

## The Nonribosomal Peptide Synthetase Enzyme DdaD Tethers $N_{\beta}$ -Fumaramoyl-L-2,3-diaminopropionate for Fe(II)/ $\alpha$ -Ketoglutarate-Dependent Epoxidation by DdaC during Dapdiamide Antibiotic Biosynthesis

Marie A. Hollenhorst,<sup>†</sup> Stefanie B. Bumpus,<sup>‡</sup> Megan L. Matthews,<sup>§</sup>  
J. Martin Bollinger, Jr.,<sup>||</sup> Neil L. Kelleher,<sup>‡,⊥</sup> and Christopher T. Walsh<sup>\*,†</sup>

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, Department of Chemistry and Institute for Genomic Biology, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, and Departments of Chemistry, Biochemistry, and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802, United States

Received August 11, 2010; E-mail: christopher\_walsh@hms.harvard.edu

**Abstract:** The gene cluster from *Pantoea agglomerans* responsible for biosynthesis of the dapdiamide antibiotics encodes an adenylation–thiolation didomain protein, DdaD, and an Fe(II)/ $\alpha$ -ketoglutarate-dependent dioxygenase homologue, DdaC. Here we show that DdaD, a nonribosomal peptide synthetase module, activates and sequesters  $N_{\beta}$ -fumaramoyl-L-2,3-diaminopropionate as a covalently tethered thioester for subsequent oxidative modification of the fumaramoyl group. DdaC catalyzes Fe(II)- and  $\alpha$ -ketoglutarate-dependent epoxidation of the covalently bound  $N_{\beta}$ -fumaramoyl-L-2,3-diaminopropionyl-S-DdaD species to generate  $N_{\beta}$ -epoxysuccinamoyl-DAP (DAP = 2,3-diaminopropionate) in thioester linkage to DdaD. After hydrolytic release,  $N_{\beta}$ -epoxysuccinamoyl-DAP can be ligated to L-valine by the ATP-dependent ligase DdaF to form the natural antibiotic  $N_{\beta}$ -epoxysuccinamoyl-DAP-Val.

### Introduction

The dapdiamide antibiotics are a family of five *N*-acylated dipeptides produced by *Pantoea agglomerans* (Figure 1A).<sup>1</sup> The “dap” prefix refers to the presence of the nonproteinogenic amino acid 2,3-diaminopropionate (DAP; blue in Figure 1), while the “diamide” suffix reflects the two backbone amide bonds. The DAP moiety, which can be acylated either on  $N_{\beta}$  or on  $N_{\alpha}$ , is the first residue of the dipeptide and is attached via a standard peptide linkage to a terminal valine (Val), isoleucine (Ile), or leucine (Leu) (red in Figure 1). The *N*-acyl moiety (green in Figure 1) is a fumaramoyl group in dapdiamides A–D and an epoxysuccinamoyl group in dapdiamide E. The fumaramoyl or epoxysuccinamoyl functionality most likely provides the electrophilic moiety that accounts for the antibiotic activity of this class of compounds.<sup>2</sup> The dapdiamides are likely cleaved intracellularly to generate acyl-DAP warheads that target glucosamine-6-phosphate synthase via capture of the nucleophilic active site cysteine (Cys).<sup>2,3</sup>

The dapdiamides A–E were isolated by activity-based cloning of dapdiamide biosynthetic genes from *P. agglomerans* CU0119 into *Escherichia coli*.<sup>1</sup> This allowed for sequencing of the responsible gene cluster, which revealed nine genes, annotated as *ddaA–I* (Figure 1B), that are necessary and sufficient for *E. coli* to make the dapdiamides. In our initial studies on the Dda enzymes, we determined that DdaG and DdaF are ATP-dependent ligases that build the *N*-acyldipeptide scaffolds.<sup>4</sup> DdaG is an AMP-generating ligase that makes the regiospecifically *N*-acylated  $N_{\beta}$ -fumaramoyl-DAP ( $N_{\beta}$ FmDAP). Our data indicated that DdaF catalyzes the last step in the pathway and forms the dipeptide linkage of *N*-acyl-DAP with Val, Ile, or Leu. DdaF cleaves ATP to ADP (not AMP), generating the *N*-acyl-DAP-phosphate as an activated intermediate for capture by Val, Ile, or Leu. This enzyme accepts only  $N_{\beta}$ -fumaramoyl-DAP ( $N_{\beta}$ FmmDAP), not  $N_{\beta}$ FmDAP, as the carboxylate substrate, suggesting DdaH, the putative fumaramoyl to fumaramoyl amide synthase, acts after DdaG but before DdaF.

In this study, we have turned our attention to the epoxidation event in the dapdiamide biosynthetic pathway. An epoxysuccinamoyl moiety is present in both dapdiamide E and its  $N_{\beta}$ -acyl-DAP isomer  $N_{\beta}$ -epoxysuccinamoyl-DAP-Val ( $N_{\beta}$ EpSmDAP-Val) [this compound has been referred to in the literature variously as CB-25-I, [2-amino-3-(oxirane-2,3-dicarboxamido)propanoyl]valine, and herbicolin I], a natural product produced by *Serratia plymuthica* and *P. agglomerans* strains

<sup>†</sup> Harvard Medical School.

