The *dddP* gene of *Roseovarius nubinhibens* encodes a novel lyase that cleaves dimethylsulfoniopropionate into acrylate plus dimethyl sulfide

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The cloned *dddP* gene of the marine bacterium *Roseovarius nubinhibens* allows *Escherichia coli* to form the volatile dimethyl sulfide (DMS) from dimethylsulfoniopropionate (DMSP), an abundant anti-stress compatible solute made by many marine plankton and macroalgae. Using purified DddP, we show here that this enzyme is a DMSP lyase that cleaves DMSP to DMS plus acrylate. DddP forms a functional homodimeric enzyme, has a pH optimum of 6.0 and was a K_m of ~14 mM for the DMSP substrate. DddP belongs to the M24B family of peptidases, some members of which have metal cofactors. However, the metal chelators EDTA and bipyridyl did not affect DddP activity *in vitro* and the as-isolated enzyme did not contain metal ions. Thus, DddP resembles those members of the M24B family, such as creatinase, which also act on a non-peptide substrate and have no metal cofactor. Site-directed mutagenesis of the active-site region of DddP completely abolished its activity. Another enzyme, termed DddL, which occurs in other alphaproteobacteria, had also been shown to generate DMS plus acrylate from DMSP. However, DddL and DddP have no sequence similarity to each other, so DddP represents a second, wholly different class of DMSP lyase.

Received 8 March 2010

Accepted 4 April 2010

INTRODUCTION

The compatible solute dimethylsulfoniopropionate (DMSP) is made in large amounts (~10⁹ tons per annum, worldwide) as an anti-stress compound by many marine phytoplankton and some higher plants (Stefels, 2000). This molecule acts as a substrate for a range of different catabolic pathways and has major environmental significance in the global sulfur cycle (Sievert *et al.*, 2007). Some of the DMSP can be broken down by the producing algae themselves (Cantoni & Anderson, 1956; Steinke *et al.*, 1998) but the DMSP that is released via viral lysis, predation or leakage is metabolized by other marine microbes (see Yoch, 2002).

At a global level, most of the DMSP is catabolized by bacteria that contain a DMSP demethylase, encoded by the *dmdA* gene, which occurs in many abundant marine

alphaproteobacteria (Howard *et al.*, 2006; Reisch *et al.*, 2008). However, other microbial pathways generate dimethyl sulfide (DMS) as one of the products. This volatile molecule has significant properties in its own right, since DMS oxidation products can act as cloud condensation nuclei that can initiate cloud cover over the oceans, affecting global albedo and providing a major route for the transfer of sulfur back to land through precipitation (Simo, 2001). DMS also acts as an attractant for many marine animals, since it is a potent chemical signal for the presence of potential food supplies (DeBose & Nevitt, 2008).

Recent genetic work has shown that different bacteria and fungi can generate DMS by several different ways, via enzymes encoded by different *ddd* (DMSP-dependent DMS) genes. Thus, the *dddD* gene was originally found in some gammaproteobacteria, including *Halomonas*, *Marinomonas*, *Psychrobacter* and *Pseudomonas* (Todd *et al.*, 2007, 2009b; Curson *et al.*, 2010), but was also likely transferred by horizontal gene transfer (HGT) to other bacteria, including some 'terrestrial' strains of rhizobia and *Burkholderia* (Todd *et al.*, 2007). The DddD polypeptide product is in the family of class III CoA-transferases, and its action on DMSP generates DMS plus 3-hydroxypropionate

Abbreviations: DMS, dimethyl sulfide; DMSP, dimethylsulfoniopropionate; ICP-OES, inductively coupled plasma, optical emission spectrometry; HGT, horizontal gene transfer; MBS, mixed buffer solutions; MES, 2-(*N*-morpholino) ethanesulfonic acid; SDM, site-directed mutagenesis.

A supplementary table of primer sequences is available with the online version of this paper.

(Todd *et al.*, 2009b). In contrast, Curson *et al.* (2008) found that *dddL*, which occurs in several marine alphaproteobacteria, encodes a 'DMSP lyase', an enzyme that cleaves DMSP into acrylate plus DMS and a proton (Cantoni & Anderson, 1956; Fig. 1).

