-bond network, deprotonate the residue Tyr271 and get ready for the next reaction.

Among DMSP lyases that belong to the cupin superfamily, the catalytic mechanisms of DddQ and DddW have been studied. The catalytic base of DddQ was proposed to be Tyr131, which maintains its deprotonation state by forming a coordination bond with Zn^{2+} [21]. When DMSP enters the catalytic pocket, Tyr131 of DddQ generates a conformational change to attack DMSP [21]. In DddW, residues His81, Tyr89 and an activated water molecule were the

candidates to be the catalytic base [22]. The catalytic base of DddY, Tyr271, corresponds to Tyr131 of DddQ and Tyr89 of DddY (Fig. 2B), suggesting that DMSP lyases belonging to the cupin superfamily may adopt similar strategies to cleave DMSP.

Universality of the catalytic mechanism of DddY

The DddY gene occurs in strains of β -, γ -, δ - and ϵ -proteobacteria from varied marine, terrestrial and clinical/fecal environments [15]. To investigate the ubiquity of the DddY catalytic mechanism to cleave DMSP into DMS and acrylate, we performed sequence alignment of DddY proteins. Although the DddY proteins from different bacteria display considerable variability in their primary amino acid sequences (from 31% to 87%), most of the residues involved in forming the acidic zone in the cavity for DMSP entry (Tyr225, Glu227 and Glu248), coordinating Zn^{2+} (His265, Glu269 and His338), binding DMSP (Phe207, Tyr225, His263, Tyr271, Trp359 and Arg361), and participating in the catalysis reaction (Tyr223 and Tyr271) are highly conserved among DddYs from β -, γ -, δ - and ϵ -proteobacteria (Fig. 8). Although the residue Phe207, which participates in forming the hydrophobic box to accommodate the tertiary sulfonium group of DMSP, exhibits a relatively lower conservation, its corresponding residues in different species are all hydrophobic residues (Fig. 8), which all have similar ability to constitute the hydrophobic box. These analyses indicates that the proposed catalytic mechanism of DddY to cleave DMSP may have universal significance in β -, γ -, δ - and ϵ -proteobacteria containing DddY.

Conclusion

DMSP and DMS play important roles in global sulfur cycle. Among the eight DMSP lyases identified, DddY is the only one that locates in periplasm. The sequence of DddY presents no

overall similarity to any polypeptide with known function [15]. In this study, we solved the crystal structure of DddY from *A. bereziniae*, and performed detailed biochemical and structural analyses. We demonstrate that DddY is the most efficient of the known DMSP lyases, likely enabling effective catabolism of DMSP in varied environments. The results demonstrated that DddY is a metal-containing cupin DMSP lyase with a novel two-domain fold, which forms a distinct group from the other DMSP lyases in the cupin superfamily. Based on structural and mutational analyses, the catalytic mechanism of DddY was proposed, which is most likely also functions in diverse β -, γ -, δ - and ϵ -proteobacteria containing DddY. Our results provide novel insights into how DMS is generated through DMSP cleavage, shedding light on global sulfur cycling.

Materials and methods

Bacterial strains and growth conditions

E. coli strains DH5 α and BL21 (DE3) were grown in Lysogeny Broth (LB) medium at 37°C.

Gene cloning, point mutation and protein expression and purification

The 1206 bp full length *dddY* gene from *A. bereziniae* was synthesized by the Beijing Genomics Institute. The gene was then subcloned into the pET22b (Novagen, America) vector with a C-terminal His tag. All of the point mutations in DddY were introduced using PCR-based methods and were verified by DNA sequencing. The DddY protein and all of its mutants were expressed in *E. coli* strain BL21 (DE3). The cells were cultured at 37°C in LB medium to an OD₆₀₀ of 0.8-1.0 and then induced at 20°C for 16 h with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The proteins were purified first with Ni²⁺-NTA resin (Qiagen, Germany) and then fractionated by gel filtration on a Superdex-200 column (GE

Healthcare, America). The Se derivative of DddY was overexpressed in *E. coli* strain BL21 (DE3) under 0.5 mM IPTG induction in the M9 minimal medium supplemented with selenomethionine, lysine, valine, threonine, leucine, isoleucine, and phenylalanine. The recombinant selenomethionine derivative was purified in a manner similar to that for WT DddY.

