Structures of dimethylsulfoniopropionate-dependent demethylase from the marine organism *Pelagabacter ubique*

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Abstract: Dimethylsulfoniopropionate (DMSP) is a ubiquitous algal metabolite and common carbon and sulfur source for marine bacteria. DMSP is a precursor for the climatically active gas dimethylsulfide that is readily oxidized to sulfate, sulfur dioxide, methanesulfonic acid, and other products that act as cloud condensation nuclei. Although the environmental importance of DMSP metabolism has been known for some time, the enzyme responsible for DMSP demethylation by marine bacterioplankton, dimethylsulfoniopropionate-dependent demethylase A (DmdA, EC 2.1.1.B5), has only recently been identified and biochemically characterized. In this work, we report the structure for the apoenzyme DmdA from *Pelagibacter ubique* (2.1 Å), as well as for DmdA co-crystals soaked with substrate DMSP (1.6 Å) or the cofactor tetrahydrofolate (THF) (1.6 Å). Surprisingly, the overall fold of the DmdA is not similar to other enzymes that typically utilize the reduced form of THF and in fact is a triple domain structure similar to what has been observed for the glycine cleavage T protein or sarcosine oxidase. Specifically, while the THF binding fold appears conserved, previous biochemical studies have shown that all enzymes with a similar fold produce 5,10-methylene-THF, while DmdA catalyzes a redox-neutral methyl transfer reaction to produce 5-methyl-THF. On the basis of the findings presented herein and the available biochemical data, we outline a mechanism for a redox-neutral methyl transfer reaction that is novel to this conserved THF binding domain.

Keywords: global warming; oceanic sulfur cycling; methyl transfer; oceanic carbon cycling; enzymology; structural biology

Introduction

In some areas of the surface ocean, the production of dimethylsulfoniopropionate (DMSP) by phytoplankton may account for as much as 10% of the total carbon fixation.^{1,2} Although DMSP is used primarily as an intracellular osmolyte by phytoplankton, the com-

pound has also been recognized as an antioxidant and predator deterrent.^{3,4} While some phytoplankton can convert DMSP to dimethyl sulfide (DMS), upon phytoplankton lysis a considerable amount of DMSP is released and rapidly metabolized by the marine microbial food web.⁵ Marine bacterioplankton are considered to be the primary mediators of DMSP conversion in seawater and the consumption of DMSP by these organisms accounts for 1–15% of their carbon demand and most of their demand for sulfur.^{6–8} The degradation of DMSP by marine bacteria occurs through two competing pathways, with either cleavage or demethylation as the first step.