A third gene, termed *dddP*, also occurs in some strains of alphaproteobacteria, including *Roseovarius nubinhibens* ISM. More surprisingly, functional DddP homologues were also found in some *Aspergillus* and *Fusarium spp.*, pointing to inter-Domain HGT of *dddP* from bacteria to these eukaryotic ascomycete fungi (Todd *et al.*, 2009a; Kirkwood *et al.*, 2010). The advent of large-scale metagenomic sequencing of microbial subgenomic fragments allows the abundance of any given gene in natural populations to be estimated. Thus, in the huge Global Ocean Sampling dataset (Rusch *et al.*, 2007) the *dddP* gene is more abundant than *dddL* or *dddD* and occurs in a significant minority of all the bacteria in oceanic surface waters (Todd *et al.*, 2009a), though it is not as common as *dmdA*, which encodes the DMSP demethylase (Howard *et al.*, 2008). Interestingly, *dddP* also occurs in the genomes of phages isolated from environments as diverse as the Arctic Ocean and a coral reef on Kiritimati, an island in the Pacific Ocean (Raina *et al.*, 2010). Thus, *dddP* is widespread, both geographically and taxonomically.

The DddP polypeptide is in the M24B family of peptidases, several members of which have been shown to bind metals, such as manganese and cobalt as cofactors (see Graham *et al.*, 2005; Rawlings *et al.*, 2010; and MEROPS, at http://merops.sanger.ac.uk/cgi-bin/organism_index?type=P;id=A). To date, the mechanism of DMSP breakdown via DddP has not been documented. Here, we describe several of the properties of the purified DddP enzyme of *R. nubinhibens* ISM and show that it, too, is a DMSP lyase.

METHODS

Molecular genetic techniques and site-directed mutagenesis (SDM). Routine handling of DNA was done as described by Wexler *et al.* (2001). The *dddP* gene (ISM_05385) of *R. nubinhibens* ISM (González *et al.*, 2003) had been cloned into the expression vector pET21a (Novagen) to form pBIO1658 (Todd *et al.*, 2009a). This plasmid was used as the template for SDM with a Quikchange XL kit (Stratagene) according to the manufacturer's instructions. Six site-directed mutants of DddP, namely D295A, D297A, D307A, H371A, E406A and E421A were made using the mutagenic oligonucleotides shown in Supplementary Table S1 (available with the online version of this paper). Mutated plasmids were confirmed by sequencing.

Overexpression and purification of DddP protein. During all DddP purification procedures, enzyme activity was monitored by

assaying DMSP-dependent DMS production (see below) and by monitoring the presence of the 50 kDa DddP polypeptide, seen following protein separation by using SDS-PAGE and staining with Coomassie Blue.

Cultures (100 ml) of *Escherichia coli* strain BL21 (DE3) pLysS (Miroux & Walker, 1996) containing plasmid pBIO1658 were grown at 37 °C in Luria–Bertani broth containing 100 µg ampicillin ml⁻¹ to an OD₆₀₀ of 0.4–0.6. To induce *dddP* expression, 10 µM IPTG was added, followed by incubation at 25 °C until cells reached the stationary phase. Cells were pelleted by centrifugation and resuspended in 0.1 vols 20 mM Tris buffer (pH 8) at 4 °C. Cells were lysed by sonication (6 × 10 s) and cell debris was removed by centrifugation. Then, 25 % (w/v) (NH₄)₂SO₄ was added to the lysate and the resulting precipitate was removed by centrifugation. The supernatant was loaded onto a phenyl sepharose high-performance column (xk16/20; GE Healthcare) equilibrated with 20 mM Tris containing 25 % (w/v) (NH₄)₂SO₄. Proteins were eluted using a 25–0 % (NH₄)₂SO₄ gradient (flow rate 3 ml min⁻¹). Fractions that contained DddP polypeptide were then applied to a DEAE (HiTrap, 5 ml; GE Healthcare) column equilibrated with 20 mM Tris buffer, pH 8 (flow rate 5 ml min⁻¹). Proteins were eluted in the same buffer with a linear gradient of 0–1 M NaCl. Fractions containing DddP were loaded onto a Superdex 200 gel filtration column (10/300GL; GE Healthcare) equilibrated with 50 mM MES buffer, pH 6 (flow rate 1 ml min⁻¹). DddP-containing fractions were pooled and stored at 4 °C. DddP concentration was estimated from A₂₈₀ measurements, using ε_{280 nm} = 76 860 M⁻¹ cm⁻¹, as calculated from the numbers of tryptophan, tyrosine and cysteine residues in the protein (Gill & von Hippel, 1989).