Enzyme assays and characterization

The enzymatic activity of DddY was determined as described by Wang *et al.* [23]. DddY (at a final concentration of 0.5 nM) and DMSP (at a final concentration of 20 mM) were mixed with reaction buffer containing 100 mM Tris-HCl (pH 8.0) in a total volume of 200 μ l. After the mixture was incubated at 60°C for 10 min, the reaction was stopped by perchloric acid, and the amount of acrylate in the reaction mixture was detected by high-performance liquid chromatography (HPLC) on a Sunfire C18 column (Waters, Ireland). To determine the optimal temperature of DddY, reaction mixtures were incubated at 30°C, 40°C, 50°C, 60°C or 70°C for 10 min. The optimum pH for DddY was examined using Britton-Robinson buffer at pH values of 5.0-10.0. Britton-Robinson buffer is a mixture of 0.04 M H₃BO₃, 0.04 M H₃PO₄ and 0.04 M CH₃COOH [37]. To determine the effects of metal ions on the activity of DddY, different metal ions at a final concentration of 2 mM were individually added to the reaction mixture. To chelate the metal ion in DddY, the metal chelator EDTA was added at a final concentration of 10 mM. The kinetic parameters of DddY were determined by non-linear analysis based on the initial rates determined with 0.5 nM DddY and 1-20 mM DMSP. All the measurements were performed under the optimal pH and temperature of DddY.

Crystallization and data collection

The purified DddY protein was concentrated to ~7 mg/ml in 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl. Initial crystallization trials for DddY were performed at 20°C using the sitting drop vapor diffusion method. Diffraction-quality crystals of DddY were obtained in hanging drops containing 0.2 M ammonium acetate, 0.1 M Bis-Tris (pH 6.5) and 25% (wt/vol) polyethylene glycol (PEG) 3350 at 20°C after 2-weeks incubation. Crystals of the DddY Se derivative were obtained in hanging drops containing 0.2 M ammonium acetate, 0.1 M Tris-HCl (pH 8.5) and 25% (wt/vol) PEG 3350 at 20°C after 1-week incubation. To obtain the structure of DddY/DMSP complex, an inactive mutant Y260A was co-crystallized with DMSP (1 mM). However, after we solved the structure of Y260A, only an acrylate molecule in the active site was identified. Crystals of Y260A/acrylate complex were obtained in hanging drops containing 0.1 M Tris-HCl (pH 8.5) and 25% PEG 3350 at 20°C after 1-week incubation. X-ray diffraction data were collected on the BL18U1&BL19U1 beamlines at the Shanghai Synchrotron Radiation Facility. The initial diffraction data sets were processed by the HKL3000 program [38].

Structure determination and refinement

The crystals of both WT DddY and the Y260A/acrylate complex belong to the $P2_12_12_1$ space group. The structure of DddY Se derivative was determined by SAD phasing. The crystal structures of WT DddY and the Y260A/acrylate complex were determined by molecular replacement using the CCP4 program Phaser [39] with the structure of DddY Se derivative as the search model. The refinements of these structures were performed using Coot [40] and Phenix [41]. All the structure figures were processed using the program PyMOL (<http://www.pymol.org/>).

Detection of metal ions

ICP-AES measurement was performed using an IRIS Intrepid II XSP (Thermo Electron, America). To detect the metal ions in DddY, 1 ml of DddY protein (7 mg/ml) was mixed with 10 ml nitric acid. The sample was incubated at 120°C overnight until the digestion was complete. The sample was then diluted to 5 ml with distilled water and filtered through a 0.22- μ m filter membrane before detection.

CD spectroscopic assays

WT DddY and its mutants were subjected to CD spectroscopic assays at 25°C on a J-810 spectropolarimeter (Jasco). CD spectra of the samples at a final concentration of approximately 6 μ M were collected from 250 to 197 nm at a scan speed of 200 nm min⁻¹ with a band width of 2 nm. All of the samples were in the buffer containing 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl.

Accession numbers

The structures of DddY, DddY Se derivative and the Y260A/acrylate complex have been deposited in the Protein Data Bank (PDB) under the accession codes 5XKX, 5XKY and 5Y4K, respectively.

Acknowledgements

We thank the staffs from BL18U1&BL19U1 beamlines of National Facility for Protein Sciences Shanghai (NFPS) and Shanghai Synchrotron Radiation Facility, for assistance during data collection. This work was supported by the National Science Foundation of China

(31630012, 31290231, 31290230 and 31470541), the National Key Research and Development Program of China (2016YFA0601303), the Aoshan Talents Program, the Program of Shandong for Taishan Scholars (TS20090803), and the National Postdoctoral Program for Innovative Talents (BX201600095, BX201700145).

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure captions

Figure 1. Characterization of recombinant DddY. A. Effect of temperature on the enzymatic activity of DddY. The activity of DddY at 60°C was defined as 100%. B. Effect of pH on the enzymatic activity of DddY. The activity of DddY at pH 8.0 was defined as 100%. C. Non-linear fit curve for DMSP cleavage by DddY. Initial rates of acrylate generation were determined with 0.5 nM DddY and 1-20 mM DMSP in the reaction buffer.