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<i>P. ubique</i> DmdA	37	-LPAAFG SI EDSYKHLKEHVQ IQ WDVAAERQVEISGKDS AE LVQLMTCRDLSKSKIGRCY YC -	96
<i>T. Maritima</i> GcvT	25	-MPLYYT SI FEEVMVRKS V GMFDVSHMG FL VKGPEAVSFIDFLITNDFSSLPDGKAI YS -	84
<i>A. globiformis</i> DmgO	497	-RTAVAMYDMTP L KRL EV SGPGAL KL LQELTADLAK-KPGAVTY T -	540
<i>Corynebacterium</i> SoxA	620	-VRDS V GM L DA ST L G KIE IR GKDA AE FLNRM Y TNGYTKLKVGMGR YG -	665
<i>P. ubique</i> DmdA	97	-PIIDEN GN LVNDPVVLKLDEN KW ISIAD---SDVIFFA KG ---LASGH--K-FD V KI--	145
<i>T. Maritima</i> GcvT	85	-VMCNEN GG IIDDLVVYKVPDEALMVVNA---ANIEKDFNW---IKSHS---KNF D VEV--	134
<i>A. globiformis</i> DmgO	541	-LLLDHAG G VRS D ITARLSEDT F QLGANGN---IDTAYFERAARHQTQSGS--A-TD V WQ--	595
<i>Corynebacterium</i> SoxA	666	-VMCKAD GM IFDDGVTLRLAEDR FL MHTTTGGAADVLDWLEE---WLQTEWPE-LD V TCT- 720	
<i>P. ubique</i> DmdA	146	---VEPVVDIMAIQ GP KSFALMEKVFGK---KITE--LK FF GFDY FD FE G TKHLIARSGW SK QG- 201	
<i>T. Maritima</i> GcvT	135	-SNISDTTALIA FQ PKARDLAKDL FG I---DINE--MW WF QARWVELD G IKMLLSRSGY TG ED- 192	
<i>A. globiformis</i> DmgO	595	-VRTTGGTCCIGLW GP LARDLVSKVSD---DFTNDGLKY FR AKNVVIG G IPVTAMRLSY VG EL- 655	
<i>Corynebacterium</i> SoxA	722	---VTEQLATVAV V GP SR SD VI AKLASSLDVSNDAFK MA FQD VT LDS-GIEARIS RI S FS GEL- 780	
<i>P. ubique</i> DmdA	202	-GY E VYVENTQSGQ KL YDHLFEV G KEFN---VGP-GCPNL-IER--IESALLSY CN DFDNDNP- 260	
<i>T. Maritima</i> GcvT	196	-G F ELML--AKNAPKVWDALM N LLRKIDGR PAG L-GARDV-C-R--LEATYLLY GQ DMDE NT NP- 252	
<i>A. globiformis</i> DmgO	659	-GW E LYT-SADNGQRLWDALWQ AG Q PF G---VIAAGRAA--FSSLRL E KGYRSWGTDMTTEHD P - 715	
<i>Corynebacterium</i> SoxA	781	-A F EIAI-PAWHGLQ V WED V YAA AG Q EF N---ITPYGTETMHVLR--AEKGFIIV GQ DTDGT VT P- 809	

Figure 1. Primary structure alignment for the dimethylsulfoniopropionate-dependent demethylase (DmdA) from *P. ubique*, the dimethylglycine oxidase from *A. globiformis*, the T protein of the glycine cleavage system from *T. maritima*, and the sarcosine oxidase from *Corynebacterium* Sp. Identical residues are colored red and similar residues are colored blue. The asterisks highlight residues that have a conserved interaction with THF. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The cleavage pathway, whether in phytoplankton or bacterioplankton, degrades DMSP to acrylate (or another C-3 compound) and DMS, with the latter compound serving as a major source of sulfur in the atmosphere.^{7,9,10} The aerosols that are formed in the atmosphere through interaction with DMS can influence global weather patterns through cloud condensation and increased backscatter of solar radiation.^{11,12}

The alternative to the cleavage pathway is the demethylation of DMSP to methyl-mercaptopropionate (MMPA). Dimethylsulfoniopropionate-dependent demethylase A (DmdA), the enzyme responsible for the demethylation of DMSP, has been recently isolated and characterized from two marine bacterial species *Ruegeria pomeroyi* and *Pelagibacter ubique*.⁶ In subsequent steps, MMPA can be further demethylated or demethiolated to form either mercaptopropionate or methanethiol.^{6,8,13} The rapid incorporation of methanethiol into the proteins of marine bacteria is well established.¹³

Initial insight into the function of DmdA comes from sequence searches that indicate that this enzyme is most closely related to the glycine cleavage system T-protein, dimethylglycine oxidase, and sarcosine oxidase.⁶ Structures for most of the latter enzymes are available in the PDB, in both the apo form and with various ligands bound.^{14–17} Although the residues of identity vary for each comparison, the sequence identity for the glycine cleavage system T-protein, dimethylglycine oxidase, and sarcosine oxidase is 26, 24, and 22%, respectively. As expected, many of the residues that are conserved are also involved in binding THF (see Fig. 1). As expected, it has been demonstrated that DmdA also has a requirement for the cofactor tetrahydrofolate

(THF).^{6,18–20} However, in comparison to these previously characterized enzymes a significant mechanistic difference is the observation that 5-methyl-THF is the product of the DmdA reaction.