DMS production assays. For *in vitro* assays with purified DddP, the protein (~10 µM) was added to an appropriate buffer solution (see below) containing different concentrations of the substrate DMSP in a sealed 1 ml vial (Alltech Associates). For routine assays, DMSP was used at a final concentration of 5 mM. Following incubation at 30 °C, the DMS in the headspace was quantified by gas chromatography as described by Todd *et al.* (2009a).

To determine the pH optimum for DddP activity, a mixed buffer solutions (MBS) of 50 mM K₂HPO₄, sodium citrate, Tris and *N*-cyclohexyl-2-aminoethanesulfonic acid was used to generate solutions in the pH range 2.0–9.0. HCl or NaOH were used to achieve the desired pH before assaying DMS production.

The effects of temperature on DddP activity were examined by incubating the reaction mixtures in 50 mM MES buffer, pH 6.0 over a range from 25 °C to 70 °C, at 5 °C intervals.

To examine the effects of metal availability on DddP function, the metal chelators 2,2'-bipyridyl (2.5 mM) or EDTA (25 mM) were added to DddP in MES buffer pH 6 and incubated for 15 min at room temperature before adding DMSP substrate and assaying as above.

K_m and V_{max} studies were done with ~0.3 µM DddP in MES buffer, pH 6, with DMSP concentrations ranging from 1 to 20 mM. Samples were incubated at 30 °C and DMS headspace measurements were taken at regular time intervals.

Analytical ultracentrifugation. Analytical ultracentrifugation was done at 12 000 r.p.m., 20 °C, in a Beckman Optima XL-I analytical

Fig. 1. Representation of the cleavage of DMSP to form DMS plus acrylate plus a proton.

ultracentrifuge, with absorbance optics and an An50Ti rotor. Partial specific volumes were estimated from DddP amino acid sequences using SEDNTERP software, version 1.05 (Philo, 1997). Scans were recorded every 4 h to determine when protein samples had reached equilibrium, when five scans were recorded per sample. DddP (~5 µM) was in 20 mM Tris, 100 mM NaCl, pH 8. Data were analysed using Ultrascan (Demeler, 2005) and fitted to a one-component model.

Detection of substrates and catabolites. Labelled [1-¹⁴C]DMSP (2.7 kBq) or [1-¹³C]DMSP were added to cell-free extracts of *E. coli* strain BL21 (DE3) pLysS containing plasmid pBIO1658, or to preparations of DddP, to a final concentration of 1 mM and incubated for 2 h. The metabolites of [1-¹⁴C]DMSP were resolved by HPLC and detected by tandem suppressed ion conductivity, UV absorption at 210 nm and online scintillation counting. Proton-decoupled ¹³C NMR spectra of the [1-¹³C]DMSP catabolites were measured at 75 MHz with a Varian Gemini 2000 in distilled water as described by Todd *et al.* (2009b).

Inductively coupled plasma, optical emission spectrometry (ICP-OES). ICP-OES was carried out on samples of 10 µM DddP in 2.5% (v/v) nitric acid. A varian vista pro CCD simultaneous ICP-OES, with axial torch, concentric seaspray nebuliser (Glass Expansion) and 50 ml cyclonic spray chamber was used to analyse triplicate samples. The power was 1.2 kW and the analysed wavelengths were: Co, 228.62 nm; Cu, 324.75 nm; Mn, 259.37 nm; Ni, 230.30 nm; Zn, 213.86 nm. The limits of detection for Co, Cu, Mn, Ni and Zn were 7.3, 5.3, 9.1, 206 and 10.0 nmol, respectively.