<sup>‡</sup> University of Illinois at Urbana–Champaign.

<sup>§</sup> Department of Chemistry, The Pennsylvania State University.

<sup>||</sup> Departments of Chemistry, Biochemistry, and Molecular Biology, The Pennsylvania State University.

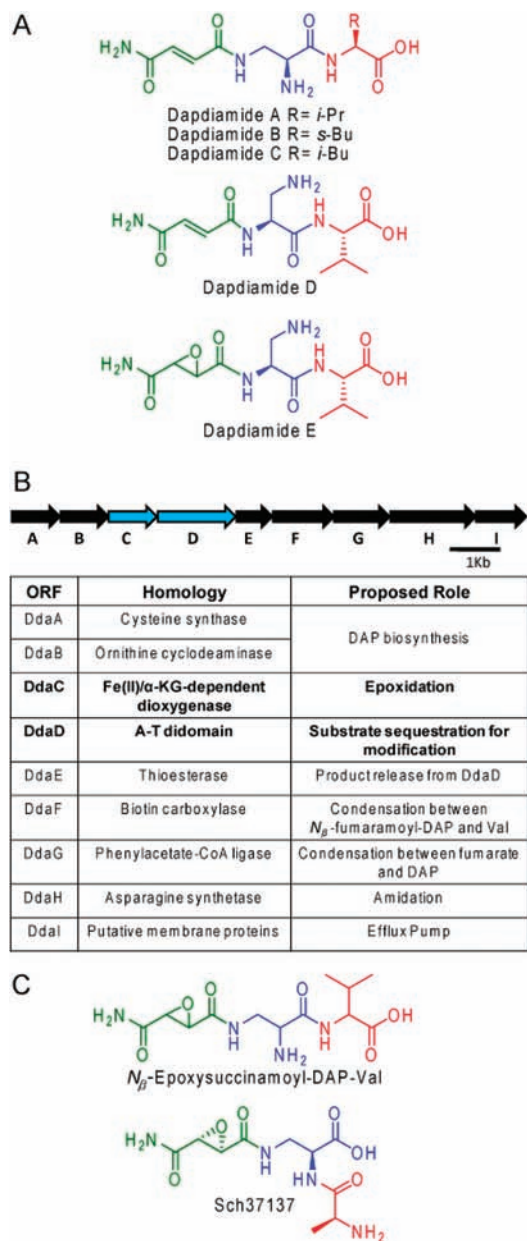
<sup>⊥</sup> Current address: Department of Chemistry, Northwestern University, Evanston, IL 60208, United States.

(1) Dawlaty, J.; Zhang, X.; Fischbach, M. A.; Clardy, J. *J. Nat. Prod.* **2010**, *73*, 441–446.

(2) Kucharczyk, N.; Denisot, M. A.; Le Goffic, F.; Badet, B. *Biochemistry* **1990**, *29*, 3668–3676.

(3) Milewski, S.; Andruszkiewicz, R.; Kasprzak, L.; Mazerski, J.; Mignini, F.; Borowski, E. *Antimicrob. Agents Chemother.* **1991**, *35*, 36–43.

(4) Hollenhorst, M. A.; Clardy, J.; Walsh, C. T. *Biochemistry* **2009**, *48*, 10467–10472.



**Figure 1.** (A) Dapdiamide family of antibiotics. (B) Dapdiamide gene cluster. (C) Dapdiamide-related epoxide natural products.

48b/90, 39b/90, and C9-1 (Figure 1C).<sup>1,5,6</sup> NMR evidence suggests that each of these compounds contains a *trans*-epoxide, but the absolute stereochemistry of the epoxide carbons has not been determined.<sup>1,5,6</sup> The most similar epoxysuccin(am)ate-containing natural product for which the epoxide stereochemistry has been determined is Sch37137 (Figure 1C), and in this compound the epoxide carbons have an (*R,R*) configuration.<sup>7,8</sup>

However, there are other known natural products that contain both (*R,R*)- and (*S,S*)-*trans*-epoxysuccin(am)ate.<sup>9,10</sup>

Here we report that DdaF catalyzes ATP-dependent ligation of both of the  $N_\beta$ -*trans*-epoxysuccinamoyl-DAP ( $N_\beta$ -*trans*-EpSmDAP) diastereomers and Val, with substrate specificity for the (*R,R*) over the (*S,S*) diastereomer. Our studies were based on the hypothesis that DdaC and DdaD are required to form  $N_\beta$ -*trans*-EpSmDAP from an olefin-containing acyl-DAP intermediate. DdaD is a third type of ATP-utilizing enzyme in this pathway, a predicted nonribosomal peptide synthetase (NRPS) module,<sup>11</sup> while DdaC has homology to mononuclear nonheme iron oxygenases.<sup>12</sup> In this study, we demonstrate that DdaC and DdaD are the relevant catalysts and determine the timing of epoxidation during  $N_\beta$ -acyl-DAP-Val assembly (Scheme 1).