Folate-dependent single carbon transfer reactions are widespread in metabolism, and include the biosynthesis of nucleotides, vitamins and amino acids.²¹ The THF N5 and N10 positions can accept single carbon groups that may consist of methyl, formyl, formimino, methylene, or methenyl. This leads to a number of distinct products and redox states for the THF cofactor. Of importance to this work is the observation that all the reactions of related proteins glycine cleavage T protein, dimethylglycine oxidase, and sarcosine oxidase, result in production of 5,10-methylene-THF (30,31) while the DmdA reaction results in production of 5-methyl-THF.^{6,20} Therefore differences can be expected in the reaction mechanisms of these proteins. In fact, the reaction that is catalyzed by DmdA must be explained in the context of a redox-neutral methyl transfer reaction from a sulfonium atom to THF. Similar to the methyl transfer reaction of methionine synthase, which involves formation of a methyl-cobalamin intermediate, there is no net change in the oxidation state of the THF cofactor.

To provide new insight into the mechanism of DMSP demethylation and insight into how a similar protein fold, with similar determinants for THF binding, would catalyze a notably different reaction, we have determined the crystal structure of DmdA from *P. ubique* (HTCC1062) alone, as well as in the presence of DMSP or THF. The models presented here provide further evidence for an evolutionarily conserved THF binding fold but also demonstrate

how the function of this binding motif can be expanded. Specifically, although the THF binding fold is conserved, the mechanism supported by the data discussed herein is unique in comparison to what has previously been reported.

Experimental Procedures

Enzyme isolation, purification, and crystallization

Expression and isolation of the dimethylsulfoniopropionate-dependent demethylase (DmdA) from recombinant *E. coli* that expressed the *P. ubique* DmdA (SAR11_0246) gene was performed as previously described.⁶ Because of the sensitivity of THF to oxygen, all crystal screening of DmdA was performed in an anaerobic chamber consisting of an atmosphere of 95% nitrogen and 5% hydrogen using the microbatch diffusion technique and an IMPAX I-5 (Douglas Instruments, East Garston, United Kingdom) crystallization robot. Prior to screening, the 48-well microbatch diffusion trays were coated with a mixture of 5% silicon and 95% paraffin oil (v/v). The robot then dispensed and mixed 1 μ L of protein solution with 1 μ L of precipitating solution. Screening with commercially available sparse matrix conditions, following by pH and precipitate optimization led to robust, diffraction quality crystals for the apo enzyme. The final mother liquor consisted of 325 mM NaCl, 30% PEG 2000, 25 mM HEPES pH 6.8. The initial concentration of PEG 2000 was 20%, but the transfer of crystals to higher concentrations in a step-wise manner resulted in lower mosaicity after freezing the crystals by rapid immersion in liquid nitrogen.

In addition to the native crystals, data was also collected on crystals that had been treated with either the substrate (2 mM DMSP) or the cofactor, tetrahydrofolate (2 mM THF). In both cases a solution of mother liquor was prepared containing either DMSP or THF. These solutions were prepared under strictly anaerobic conditions. After exposure to either DMSP or THF, the crystals were back-soaked in mother liquor containing increasing amounts of PEG 2000 as described above. The latter two treatments appeared to improve the diffraction quality of the crystals (See Table I). Attempts were made to try and turn the enzyme over in the crystal lattice by soaking DmdA crystals in both THF and DMPS for 30 min prior to cryo-soaking and freezing, but were inconclusive.

Data collection, phasing, and refinement

All data sets were collected at the Advanced Photon SER-CAT on beamline 22-ID. To find an appropriate model to use for molecular replacement and phasing, the DmdA protein sequence was used to search for proteins with similar sequences and known struc-

Table I. Data Collection and Refinement Statistics

	Data set		
	Apo ^a	DMSP soak	DMSP/THF soak
Resolution range ($\text{\AA}$)	50.0–2.1	50.0–1.6	50.0–1.6
R_{sym} (%) ^b	6.0 (28.5) ^c	8.0 (15.1) ^c	11.7 (23.1)
I/s	15.9 (4.4)	19.1 (13.2)	29.1 (7.4)
$\Delta F/\Delta\sigma$ (ΔF)			0.79 (0.8)
Redundancy	5.4 (3.7)	6.0 (4.5)	14.1 (6.2)
Unique reflections	76,276	179,466	190,789
R_{cryst} (%)	16.6	13.0	12.6
R_{free} (%)	23.0	18.4	17.6
RMSD bonds ($\text{\AA}$)	0.020	0.024	0.024
RMSD angles ($^\circ$)	1.71	1.96	1.95
Average B factor			
Protein	42.6	25.8	27.6
Ligand	—	27.5	38.7
Solvent	54.9	47.9	54.1

^a Data for the apo crystals was collected at an X-ray energy of 1.9 $\text{\AA}$ in order to increase the anomalous signal for sulfur.