RESULTS AND DISCUSSION

DddP is a DMSP lyase that cleaves DMSP to DMS plus acrylate

The cloned *dddP* gene of *R. nubinhibens* and of some strains of *Aspergillus* and *Fusarium* fungi had previously been shown to confer on *E. coli* the ability to generate DMS from DMSP (Ddd⁺ phenotype), but the identity of the other catabolite(s) was not established (Todd *et al.*, 2009a; Kirkwood *et al.*, 2010).

We therefore presented two forms of labelled DMSP, either [1-¹³C]DMSP or [1-¹⁴C]DMSP, to cell-free extracts of *E. coli* strain BL21 (DE3) pLysS containing plasmid pBIO1658, in which *dddP* is expressed from a promoter in the pET21a vector. The catabolic fate of the ¹³C was determined by assaying samples by ¹³C-NMR, and the ¹⁴C was detected by scintillation counting following HPLC. In both cases, the only newly formed labelled compound was identified as acrylate. Similarly, when [1-¹³C]DMSP was added to cell-free extracts of *E. coli* containing plasmid pBIO1660, in which *dddP* of the fungus *Fusarium graminearum* is cloned in pET21a (Todd *et al.*, 2009a), these bacteria also made [1-¹³C]acrylate plus DMS.

Thus, DddP is a DMSP lyase, as defined by the enzymic activity that cleaves the S–C bond in DMSP to form DMS plus acrylate plus an (inferred) proton. This is the same bioconversion that is mediated by DddL, another DMSP lyase found in several other marine alphaproteobacteria (Curson *et al.*, 2008). It is important to note, though, that

DddP and DddL belong to wholly different polypeptide families (Curson *et al.*, 2008; Todd *et al.*, 2009a).

Purification of dimeric DddP enzyme

DddP polypeptide was purified (see above) from *E. coli* BL21 (DE3) pLysS containing pBIO1658. Fig. 2 shows the enrichments of DddP using a 25% (NH₄)₂SO₄ cut, followed by three chromatographic separations, namely by hydrophobic interaction, anion exchange and gel filtration. Throughout the purification, the DMSP-dependent production of DMS in different fractions was correlated with the presence of a Coomassie-stained polypeptide of approximately 50 kDa, seen by separation using PAGE (the predicted molecular weight of DddP is 49 972 Da). This polypeptide was >95% pure, with a yield of ~50 mg per litre of initial *E. coli* culture. The purified preparations of DddP were routinely stored in 50 mM MES, pH 6 at 4 °C, without detectable loss of activity over a period of several weeks.

Other M24B family members, such as creatinase from *Actinobacillus*, are known to be dimeric (Padmanabhan *et al.*, 2002). To determine the association state of DddP, the purified protein was analysed by sedimentation equilibrium analytical ultracentrifugation. Data obtained at 12 000 r.p.m. (Fig. 3) fitted well to a single component model, yielding a molecular mass of 95 300 ± 8000 Da. Thus, DddP is clearly a homo-dimer in solution. Similar analyses in the presence of 0.45 M NaCl did not affect the

Fig. 2. Purified *R. nubinhibens* ISM DddP protein from *E. coli* cultures visualized by SDS-PAGE. Proteins were separated by SDS-PAGE on 12% acrylamide gels and stained with Coomassie Blue. Lanes: 1, broad-range protein marker (Bio-Rad); 2, crude cell extract after sonication; 3, supernatant remaining after treating the cell extract with 25% (NH₄)₂SO₄; 4, phenyl sepharose column eluate; 5, DEAE column eluate; 6, gel filtration column eluate.

Fig. 3. Sedimentation equilibrium ultracentrifugation of purified DddP protein. Ultracentrifugation scans of absorbance (at 260 nm) against $\text{radius}^2 - \text{radius}^2(\text{ref})$ following equilibration at 20 °C of DddP (5.1 μM in 20 mM Tris pH 8) at 12 000 r.p.m. The data were fitted to a single species with a mean molecular weight of $95\,300 \pm 8000$ Da (solid line; lower plots). Plot of residual difference between experimental data and fitted curve (upper plot).

association state, indicating that the interaction between the two DddP polypeptides in the dimer is stable.