## Materials and Methods

**Materials and General Methods.** Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). Polymerase chain reaction (PCR) was performed with Phusion High-Fidelity PCR Mastermix (New England Biolabs). Cloning was performed using the Gateway System (Invitrogen). One Shot Chemically Competent TOP10 *E. coli* (Invitrogen) and Nova-Blue(DE3) (Novagen) were used for routine cloning and propagation of DNA vectors. Recombinant plasmid DNA was purified with a Qiaprep kit (Qiagen). DNA sequencing was performed at the Molecular Biology Core Facilities of the Dana Farber Cancer Institute (Boston, MA). Nickel–nitrilotriacetic acid agarose (Ni–NTA) superflow resin and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels were purchased from Qiagen and Biorad, respectively. Protein concentrations were determined by Bradford assay<sup>13</sup> with bovine serum albumin (BSA) as a standard or with a Nanodrop 1000 spectrophotometer (Thermo Scientific) on the basis of the absorbance at 280 nm with the predicted molar extinction coefficient.

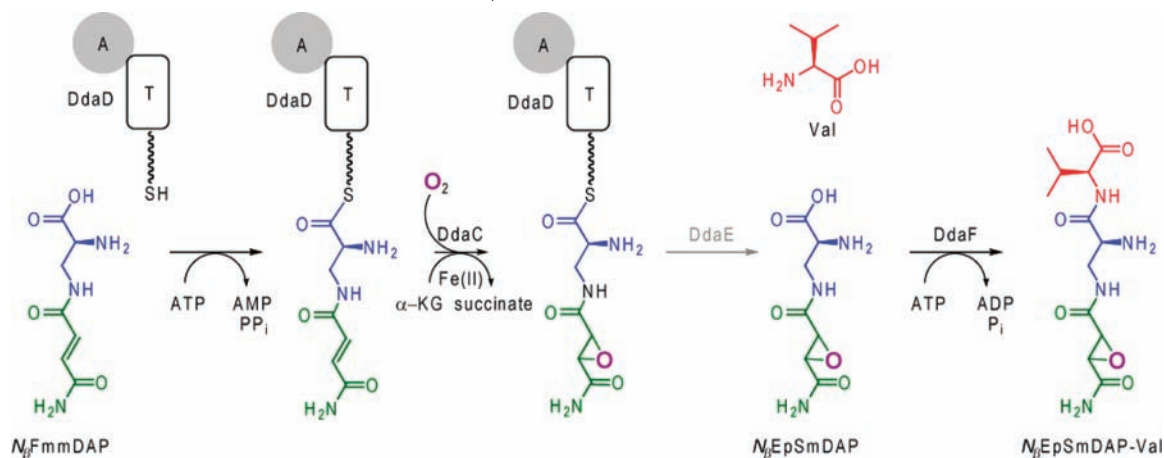
Anaerobic manipulations were performed under a nitrogen atmosphere using an Mbraun Labmaster glovebox (Stratham, NH) maintained at 2 ppm O<sub>2</sub> or less. Buffers were sparged with argon for 20–30 min and equilibrated overnight with the nitrogen atmosphere in the glovebox before use.

A pyruvate kinase/lactate dehydrogenase (PK/LDH) enzyme mix from rabbit muscle was purchased from Sigma as a buffered aqueous glycerol solution. Synthetic dapdiamide A and the plasmid containing the dapdiamide gene cluster, pUC19 A10A, were provided by Jessica Dawlaty (Harvard Medical School, Boston, MA).<sup>1</sup> BODIPY–CoA<sup>14</sup> and Sfp<sup>15,16</sup> were prepared according to published procedures.  $N$ -His<sub>6</sub>-tagged DdaF was purified as described previously.<sup>4</sup>  $N_\beta$ FmDAP,  $N_\alpha$ FmDAP,  $N_\beta$ FmmDAP,  $N_\alpha$ FmmDAP, and fumaramate (Fmm) were synthesized as described previously.<sup>4</sup>

**DdaF ADP Production Assay.** The reaction mixtures (250  $\mu$ L) were incubated at room temperature and contained 250 nM DdaF, 5 mM Val, 10 mM ATP, 12 mM MgCl<sub>2</sub>, 200  $\mu$ M NADH, 500  $\mu$ M phosphoenolpyruvate, 59 units/mL pyruvate kinase, 41 units/mL lactate dehydrogenase, 5 mM borate·NaOH (pH 9.5), and 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 8). The consumption of NADH was monitored continuously in

- (9) Martin, W. R.; Foster, J. W. *J. Bacteriol.* **1955**, *70*, 405–414.
- (10) Hanada, K.; Tamai, M.; Ohmura, S.; Sawada, J.; Seki, T.; Tanaka, I. *Agric. Biol. Chem.* **1978**, *42*, 529–536.
- (11) Fischbach, M. A.; Walsh, C. T. *Chem. Rev.* **2006**, *106*, 3468–3496.
- (12) Hausinger, R. P. *Crit. Rev. Biochem. Mol. Biol.* **2004**, *39*, 21–68.
- (13) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
- (14) La Clair, J. J.; Foley, T. L.; Schegg, T. R.; Regan, C. M.; Burkart, M. D. *Chem. Biol.* **2004**, *11*, 195–201.
- (15) Quadri, L. E. N.; Weinreb, P. H.; Lei, M.; Nakano, M. M.; Zuber, P.; Walsh, C. T. *Biochemistry* **1998**, *37*, 1585–1595.
- (16) Lambalot, R. H.; Gehring, A. M.; Flugel, R. S.; Zuber, P.; LaCelle, M.; Marahiel, M. A.; Reid, R.; Khosla, C.; Walsh, C. T. *Chem. Biol.* **1996**, *3*, 923–936.