^b $R_{\text{sym}} = \sum_{\text{hkl}} [\sum_i (I_{\text{hkl},i} - \langle I_{\text{hkl}} \rangle)] / \sum_{\text{hkl},i} I_{\text{hkl},i}$, where I_{hkl} is the intensity of an individual measurement of the reflection with indices hkl and $\langle I_{\text{hkl}} \rangle$ is the mean intensity of that reflection.

^c Numbers in parenthesis refers to the values in the outer resolution shell.

tures. The following coordinates were taken from the protein data bank and used as search models: 1YX2, a probable aminomethyltransferase from *Bacillus subtilis* (Unpublished, PDB code 1YX2); 1WOO, the T protein of the glycine cleavage system from *Thermotoga maritima*,¹⁵ 1V5V, the T protein of the glycine cleavage system from *Pyrococcus horikoshii*,¹⁶ and 1PJ5, the dimethylglycine oxidase from *Arthobacter globiformis*.²² Several programs from the CCP4 program suite were employed during structure determination and refinement.²³ Various molecular replacement attempts were made using the program PHASER,²⁴ with resolution from 3–5 $\text{\AA}$, and intact polypeptide vs. poly-alanine and poly-serine truncations, and with ensembles of multiple search models. Successful solutions were encountered with 1WOO residues 25–329, 1V5V residues 29–373, and with ensembles of those two models superimposed. The top translational log-likelihood score (TFZ) was 10.3. Polyalanine chains were placed according to the MR result and used to calculate model phases, which were run through density modification with solvent flattening, histogram matching and two-fold NCS averaging using MAGICSQUASH.²⁵ The resulting phases were used to generate an anomalous Fourier difference map that was of sufficient quality to identify the positions of the sulfur atoms. The sulfur positions were used to generate SAD phases, which were then combined with the MR model phases. Density modification and phase combination were interspersed with map fitting using Coot,²⁶ and refinement based on

maximum likelihood with REFMAC5.²⁷ The sulfur positions from the anomalous Fourier difference map served as guideposts during the map fitting. NCS restraints were maintained until the polypeptide was complete, and then the NCS restraints were removed for additional model building, water and ligand placement, and refinement of the native DmdA structure. The native structure of DmdA was then used as a molecular replacement model for determination of the cocrystal structures of DMSP or THF. In both of the latter cases, better diffraction was obtained (1.60 Å), and anisotropic thermal factors were allowed.

Results and Discussion

Domain architecture of DmdA

DmdA belongs to a diverse family of enzymes that includes the glycine cleavage T-protein as well as specific domains of dimethylglycine oxidase and sarcosine oxidase, with the strongest similarity to the glycine cleavage system. A common trait amongst these family members is requirement for a THF cofactor. However, both dimethylglycine oxidase and sarcosine oxidase are larger proteins with additional domains as well as flavin binding sites and therefore the glycine cleavage T-protein serves a better example for comparison with DmdA as judged by overall sequence and fold comparison. The sequence alignment for *P. ubique* DmdA with that of the *A. globiformis* dimethylglycine oxidase, and the T-protein of the glycine cleavage system from *T. maritima* is shown in Figure 1. The sequence identity between DmdA and T-proteins of known glycine cleavage systems averages about 25%.⁶ While this may suggest a conserved structure, phylogenetic analyses has shown that proteins with a confirmed function as glycine cleavage T-proteins from prokaryotes, eukaryotes, and archaea cluster together but are distinct from proteins that demonstrate DMSP demethylase activity.⁶ This strongly suggests that DmdA, while structurally similar, is evolutionarily more distant from the T-proteins. Consistent with this view, is the observation that relatively few amino acids, mostly those that interact with THF, are conserved (highlighted by asterisks in Fig. 1).