Enzymic properties of DddP *in vitro*

To optimize conditions to assay DddP *in vitro*, its activity was measured initially in MBS buffer over a range of pH values. Maximal activity was at pH 6.0, at which the rates of DMS production were $\sim 30\%$ greater than at either pH 5.0 or pH 7.0 (Fig. 4a). All subsequent assays were done in MES buffer, pH 6.0. The DddP enzyme was active over a range of temperatures, the highest rates being at 60 °C, above which there was rapid loss of activity (Fig. 4b). All subsequent assays were done at 30 °C, the temperature used by González *et al.* (2003) in their initial descriptions of *R. ruber* ISM.

Under these conditions, DddP generated approximately equimolar amounts of DMS and of acrylate, as measured, respectively, by gas chromatography and by the accumulation of ^{14}C -labelled acrylate when the substrate was $[1-^{14}\text{C}]\text{DMSP}$. The appearance of $[1-^{13}\text{C}]$ -labelled acrylate at the expense of the $[1-^{13}\text{C}]\text{DMSP}$, revealed by NMR spectroscopy, is shown in Fig. 5. Initial rates of DMS production were measured over a range of DMSP concentrations. The data fitted reasonably well to the Michaelis–Menten equation (see Fig. 6) from which a K_m of DddP for DMSP of 13.8 ± 5.5 mM and a $V_{\max}$ of 0.31 ± 0.06 nmol DMS $\text{min}^{-1} (\mu\text{g DddP})^{-1}$ were obtained. Such a relatively high K_m value was also found for the structurally related M24B family enzyme creatinase (Schumann *et al.*, 1993) and for the DmdA DMSP demethylase in the related bacterium *Ruegeria pomeroyi* and in *Pelagibacter ubique*. It was shown that these bacteria

could efficiently import DMSP from the media, generating intracellular concentrations as high as 70 mM, even when extracellular DMSP was several orders of magnitude less abundant (Reisch *et al.*, 2008).

SDM of the predicted catalytic region of DddP

As noted by Todd *et al.* (2009a), DddP is in the M24B family (COG0006; PF00557) of peptidases (probability of $8e^{-38}$); see the MEROPS classification of proteolytic enzymes (<http://merops.sanger.ac.uk/cgi-bin/famsum?family=m24>). As its name suggests, this family includes known enzymes that cleave peptide bonds (e. g. Roderick & Matthews, 1993). However, some M24B members have non-peptide substrates (see below), so the ability of DddP to cleave the non-peptide DMSP would not be unprecedented, although this would represent the first example of the cleavage of a C–S bond by a member of this family.

The M24B enzymes, including those that act on peptides and those that cleave non-peptide bonds, contain a ‘pitta bread’ fold that includes their active sites (Bazan *et al.*, 1994). This catalytic region is located in the C-terminal half of DddP. Within this region, 6 amino acid residues (D295, D297, D307, H371, E406 and E421) were predicted by Todd *et al.* (2009a) to be at, or close to, the active site, based on their conservation and location relative to the active sites and/or predicted metal-binding sites of other M24B family members (e.g. Maher *et al.*, 2004). To determine the role of these residues, each was changed to alanine by SDM of *dddP* and cloned in plasmid pBIO1658. The mutants were verified by sequencing, and the mutated plasmids were transformed into *E. coli* strain BL21 (DE3) pLysS. It was confirmed by SDS-PAGE that these

Fig. 4. Effect of pH and temperature on DddP activity. Assays of DMS production *in vitro* with DddP preparations and 5 mM DMSP were done in solutions with different pH values (a) and in 50 mM MES buffer pH 6.0 at different temperatures (b). Values are expressed as percentages relative to the maximum activity. Error bars (SD) were calculated from duplicate experiments.

transformants made DddP polypeptide at equivalent amounts and with equivalent electrophoretic mobilities to those seen with *E. coli* containing cloned wild-type *dddP*. However, when cell-free extracts were assayed for their Ddd phenotypes, the mutants no longer generated DMS from DMSP above background levels. Thus, all these six residues in DddP are essential for the DMSP lyase activity of DddP.

DddP is not a metalloprotein

Several, but not all, polypeptides in the M24B peptidase family have been shown to have metal (usually Co or Mn) cofactors (<http://merops.sanger.ac.uk/cgi-bin/famsum?family=m24>). We therefore used two approaches to determine whether DddP also has a metal cofactor.