Figure 2. Overall structural analysis of DddY. A. The overall structure of mature DddY. The DddY molecule contains a cap domain (colored in bluewhite) and a catalytic domain (colored in orange). The metal ion in DddY is shown as grey sphere. B. Sequence alignment of the cupin regions of DddY, DddL, DddQ, DddW and DddK. Residues conserved in cupin motifs 1 and 2 are shown above the sequence. Numbers in parentheses refer to the amino acid numbers in the DddY sequence from *A. bereziniae*. Sequences DddY_1 (WP_004831354.1) and DddY_2 (WP_025821852.1) are from *A. bereziniae* and *Shewanella marina*, respectively. Sequences DddL_1 (EEB86351.1) and DddL_2 (EAV43167.1) are from *Roseobacter* sp. GAI101 and *Labrenzia aggregata* IAM 12614, respectively. Sequences DddQ_1 (AAV94883.1) and DddQ_2 (EEX08322.1) are from *Ruegeria pomeroyi* DSS-3 and *Ruegeria lacuscaerulensis* ITI-1157, respectively. Sequences DddW_1 (AAV93771.1) and DddW_2 (EAQ44306.1) are from *R. pomeroyi* DSS-3 and *Roseobacter* sp. MED193, respectively. Sequences DddK_1 (AAV93771.1) and DddK_2 (WP_014953073.1) are from *Pelagibacter* strain HTCC1062 and Alpha proteobacterium HIMB5, respectively. The alignment was done with ClustalW [42] and ESPript [43]. C. Superimposed structures of DddY and DddQ. The

cap domain and the catalytic domain of DddY are colored in bluewhite and orange, respectively. DddQ is colored in cyan.

Figure 3. The neighbor-joining phylogenetic tree of DddY, DddL, DddQ, DddW and DddK. Phylogenetic analysis was performed using MEGA version 6.0 [44].

Figure 4. Analysis of the cavity of DddY for substrate entry. A. Electrostatic surface of DddY. Red denotes negative potential, and blue denotes positive potential. B. Cross-sectional view of the cavity of DddY. The acetate molecule and Zn^{2+} are shown, Residues involved in constituting the acidic zone are colored in yellow.

Figure 5. Structural analyses of important residues in the active site of DddY. The Zn^{2+} is shown as grey sphere. A. Residues and molecules involved in coordinating Zn^{2+} in DddY. B. Residues and molecules involved in coordinating Zn^{2+} in Y260A/acrylate complex. The $2F_o-F_c$ densities for Zn^{2+} , acetate molecule, acrylate molecule and selected DddY residues are contoured in blue at 1.5σ . The F_o-F_c densities for acetate and acrylate molecules are contoured in green at 3.0σ in the insets. The possible coordination bonds are represented by dashed lines. C. Residues involved in DMSP binding and cleavage. DddY residues that may participate in forming interactions with the tertiary sulfonium group of DMSP are colored in purple. Residues involved in forming interactions with the carboxyl group of DMSP are colored in cyan. Residues that may participate in catalyzing DMSP cleavage are colored in yellow. The possible hydrogen bonds are represented by dashed lines.

Figure 6. Mutational analysis of important residues in the active site of DddY. A. Enzymatic activities of the mutants of DddY. The activity of WT DddY was defined as 100%. B. CD spectra of WT DddY and its mutants.

Figure 7. The catalytic mechanism of DddY to cleave DMSP generating DMS and acrylate. A. In the absence of DMSP, Zn^{2+} is coordinated by residues His265, Glu269, His338 and a water molecule. B. DMSP displaces the water molecule, and forms a new coordination bond with Zn^{2+} . C. The residue Tyr271 acts as the general base to initiate the reaction. D. DMS and acrylate are generated from DMSP cleavage.

Figure 8. Sequence alignment of DddY proteins from strains of β -, γ -, δ - and ϵ -proteobacteria. Black triangles indicate residues involved in DMSP binding, black stars indicate residues involved in catalyzing DMSP cleavage, white dots indicate residues involved in forming the acidic zone of the cavity for substrate entry, and black dots indicate residues involved in coordinating Zn^{2+} . A. *bereziniae*, *Acinetobacter baylyi*, *S. marina* and *Shewanella algae* belong to γ -proteobacteria. *Alcaligenes faecalis* belongs to β -proteobacteria. *Desulfovibrionaceae* belongs to δ -proteobacteria. *Arcobacter nitrofigilis* belongs to ϵ -proteobacteria. The alignment was done with ClustalW [42] and ESPript [43].