Nonetheless, the tri-domain structure observed for the T-protein of the glycine cleavage system is clearly apparent in the overall fold of DmdA (Fig. 2). Interestingly, we observed two monomers in the asymmetric unit for DmdA with some domain swapping at the N-terminus for residues 1–11. These observations are consistent with previous work that has shown DmdA functions as a dimer in solution^{5,6} (see Fig. 2, Panel A). This is a distinct functional difference in comparison to the T-proteins that do not function as a dimer and do not show any domain-swapping in their structures.

Figure 2. Cartoon representation of the overall structure of the Dimethylsulfoniopropionate-dependent demethylase (DmdA) from the marine bacterium *P. ubique*. **A**, The crystallographic dimer observed in the asymmetric unit with monomer chains A and B colored light brown and green respectively. **Panel B**; A 90° rotation from the view presented in Panel A showing a single monomer and coloring the molecule by secondary structure with helices, sheets, and random coils shown in green, cyan, and beige respectively. The individual domain structures are highlighted by the dashed areas.

The three domains of DmdA are highlighted in Figure 2, Panel B. Domain 1 (residues 1–62 and 150–248) contains a single Greek Key motif surrounded by three α -helices. Domain 2 (residues 63–149 and 249–288) consists of a five-stranded antiparallel β -sheet with α -helices on either side. The active site for DmdA is positioned in a cleft between domains 1 and 2 with the primary opening to solvent facing away from the dimer interface. The antiparallel β -sheets from domains 1 and 2 are packed

Figure 3. Wall-eyed stereo view of the model, $2F_o-F_c$ composite omit map (blue electron density mesh), and the $1F_o-F_c$ difference map (green electron density mesh) for data collected on crystals soaked with tetrahydrofolate (THF). The $2F_o-F_c$ composite omit map is contoured at 1.5 s and was generated using the simulated annealing protocol with 7% of the model omitted per cycle. The $1F_o-F_c$ (difference) map was generated after omission of the atoms for THF from the map calculation and is contoured at 3.0 s. Carbon, oxygen, and nitrogen atoms are represented by sticks and colored tan, red, and blue, respectively.

against one another on one side of the active site and solvent has substantial access throughout the active site cleft. The C-terminal domain 3 (residues 289–369) forms a distorted jellyroll that, also in contrast to the T protein from the glycine cleavage system, does not contain a significant C-terminal extension covering domains 1 and 2 (Fig. 2). The arrangement of these domains and the identification of this overall fold as a prototype for THF binding has been uncovered within the last decade^{14,28} and essentially comprises the cloverleaf-like positioning of these three domains. Despite the low sequence identity of 25% or less seen in Figure 1, the fold is conserved between these three enzymes. For clarity we have highlighted the active site cleft with an arrow in Figure 1.

The tetrahydrofolate binding site

To provide insight into the substrate and cofactor binding modes, anaerobically prepared crystals were soaked with either THF or DMSP. Multiple rounds of model building and anisotropic refinement lead to a model with occupancies values of 0.75 for the THF molecules and omission of the THF molecules lead to a substantial increase in the values of R and R_{free} , consistent with the difference density shown in Figure 3 (green electron density mesh) and Figure 4. The cofactor difference density in the cleft between domains 1 and 2 was clearly visible when no THF was modeled in the active site (see Fig. 3). These observations are consistent conservation of the overall THF binding fold. Moreover, consistent with the secondary structure alignments shown in Figure 1, there are a few strictly conserved residues positioned

in such a way as to form a hydrogen bond with the folate moiety of THF. This included residues Y206 and E204. Another conserved residue, Y95, has a role in ring stacking with the THF cofactor.