- (5) Shoji, J.; Hino, H.; Sakazaki, R.; Kato, T.; Hattori, T.; Matsumoto, K.; Tawara, K.; Kikuchi, J.; Terui, Y. *J. Antibiot.* **1989**, *42*, 869–874.
- (6) Sammer, U. F.; Volksch, B.; Mollmann, U.; Schmidtke, M.; Spitteller, P.; Spitteller, M.; Spitteller, D. *Appl. Environ. Microbiol.* **2009**, *75*, 7710–7717.
- (7) Cooper, R.; Horan, A. C.; Gentile, F.; Gullo, V.; Loebenberg, D.; Marquez, J.; Patel, M.; Puar, M. S.; Truumees, I. *J. Antibiot.* **1988**, *41*, 13–19.
- (8) Rane, D. F.; Girijavallabhan, V. M.; Ganguly, A. K.; Pike, R. E.; Saksena, A. K.; McPhail, A. T. *Tetrahedron Lett.* **1993**, *34*, 3201–3204.

**Scheme 1.** Proposed Role of DdaC–F in the Formation of  $N_{\beta}$ EpSmDAP–Val<sup>a</sup>

<sup>a</sup> The grayed-out step has not been biochemically validated.

Plastibrand micro UV cuvettes in a Varian Cary 50 UV–vis spectrophotometer by measuring the absorbance at 340 nm. Kinetic constants were derived from velocity versus substrate concentration data with GraphPad Prism using a nonlinear, least-squares fitting method for  $N_{\beta}$ FmmDAP and  $N_{\beta}$ -(*R,R*)-epoxysuccinamoyl-DAP ( $N_{\beta}$ RREpSmDAP) and a linear regression for  $N_{\beta}$ -(*S,S*)-epoxysuccinamoyl-DAP ( $N_{\beta}$ SSEpSmDAP).

**DdaD ATP–[<sup>32</sup>P]PP<sub>i</sub> Exchange Assays.** The reaction mixtures (170–350  $\mu$ L) contained 5  $\mu$ M DdaD, 125  $\mu$ M substrate, 1 mM ATP, 1 mM MgCl<sub>2</sub>, 40 mM KCl, 5 mM Na[<sup>32</sup>P]PP<sub>i</sub> (approximately  $(2-4) \times 10^7$  counts per min (cpm)/mL), and 50 mM HEPES (pH 7.5). The reactions were incubated at room temperature for 10 min, and then 50  $\mu$ L aliquots were removed and quenched with 250  $\mu$ L of a charcoal suspension (100 mM NaPP<sub>i</sub>, 350 mM HClO<sub>4</sub>, and 16 g/L charcoal). The samples were mixed using a vortex and then centrifuged at 16000g for 3 min. The pellets were washed twice with 250  $\mu$ L of wash solution (100 mM NaPP<sub>i</sub>, and 350 mM HClO<sub>4</sub>). Charcoal-bound radioactivity was measured on a Beckman LS 6500 scintillation counter.

**DdaC/D Enzymatic Assays with Anaerobically Purified DdaC.** Phosphopantetheinylation reactions (12–270  $\mu$ L) contained 25  $\mu$ M DdaD, 12  $\mu$ M Sfp, 400  $\mu$ M coenzyme A (CoA), 10 mM MgCl<sub>2</sub>, 1.5 mM dithiothreitol (DTT), and 50 mM HEPES (pH 7.5). The reactions were incubated at room temperature for 1 h. To form aminoacyl-S-DdaD, 120–250  $\mu$ M substrate and 1 mM ATP were added (final volume 12.5–156  $\mu$ L) and the reactions incubated for an additional 5–60 min at room temperature.

The following reagents were added to the  $N_{\beta}$ FmmDAP-S-DdaD preparations to the indicated final concentrations: 50  $\mu$ M Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> (stock solution 2.5 mM in 200  $\mu$ M HCl), 300  $\mu$ M  $\alpha$ -ketoglutarate ( $\alpha$ -KG) (stock solution 6 mM in 50 mM HEPES (pH 8)), 4 mM ascorbic acid (stock solution 100 mM in 50 mM HEPES (pH 8)), and 10  $\mu$ M DdaC (final volume 73–79  $\mu$ L). The reactions were incubated for 5–60 min at room temperature.

The reactions were quenched by flash freezing in N<sub>2</sub>(l) for subsequent trypsin digestion or by the addition of an equal volume of 25% formic acid followed by flash freezing in N<sub>2</sub>(l) for reversed-phase liquid chromatography (RPLC)–Fourier transform mass spectrometry (FTMS) analysis of intact DdaD.

H<sub>2</sub><sup>18</sup>O incubations were carried out as described above with the exception that the final reaction mixtures contained 63% H<sub>2</sub><sup>18</sup>O (Cambridge Isotope Laboratories).