First, we conducted the enzyme assays for DMS production in the presence of excess concentrations of the chelating agents EDTA (25 mM) and bipyridyl (2.5 mM) in buffers lacking any additional metals. Neither compound had any detectable inhibitory effect. A similar set of observations on the lack of inhibition by EDTA of the M24B protein creatinase of *Paracoccus* led Wang *et al.* (2006) to conclude that this enzyme also lacked any metal cofactor.

To confirm this, we used ICP-OES to search directly for any metals in the as-purified, functional DddP enzyme.

Fig. 5. NMR spectra of the products of DddP-mediated cleavage of [1-¹³C]DMSP. ¹³C NMR spectra for pure samples of DMSP and acrylate are shown as indicated, with peaks at chemical shifts of 173.4 and 170.2, respectively. The spectra below the reference compounds are from reaction mixes containing 5 mM DMSP plus 0.9 μM DddP enzyme, sampled after 1, 3, 5 and 16 h incubation, as indicated.

Consistent with the results obtained with the chelating agents, none of the metals Co, Cu, Mn, Ni or Zn was detected at above background levels. Taking into account the limits of sensitivity, these data indicated that none of these metals was present at a stoichiometry greater than 0.1 per DddP monomer, so we conclude that DddP does not contain or require a metal cofactor.

Concluding remarks

DddP is unusual, but not unique, among the M24B family of peptidases in cleaving a non-peptide bond. For example, creatinase of *Pseudomonas putida* generates sarcosine plus urea from water plus creatine (Bazan *et al.*, 1994), and AgcA of *Agrobacterium tumefaciens* converts one opine

Fig. 6. Determination of the DddP K_m for DMSP. Plot of initial rate (normalized to protein concentration) as a function of DMSP concentration. DddP (0.3 μ M) was in 50 mM MES, pH 6.0. The solid line represents a fit to the Michaelis–Menten equation.

(mannopine) to another (agropine) via an internal condensation reaction (Hong *et al.*, 1997). Although many well-characterized M24B enzymes, such as the cobalt-dependent methionine aminopeptidase from *E. coli* have metal cofactors, DddP does not and so resembles, for example, the creatinase of *Paracoccus*, which also lacks a metal-containing active site (Wang *et al.*, 2006). It may be significant that creatinase and DddP, the two known non-metal-dependent M24B enzymes, have structurally related substrates that carry a positive charge at or close to the catalytically cleaved bond. The work described here therefore further extends the types of enzymic activities that can be accomplished by M24B family members and, importantly, establishes that at least one of them can cleave a C–S bond. Substitution of six predicted active site residues of DddP resulted in stable enzymes without detectable lyase activity. This suggests that the DddP active site is finely tuned for DMSP turnover and cannot tolerate substitutions in either its constituent residues or its substrate.

The general term ‘DMSP lyase’ has long been used to describe those enzymes that cleave DMSP into acrylate plus DMS (Cantoni & Anderson, 1956). However, earlier biochemical and physiological studies on different bacteria that had a Ddd⁺ phenotype indicated that there was considerable heterogeneity in the behaviour of the different lyases (Yoch, 2002). Recent genetic studies have confirmed and begun to explain the marked differences among the enzymes that can liberate DMS from DMSP. Thus, the previously identified DddD enzyme is a predicted class III acyl-CoA transferase, although the first DMSP catabolic product to be identified was 3-OH-propionate (Todd *et al.*, 2009b). A second enzyme, DddL, is a DMSP lyase *sensu stricto*, cleaving DMSP into DMS plus acrylate, but the gene product corresponding to DddL had previously been ascribed to a domain of unknown function (Curson *et al.*, 2008). The finding here that a completely different

DMSP lyase, DddP, which has no sequence or predicted structural similarity to DddL, can also effect the same general reaction adds further to the biochemical diversity of the catabolism of DMSP and the emission of the important gas DMS.

ACKNOWLEDGEMENTS

We thank the University of East Anglia, BBSRC and the NERC for financial support, and Andrew Curson, Charles Brearley, Myles Cheesman, Gaye White, Rob Green and Matt Sullivan for useful discussions. Verity Lyall, Yohan Chan, Tom Clarke and Graham Chilvers provided valuable technical assistance.

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Edited by: R. J. Maier