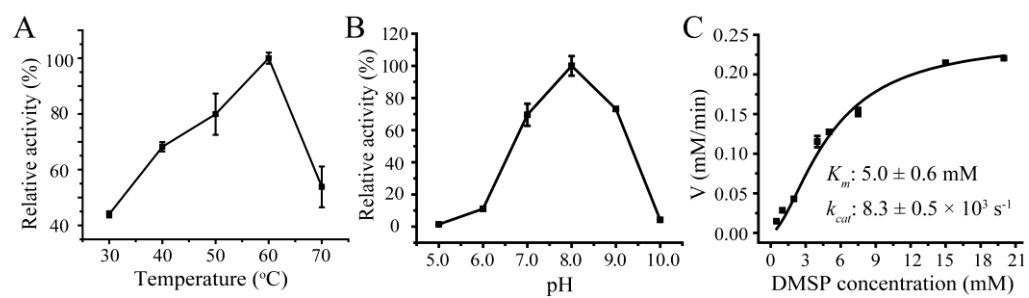


Figure 1

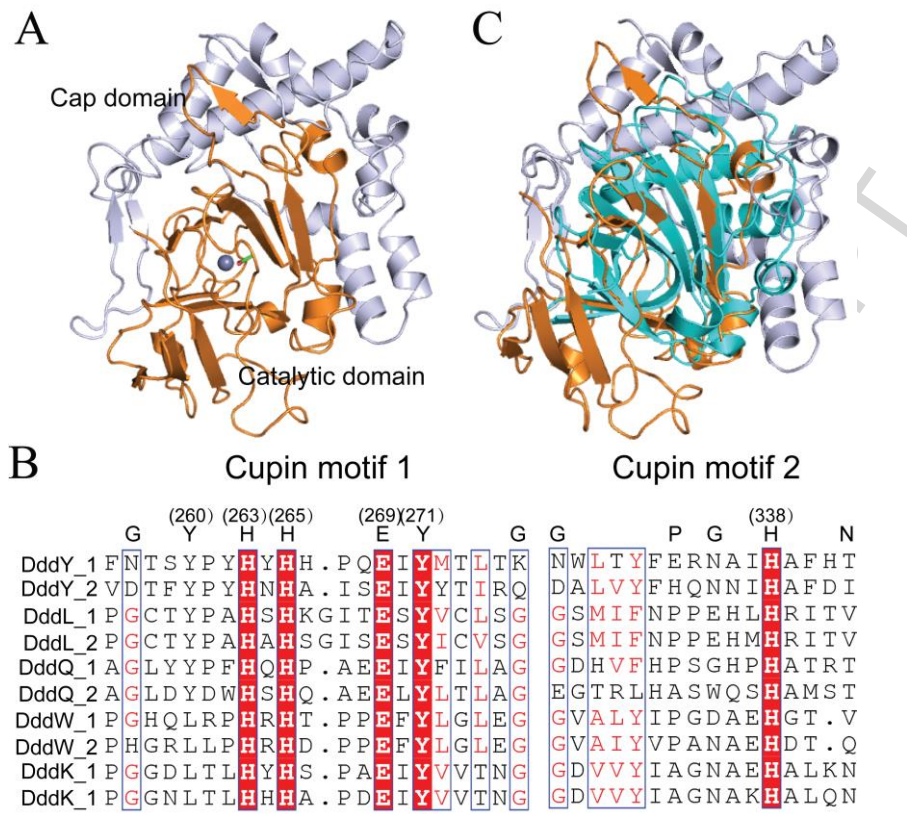


Figure 2

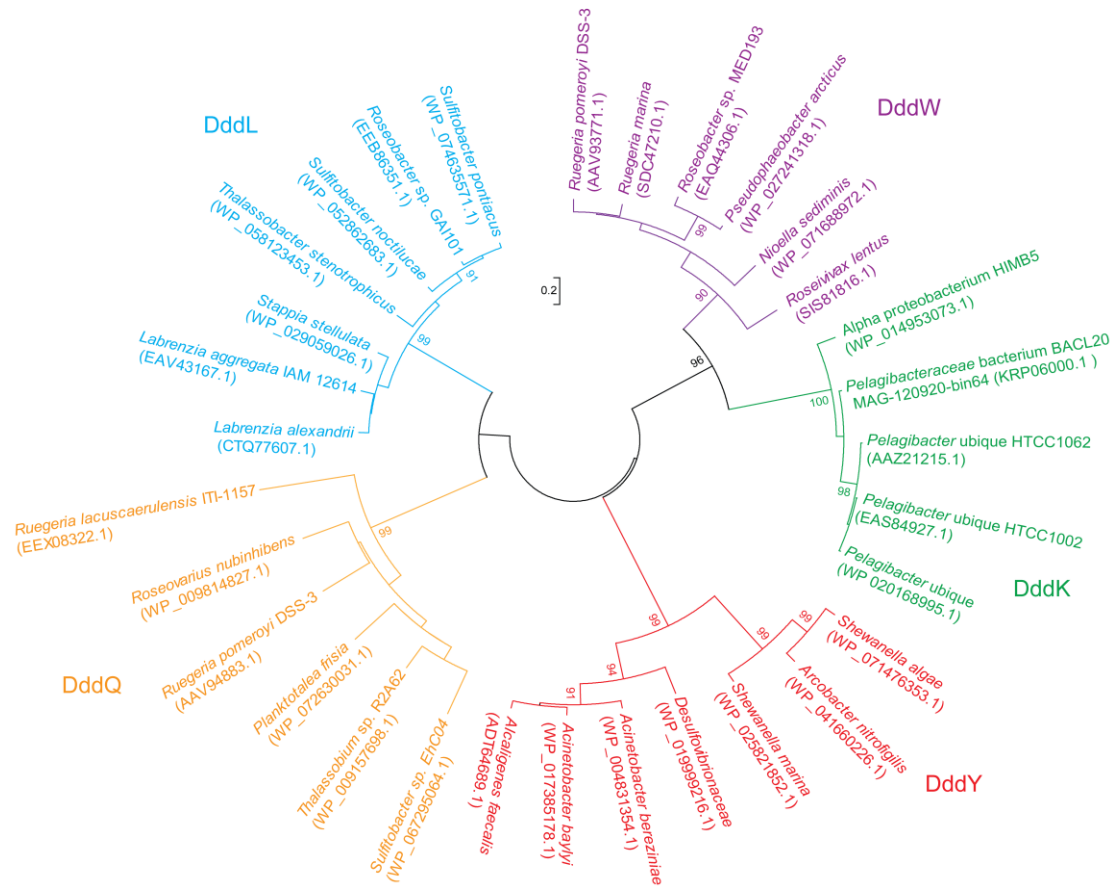


Figure 3

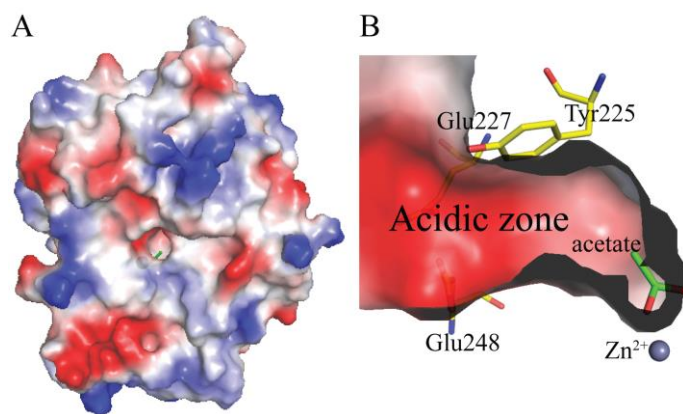


Figure 4

ACCEPTED MANUSCRIPT

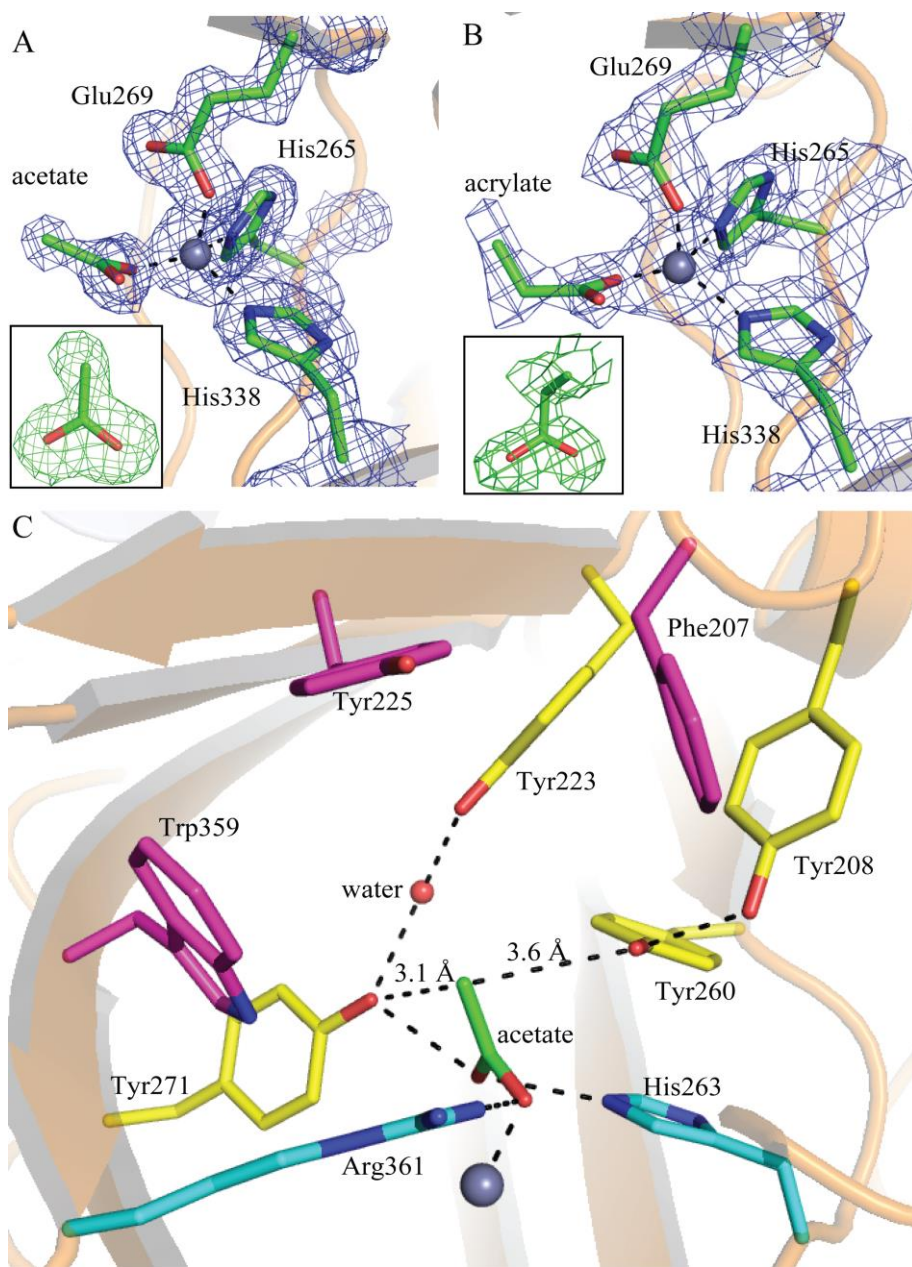


Figure 5

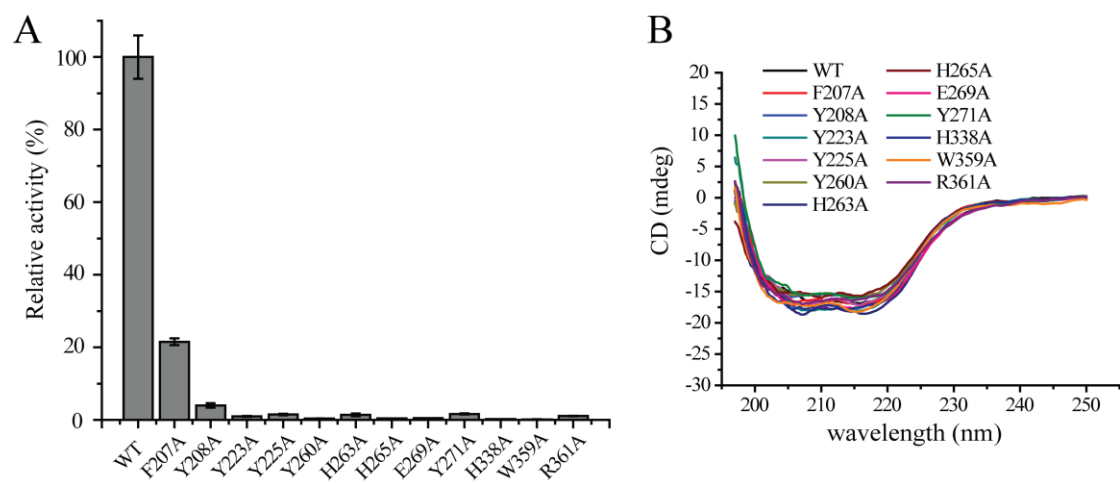


Figure 6

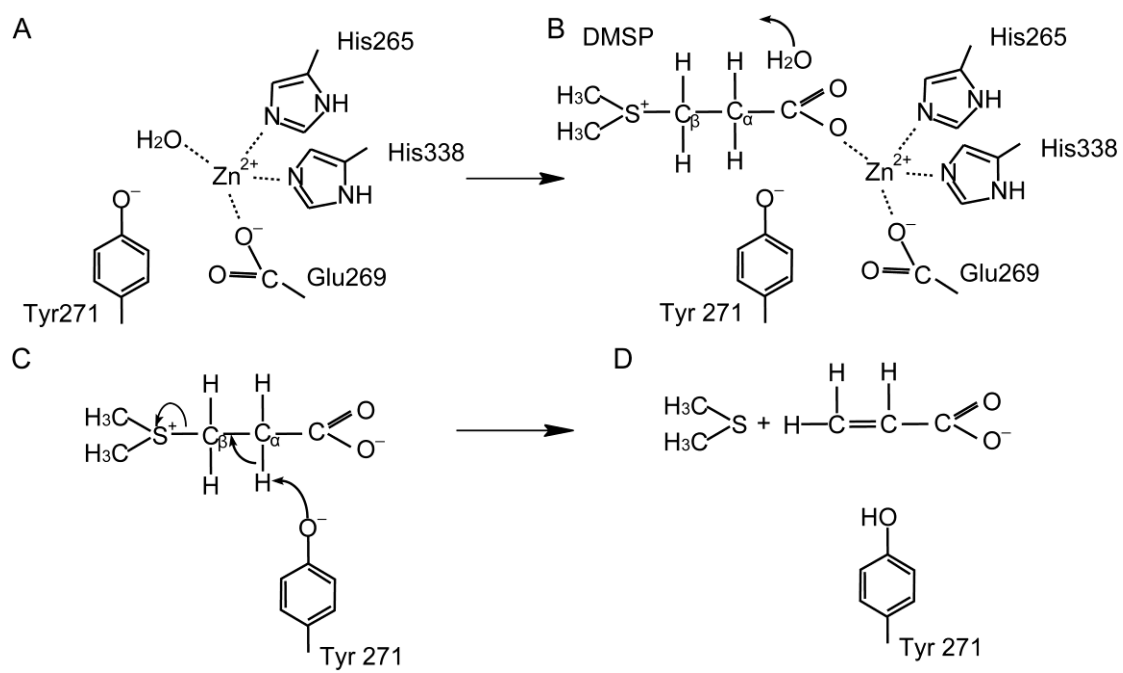


Figure 7