Although some of the interactions of THF appear to be fairly highly conserved, when the primary structure of DmdA is compared with other enzymes with a similar fold, there are significant differences as well. For example, in marked contrast to what was observed for *T. Maritima* glycine cleavage T-protein and *A. globiformis* dimethylglycine oxidase, a significant amount of acidic side chains also interact with tetrahydrofolate cofactor and line the active site around the folate moiety. This includes residues E204, Y206, S122, and D108. E63 is also within hydrogen bonding distance of E204 as well as S122. These observations are distinctly different from what has been observed previously for this THF binding fold and demonstrates that DmdA presents a rather electrostatically negative binding pocket for the folate ring of the THF. Figure 4 shows the relative positioning of THF in DmdA relative to some conserved interactions that are observed in dimethylglycine oxidase, and the glycine cleavage T protein. Interestingly, the relative positioning of Y206 in DmdA is different in comparison to the other THF dependent enzymes (see Fig. 4, Panel A) but the interaction, in this case a hydrogen bond, remains the same. Specifically, this tyrosine provides a hydrogen bond to the amine group of the folate ring on the C-2 carbon atom. In addition, there are some unique aspects of the THF binding domain in DmdA that support a novel mechanism. Specifically, there is an additional acidic residue (E63) as well as

Figure 4. Superposition of the tetrahydrofolate cofactor and surrounding residues of DmdA, dimethylglycine oxidase, and the glycine cleavage T protein showing the relative positions of conserved residues. Panel A, Oxygen atoms are in red, nitrogen in blue, and carbon atoms colored according to structure. Tan: DmdA with THF, green: dimethylglycine oxidase from *A. globiformis* with folinic acid (PDB entry 1PJ7)(23), and yellow: glycine cleavage T-protein from *T. Maritima* with THF (PDB entry 1WOO) (33). Panel B; Close up of the THF-bound DmdA model showing additional residues within hydrogen bonding distance and Van der Waals contact of the folate ring of THF. The electron density around the THF is not shown in order to show the N-5 site of methylation (highlighted by the arrow). Panel C, Close-up showing two critical hydrogen bonding interactions (dashed lines) between the THF molecule and the carbonyl oxygen atom of S122 (1) and hydroxyl group of Y206 (2). The electron density is identical to what is described in Figure 3.

another potential hydrogen bond donor/acceptor from the oxygen atom of S122. For reference, the ultimate destination of the methyl group coming from DMDA is highlighted by the black arrow in Panel B and by a red asterick in Panel C of Figure 4, respectively. As can be seen in Figure 4, Panel C,

a backbone carbonyl oxygen atom from residue 122 is within 2.8 Å of the N-8 nitrogen atom of THF. It has been well established that the planarity of the peptide bond is due to electronic resonance. In fact, not only can the carbonyl oxygen become negatively charged, but the N—H protons of protein backbones

Figure 5. Wall-eyed stereo view of the model, $2F_o - F_c$ composite omit map (blue electron density mesh), and the $1F_o - F_c$ difference map (green electron density mesh) for data collected on crystals treated with dimethylsulfoniopropionate (DMSP). The $2F_o - F_c$ composite omit map is contoured at 1.5 σ and was generated using the simulated annealing protocol with 7% of the model omitted per cycle. The $1F_o - F_c$ (difference) map was generated after omission of the atoms for DMSP from the map calculation and is contoured at 3.0 σ . Multiple conformations for S246 are shown. Carbon, oxygen, and nitrogen atoms are represented by sticks and colored tan, red, and blue, respectively.

can readily be exchanged via formation of the imidic acid.²⁹ In any case, this observation suggests that a strong hydrogen bond is shared between these two atoms. Moreover, the activity that has been reported for DmdA and the electron density of the THF molecule both support a reduced form of THF with both the N-5 and N-8 atoms of the folate moiety adopting Sp^3 hybridization (Fig. 4, Panels B and C). This is consistent with the physiological reaction catalyzed by DmdA and distinctly different from the enzymes discussed above that utilize a similar binding fold for THF in notably different metabolic reactions. Specifically, in contrast to the glycine cleavage T protein, dimethylglycine oxidase and sarcosine oxidase, DmdA catalyzes a redox-neutral methyl transfer reaction that results in production of the 5-methyl-THF. The presence of a hydrogen bond from a backbone carbonyl to the N-8 nitrogen atom (highlighted by “1” in Fig. 4, Panel C) would certainly be consistent with a mechanism for making the N-5 nitrogen atom (highlighted by the “*” in Fig. 4, Panel C) a better nucleophile in order to attack the methyl group on the sulfonium atom of dimethylsulfopropionate (DMDA).