**Trypsin Digestion of DdaD-Containing Reactions.** The following digestion procedure was used for analysis of (1) conversion of *apo*-DdaD to *holo*-DdaD (HS-DdaD), (2) loading of DdaD with  $N_{\beta}$ FmmDAP to generate  $N_{\beta}$ FmmDAP-S-DdaD, (3) conversion of  $N_{\beta}$ FmmDAP-S-DdaD to  $N_{\beta}$ EpSmDAP-S-DdaD by DdaC, (4) dependence of epoxide formation on  $\alpha$ -KG, (5) loading of dap-

diamide A onto HS-DdaD, and (6) incorporation of <sup>18</sup>O into  $N_{\beta}$ EpSmDAP-S-DdaD using H<sub>2</sub><sup>18</sup>O.

All reactions were stored at –80 °C until trypsin digestion. Prior to trypsin digestion, the samples were thawed at room temperature. Trypsin (Promega Sequencing grade) was resuspended in the buffer provided by the manufacturer to a final concentration of 1  $\mu$ g/ $\mu$ L and incubated at 30 °C for 15 min. An aliquot of the reaction containing 50  $\mu$ g of DdaD was removed and added to an equal volume of 0.1 M NH<sub>4</sub>HCO<sub>3</sub> (pH 7.8–8) and 2 mM Tris(2-carboxyethyl)phosphine (TCEP) (pH 6). This mixture was incubated for 4 min at room temperature, and trypsin was added at a mass ratio of 1:5 trypsin:total protein in the digestion reaction. After addition of the trypsin, the reaction was incubated at 30 °C for 5 min. The reaction was quenched by the addition of one-half reaction volume of 25% formic acid and stored at –80 °C until mass spectrometric analysis.

**RPLC–FTMS Analysis of Trypsin Digests.** All RPLC–FTMS analyses were conducted using an Agilent 1200 high-performance LC (HPLC) system with an autosampler coupled directly to a ThermoFisher Scientific LTQ-FT hybrid linear ion trap FTMS system operating at 11 T. The mass spectrometer was calibrated weekly using the calibration mixture and instructions specified by the manufacturer. All instrument parameters were tuned according to the manufacturer’s instructions (employing bovine ubiquitin (Sigma) for tuning purposes).

For all analyses of trypsin digests of DdaD-containing reactions, a 1 mm  $\times$  150 mm Jupiter C18 column (Phenomenex, 300 Å, 5  $\mu$ m) was connected in-line with the electrospray ionization (ESI) source (operated at ~5 kV with a capillary temperature of 200–250 °C) for the MS system. The 70 min separation gradient used for all RPLC analyses is shown in Table S1 (Supporting Information), where solvent A was H<sub>2</sub>O + 0.1% formic acid and solvent B was acetonitrile (MeCN) + 0.1% formic acid. A trypsin-digested reaction mixture was loaded onto the column using the autosampler and separated according to the gradient shown.

All ionized peptide species entering the mass spectrometer were subjected to an MS method with five MS and MS/MS events: (1) full scan measurement of all intact peptides (all ions detected in the FTMS instrument in profile mode, resolution 100 000,  $m/z$  range detected 400–2000), (2) the phosphopantetheinyl (Ppant) ejection assay using nozzle–skimmer dissociation (NSD) (all ions detected in the FTMS instrument in profile mode, resolution 50 000,  $m/z$  range 250–500, surface-induced dissociation (SID) 75 V), (3–5) data-dependent MS/MS on the first, second, and third most abundant ions from scan 1 using collision-induced dissociation (CID) (all ions detected in the FTMS instrument in profile mode, minimum target signal counts 5000, resolution 50 000,  $m/z$  range detected dependent on target  $m/z$ , default charge state 2, isolation width 5

$m/z$ , normalized collision energy (NCE) 35, activation  $q$  value 0.40, activation time 30 ms). During all analyses, dynamic exclusion was enabled with the following settings: repeat count 2, repeat duration 30 s, exclusion list size 300, exclusion duration 60 s.

All data were analyzed using Qualbrowser (Xcalibur) and ProSightPC, both provided with the LTQ-FT system. ProSightPC was used to search all MS/MS data against a database containing the apo-DdaD protein sequence for peptide identification, with an intact peptide mass tolerance of 750 Da (which allowed for observation of HS-DdaD and HS-DdaD loaded with a variety of substrates) and a fragment ion mass tolerance of 10 ppm (the  $\Delta m$  mode was enabled for all searches). A minimum of five matching fragment ions were required for peptide identification. In Qualbrowser, ions of interest were searched within a range of 0.01  $m/z$  around the isotopic peak of interest, within a tolerance of 5 ppm.

**RPLC–FTMS Analysis of Intact DdaD.** RPLC–FTMS analysis of intact DdaD by the Ppant ejection assay was employed for analyses of the loading of  $N_{\beta}RREpSmDAP$  onto HS-DdaD, the loading of  $N_{\beta}SSEpSmDAP$  onto HS-DdaD, and  $^{18}O$  incorporation into  $N_{\beta}EpSmDAP$ -S-DdaD using  $^{18}O_2$ . All reactions were quenched with an equal volume of 25% formic acid and stored at  $-80^{\circ}C$  prior to analysis.