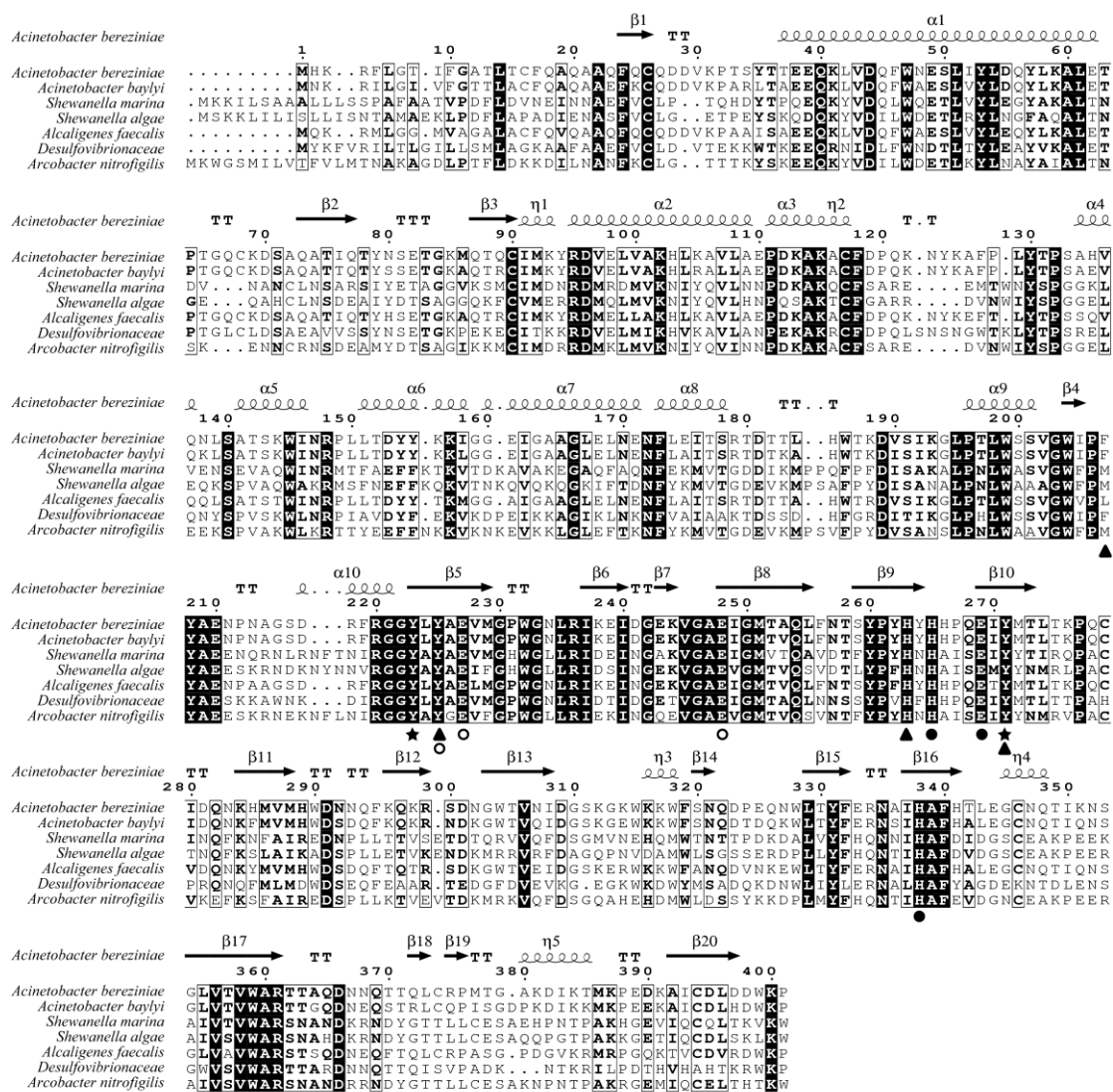
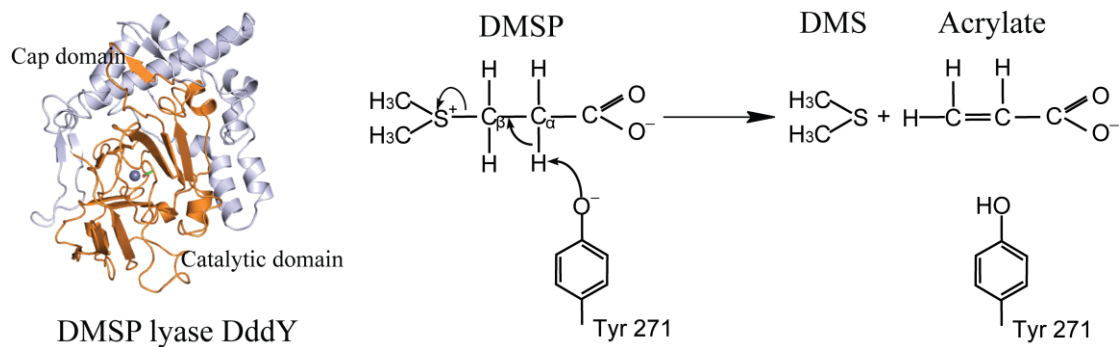


Figure 8



Graphical abstract

Table 1. Crystallographic data collection and refinement of DddY

Parameters	Se derivative	WT DddY	Y260A/acrylate
Diffraction data			
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit cell			
a, b, c (Å)	61.2, 72.4, 88.3	61.1, 72.1, 88.0	60.8, 72.6, 89.5
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution range	50.0-2.3	50.0-1.5	50.0-2.0