The substrate binding mode

If the proposal above is correct, then we would expect that the substrate for DmdA, DMSP, to bind close to W197 in such a way that the methyl group on the sulfonium ion is positioned close to the N-5 nitrogen atom of the THF molecule, as observed in

the structure above. To this end, DmdA crystals were soaked with DMSP alone and a difference map revealed density consistent with substrate binding (Fig. 5). Consistent with the observed binding mode of THF and our proposal, the side chain of W197 is significantly repositioned in order to accommodate the DMSP molecule. Similar to the THF-bound structure, iterative rounds of model building and refinement resulted in occupancy values for the atoms of the DMSP molecules of 0.6 and 0.8 for the DMSP molecules in the A and B monomers, respectively. In addition, there is clear evidence in the density maps that S246 is in multiple conformations (Fig. 5, blue electron density mesh). The positioning of the substrate and the identification of specific interactions with residues S246, R11, W197, and Y32, leaves relatively little room for movement. These observations are consistent with previous work that showed dimethylsulfoniobutanoate and dimethylsulfoniopentanoate are poor substrates for DmdA.⁶ An overall comparison of the apo DmdA structure with either the THF-bound or substrate bound form reveals that the majority of changes in the peptide are focused on primarily two amino acids, W197 and S246. While both of these residues contribute to the active site, the residues that are conserved tend to have specific interactions with the THF cofactor and appear to adopt similar conformations, regardless of the presence of substrate or THF.

Attempts were made to try and turn the enzyme over in the crystal lattice as well as to try and

Figure 6. Relative position of DMSP and THF and important hydrogen bonds in the active site of DmdA. Panel A, Stick diagram showing the relative positions of DMSP, THF, S122, Y206, and E204 in the DMSP-bound as well as the THF-bound model. Carbon, nitrogen, oxygen, and sulfur atoms are colored tan, blue, red, and yellow, respectively. The Figure was generated by simply loading both the DMSP-bound and THF-bound model into PYMOL and then displaying the selected residues in both structures. Panel B, Stick diagram and composite omit map (blue electron density mesh) for some important side chains and ordered water molecules in the active site of the THF-bound model. Dashed lines and numbers identify close interaction and the distances between the respective atoms in angstroms. In all cases, carbon, nitrogen, and oxygen atoms are colored tan, blue, and red. The composite omit map was generated as described in the previous figure legends. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

collect data on DmdA crystals that were soaked with the product, MMPA. However, in both cases an interpretation of density in the substrate binding site was not clear and suggested a low occupancy for the MMPA molecule (data not shown).

A plausible mechanism for DmdA

Given the available biochemical information, the data reported herein, and what is known about redox-neutral methyl transfer reactions involving THF as well

as methyl transfer from a sulfonium atom to a nitrogen atom, a reasonable mechanism can be identified. As a rational first step we begin by comparing the relative positions of DMSP, THF, and some of the conserved residues in both models. A reasonable starting point for this is to simply overlay the THF- and DMSP-bound models and look at the relative spatial positioning of these two molecules as well as the side chains of key residues. If this is done, it is clear that in order to accommodate the DMSP molecule in the THF-bound model, relatively little has to change. In fact, removal of some water molecules and the movement of the side chain of W197 is all that needs to occur. In the observed binding mode, the closest approach of a methyl group on DMSP is ~ 3.3 Å (Fig. 6, Panel A). In addition to the relative positions of THF and DMSP, a number of well-defined water molecules are seen in the electron density for the THF-bound model. One of these is within 2.6 Å of the N-5 nitrogen atom of THF (Fig. 6, Panel B). Again, these observations are consistent with previous biochemical data that demonstrated DmdA produces 5-methyl-THF^{11,30} and is in marked contrast to its structural relatives, specifically, the glycine cleavage T-protein, dimethylglycine oxidase, and sarcosine oxidase all produce 5,10-methylene-THF.^{31,32} This further supports the conclusion that the reaction mechanism for DmdA is substantially different from that of other enzymes with a similar THF binding fold.^{14,16}