For all analyses of intact proteins in DdaD-containing reactions, a 1 mm  $\times$  150 mm Jupiter C4 column (Phenomenex, 300 Å, 5  $\mu m$ ) was connected in-line with the ESI source (operated at  $\sim 5$  kV with a capillary temperature of 200–250  $^{\circ}C$ ) for the MS system. A reaction aliquot containing 12–15  $\mu g$  of DdaD was injected for each sample. The 45 min separation gradient used for all RPLC analyses is shown in Table S2 (Supporting Information), where solvent A was  $H_2O + 0.1\%$  formic acid and solvent B was MeCN + 0.1% formic acid. The reaction mixture was loaded onto the column using the autosampler and separated according to the gradient shown.

All ionized protein species entering the mass spectrometer were subjected to an MS method with two MS and MS/MS events: (1) full scan measurement of all intact peptides (all ions detected in the ion trap MS instrument in profile mode,  $m/z$  range detected 400–2000), (2) Ppant ejection assay using NSD (all ions detected in the FTMS instrument in profile mode, resolution 50 000,  $m/z$  range 250–500, SID = 75 V). All data were analyzed using Qualbrowser (Xcalibur), provided for analysis with the LTQ-FT system. Ppant ejection ions of interest were searched within a range of 0.01  $m/z$  around the isotopic peak of interest, within a tolerance of 5 ppm.

**Determination of the Source of the Epoxide Oxygen by Use of  $^{18}O_2$ (g).** Phosphopantetheinylation reactions (506  $\mu L$ ) contained 25  $\mu M$  DdaD, 12  $\mu M$  Sfp, 400  $\mu M$  CoA, 1 mM  $MgCl_2$ , 1.5 mM DTT, and 50 mM HEPES (pH 7.5). The reactions were incubated at room temperature for 1 h. To form  $N_{\beta}FmmDAP$ -S-DdaD, 123  $\mu M$   $N_{\beta}FmmDAP$  and 5.5 mM ATP were added (final volume 522  $\mu L$ ) and the reactions incubated for an additional 5 min at room temperature.

$O_2$ -free solutions (prepared as previously described<sup>17</sup>) (225  $\mu L$ ) containing  $N_{\beta}FmmDAP$ -S-DdaD and  $\alpha$ -KG (0.13 mM) in the absence or presence of DdaC (4.2  $\mu M$ ) were placed in septum-sealed flasks. The flasks were stirred on ice and briefly evacuated to  $\sim 30$  Torr. One flask was subsequently refilled with 1.3 atm of a gas mixture containing 80%  $^{18}O_2$ (g) (95–98% isotopic enrichment) and 20%  $N_2$ (g), and the second flask was refilled with 1.1 atm of natural-abundance  $O_2$ (g).  $Fe(NH_4)_2(SO_4)_2$  and ascorbic acid were subsequently added to the flasks (to concentrations of 21  $\mu M$  and 1.7 mM, respectively) via a gastight syringe. The solutions were stirred for 5 min at 0  $^{\circ}C$  to allow the reactions to reach completion. The flasks were opened to air, the reactions were terminated by addition of each solution to an equal volume of 25% formic acid, and the acidified mixtures were flash frozen in  $N_2$ (l).

**Table 1.** DdaF Kinetics with Respect to  $N_{\beta}FmmDAP$  and  $N_{\beta}EpSmDAP$ s<sup>a</sup>

substrate	$k_{cat}$ ( $min^{-1}$ )	$K_m$ ( $\mu M$ )	$k_{cat}/K_m$ ( $min^{-1} mM^{-1}$ )
$N_{\beta}FmmDAP$	21 $\pm$ 3	72 $\pm$ 34	290
$N_{\beta}RREpSmDAP$	181 $\pm$ 7	53 $\pm$ 7	3400
$N_{\beta}SSEpSmDAP$ <sup>b</sup>	ND	ND	82 $\pm$ 8

<sup>a</sup> The values were determined by coupled spectrophotometric ADP production assay with a fixed Val concentration of 5 mM. Data are presented as the value  $\pm$  standard deviation. ND = not determined.

<sup>b</sup> Saturation not achieved at concentrations up to 590  $\mu M$ .

**Nucleotide Sequence Accession Number.** The nucleotide sequence of the dapdiamide gene cluster from *P. agglomerans* strain CU0119 has been deposited in the NCBI GenBank database under accession number HQ130277.

## Results

**DdaF Ligates  $N_{\beta}EpSmDAP$  and Val in an ATP-Dependent Manner.** Guided by the hypothesis that the dapdiamide pathway produces an antibiotic containing a *trans*-epoxysuccinamate moiety, we synthesized both diastereomers of  $N_{\beta}$ -*trans*-EpSmDAP (see the Supporting Information for synthetic protocols and characterization) and tested the activity of DdaF in ligating these compounds to Val.