(Å)	(2.34-2.30) *	(1.55-1.50)	(2.03-2.00)
Redundancy	12.1 (12.5)	4.3 (4.2)	24.9 (23.2)
Completeness (%)	99.7 (99.7)	93.5 (97.4)	100 (100)
R_{merge}^{**}	0.1 (0.2)	0.1 (0.3)	0.2 (0.3)
$\ \sigma \ $	48.1 (27.1)	27.8 (7.0)	41.6 (18.0)
Refinement statistics			
R-factor	0.14	0.13	0.14
Free R-factor	0.20	0.18	0.19
RMSD from ideal geometry			
Bond lengths (Å)	0.007	0.005	0.007

Bond angles	0.88	0.79	0.79
$\overset{\circ}{\frown}$			
Ramachandran			
plot (%)			
Favoured	96.8	98.2	97.1
Allowed	3.2	1.8	2.7
Outliers	0	0	0.2
B-factors (\AA^2)			
Protein	17.5	17.5	17.7
Metal	26.4	13.4	13.5
Ligand	-	20.5	21.6
Water	22.8	32.5	26.3
All atoms	18.0	19.8	18.8

*Numbers in parentheses refer to data in the highest resolution shell.

** $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I(hkl)_i - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i \langle I(hkl) \rangle}$.

Highlights

- DddY is the only known periplasmic dimethylsulfoniopropionate (DMSP) lyase
- Crystallographic studies indicate that DddY belongs to the cupin superfamily
- The catalytic mechanism is proposed based on structural and mutational analyses
- The proposed mechanism of DddY may have universal significance