Considering the observations above, the accessibility water has to the active site, the number of acidic side chains in the active site (E63, D108, and E204), and the binding mode of DMSP relative to THF, all of the available evidence points to a concerted mechanism in which methyl transfer is coupled to proton transfer. This would be a concerted reaction, much like what has been proposed for *S*-adenosylmethionine (SAM)-dependent *N*-methyltransferases. Similar to SAM, the substrate for DmdA, also contains a sulfonium atom, making the methyl group a fairly good leaving group already. As outlined in Figure 7, and consistent with the literature on these enzymes,^{22,31} we propose that the methyl transfer reaction proceeds via an S_N2 intermediate with concerted methyl and proton transfer that is mediated by a water molecule in the active site (Fig. 7). Further support for this proposal comes from the observation that a number of SAM-dependent methyltransferases, such as protein arginine, glycine-N, and phenylethanolamine-N methyltransferase also help facilitate such a mechanism through hydrogen bonds involving acidic residues,^{30,32,33} and polarization of the nitrogen atom targeted for methyl transfer. It has been proposed that polarization of the substrate lowers the energy barrier to the S_N2 -like methyl transfer reaction.^{34,35} For DmdA, a number of acidic residues, including E63, D108, and E204 are all within hydrogen bonding range of THF

Figure 7. Proposed reaction mechanism for the redox-neutral methyl transfer reaction catalyzed by DmdA. The hydrogen bond from Y206 is a conserved feature of the THF binding motif. In addition, DmdA also contains a number of acidic side chain that line the interior of the active site and an additional hydrogen bond from the backbone carbonyl oxygen atom of S122 (see Fig. 4, Panel C). The Sp^3 hybridized N-5 atom of THF is already nucleophilic and, similar to what has been reported for the SAM-dependent *N*-methyltransferases, interactions in the active site support a concerted methyl/proton transfer mechanism facilitated by water.

or water molecules that are. Another important observation is the hydrogen bonding interaction with the backbone carbonyl of S122. This interaction will impact the nucleophilic character of the N-5 atom of THF, which is Sp^3 hybridized and fairly nucleophilic already. At this time we can not conclusively identify the side chain that acts as a general base in our proposed reaction mechanism, but one would expect a significant kinetic isotope effect in D_2O and identification of the general base could be made through site directed mutagenesis studies.

The mechanism proposed in Figure 7 is also supported by mutational and theoretical studies of the SAM-dependent *N*-methyltransferases and the proposed role of acidic residues and ordered solvent molecules in facilitating the concerted methyl and proton transfer reaction via the S_N^2 intermediate.²² The presence of a tryptophan residue parallel to the sulfonium atom of the DMSP substrate in DmdA also makes sense (see Fig. 5). Specifically, in light of the proposed mechanism, partial stabilization of the positively charged sulfonium moiety may be beneficial. This is accomplished by stacking against the partial negative charge emanating from the ring current of the W197 side chain (see Fig. 5). W197 is also unique to DmdA when compared with other enzymes with the same THF binding fold (Fig. 1).

In conclusion, our results show that the overall fold of DmdA and its THF binding site resembles that of its closest relatives, while the reaction mechanism more closely resembles the SAM-dependent methyl transferases that perform *N*-methylation reactions. This clearly provides new evidence for the

catalytic plasticity of this fold. In the case of the ecological niche occupied by DmdA, the reaction mechanism proposed for DmdA also makes sense as the product of methyl transfer reaction (in this case is *N*5-methyl-THF) is a less oxidized compound compared with 5,10 methylene-THF, and thus the DmdA reaction provides a significant selective advantage for these marine organisms living on meager energetic feedstocks. This is particularly important for the marine oligotroph *P. Ubique*, as this demethylation reaction allows the bacterium to recoup and utilize the carbon that would otherwise be lost during the DMSP cleavage reaction.

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