Analysis of the enzymatic assays by LC–MS (see the Supporting Information for the method) revealed that DdaF can catalyze the ligation of both  $N_{\beta}$ -*trans*-EpSmDAP diastereomers to Val to produce the  $N_{\beta}EpSmDAP$ -Val dipeptide antibiotics (Figure S1, Supporting Information). A coupled spectrophotometric ADP production assay was used to kinetically characterize the activity of DdaF with respect to the two  $N_{\beta}$ -*trans*-EpSmDAP diastereomers. DdaF was found to use  $N_{\beta}RREpSmDAP$  as a saturable substrate, whereas saturation was not achieved with  $N_{\beta}SSEpSmDAP$  at concentrations up to 590  $\mu M$  (Table 1; Figure S2, Supporting Information). These findings suggested that  $N_{\beta}RREpSmDAP$  may be an on-pathway intermediate, with DdaF catalytic efficiency for this substrate approximately 40-fold greater than that for the corresponding (*S,S*)-epoxide.

**Expression and Purification of DdaC and DdaD in *E. coli*.** The *ddaC* and *ddaD* genes were amplified from pUC19 A10A, a plasmid containing the dapdiamide gene cluster,<sup>1</sup> and cloned into an expression vector encoding an N-terminal His<sub>6</sub> tag for DdaC or a C-terminal His<sub>6</sub> tag for DdaD. The proteins were overexpressed in *E. coli* BL21(DE3) and purified by Ni–NTA affinity chromatography (see the Supporting Information for the methods, Figure S3). Yields ranged from 4 to 6 mg/L for DdaC and from 11 to 14 mg/L for DdaD.

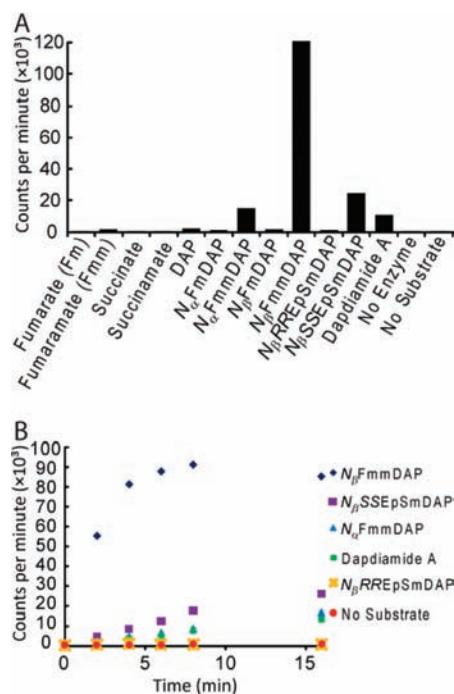
Following Ni–NTA chromatography, DdaC was either flash frozen to yield an aerobic preparation or gel filtered into an anaerobic atmosphere. The anaerobic preparations were incubated with  $Fe(NH_4)_2(SO_4)_2$ ,  $\alpha$ -KG, and DTT and subsequently desalted, giving a preparation with an iron occupancy of 47  $\pm$  4% (average  $\pm$  standard deviation, data from two independent experiments) by ferene spectrophotometric assay.<sup>18</sup>

**DdaD Activates and Covalently Tethers  $N_{\beta}FmmDAP$ .** The substrate specificity of the DdaD adenylation (A) domain was probed using ATP–[<sup>32</sup>P]PP<sub>i</sub> exchange assays,<sup>19</sup> which revealed that  $N_{\beta}FmmDAP$  is the preferred substrate (Figure 2). The kinetics of  $N_{\beta}FmmDAP$  adenylation by DdaD were determined

(17) Price, J. C.; Barr, E. W.; Tirupati, B.; Bollinger, J. M., Jr.; Krebs, C. *Biochemistry* **2003**, *42*, 7497–7508.

(18) Hennessy, D. J.; Reid, G. R.; Smith, F. E.; Thompson, S. L. *Can. J. Chem.* **1984**, *62*, 721–724.

(19) Lee, S. G.; Lipmann, F. *Methods Enzymol.* **1975**, *43*, 585–602.



**Figure 2.** DdaD ATP– $[^{32}\text{P}]\text{PP}_i$  exchange data. In these experiments, DdaD,  $[^{32}\text{P}]\text{PP}_i$ , unlabeled ATP, and substrate were incubated, and then ATP separated from  $\text{PP}_i$  by its specific adsorption to charcoal. The graph shows counts per minute arising from scintillation of the charcoal. (A) Adenylation activity with a variety of potential substrates. The reactions were quenched after 10 min. (B) Time course with  $N_\beta$ FmmDAPs, and dapdiamide A.

by this assay; the  $K_m$  value is  $420 \pm 80 \mu\text{M}$ , and the  $k_{\text{cat}}$  value is  $64 \pm 5 \text{ min}^{-1}$  (Figure S4, Supporting Information). DdaD was also active with  $N_\beta$ SSEpSmDAP but to a much lesser extent, with a  $k_{\text{cat}}/K_m$  of  $2.3 \text{ min}^{-1} \text{ mM}^{-1}$  compared with  $150 \text{ min}^{-1} \text{ mM}^{-1}$  for  $N_\beta$ FmmDAP (Figure S4). No exchange was observed with  $N_\beta$ RREpSmDAP.

The competence of the DdaD T domain to be phosphopantetheinylated (PPTated) was initially validated by the observation that it could be modified by the promiscuous *Bacillus subtilis* phosphopantetheinyl transferase (PPTase) Sfp<sup>15,16</sup> with BODIPY–CoA<sup>14</sup> to produce a fluorescent band during SDS–PAGE analysis (Figure S5, Supporting Information).

Next we turned to FTMS and the Ppant ejection assay<sup>20,21</sup> (Scheme S1, Supporting Information) to further characterize intermediates tethered to DdaD. The experiments were carried out in one of two fashions. For some analyses, enzymatic incubations were subjected to trypsin digestion, and tryptic peptides were separated by RPLC coupled directly to a hybrid linear ion trap FTMS system (ThermoFisher Scientific LTQ–FT), allowing determination of the masses of intact peptides, identification of the DdaD active site peptide (containing the site of serine (Ser) phosphopantetheinylation (PPTation)), and observation of Ppant ejection products. In other experiments, undigested reactions were subjected to RPLC–MS, and Ppant ejection was observed directly from intact DdaD.

During LC–MS analysis of a tryptic digest of apo–DdaD incubated with Sfp and CoA, we observed a thiolation (T)

domain tryptic peptide that underwent the expected 340.09 Da mass shift for PPTation (Figure 3A,B; Table S3, Supporting Information). The PPTated peptide was subjected to two different types of MS/MS fragmentation; NSD resulted in the formation of the predicted small molecule Ppant ejection product (a 1+ ion at  $m/z$  261.127) (Figure 3C, Table S3), and CID resulted in the formation of the predicted Ppant ejection peptide marker ions (Figure 3D). These are the species corresponding to apo–DdaD – 18 Da, from the formation of dehydroalanine (Dha) at the active site Ser (brown in Figure 3D, 2+ ion at  $m/z$  1451.6994, with an intact mass of 2901.3828 Da, an error of –7 ppm from the theoretical mass), and to apo–DdaD + 80 Da, from the retention of the phosphate moiety on the active site Ser during loss of the small molecule ejection ion with  $m/z$  261.127 (tan in Figure 3D, 2+ ion at  $m/z$  1500.7010, with an intact mass of 2999.386 Da, an error of 1 ppm from the theoretical mass). Localization of the site of Ppant modification was achieved by CID MS/MS fragmentation of the PPTated T domain active site tryptic peptide. This analysis demonstrated that Ser533, which aligns with known T domain active site Ser residues, is indeed the site of PPTation (Figure S7, Supporting Information).

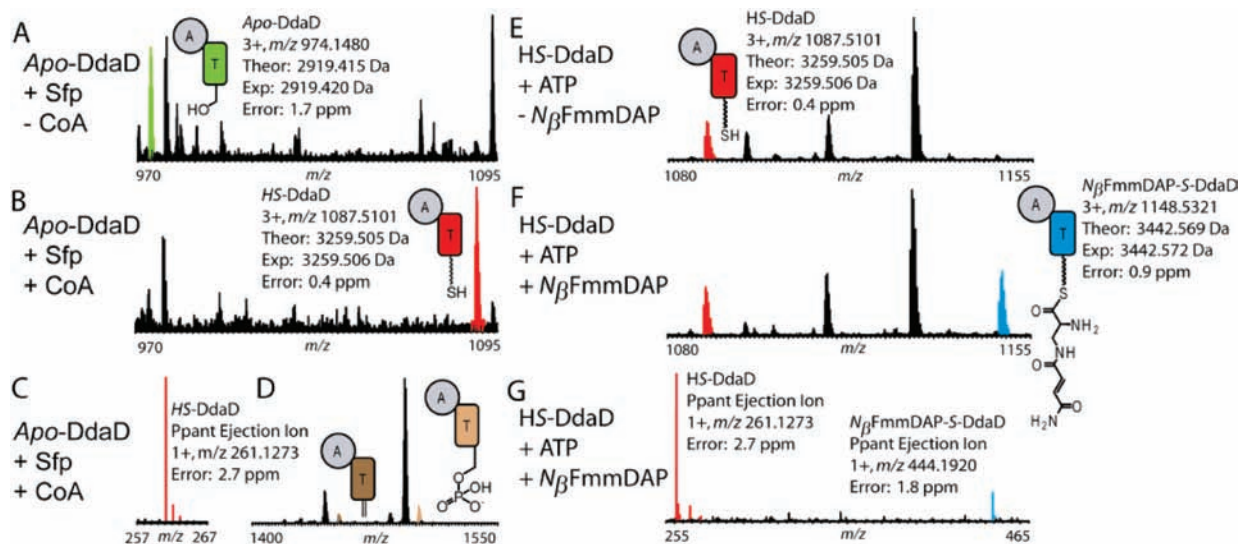
When DdaD, ATP, and  $N_\beta$ FmmDAP were incubated, we observed loading of the substrate onto the T domain both by the mass shift of the intact peptide (a mass shift of +183.066 Da from the HS–DdaD active site peptide, where the theoretical mass shift is +183.064 Da) and by Ppant ejection to produce an ion at  $m/z$  444.2 (Figure 3E–G; Table S3, Supporting Information). Additional confirmation that  $N_\beta$ FmmDAP is covalently tethered to the DdaD T domain came from LC–MS analysis of the  $N_\beta$ FmmDAP compound following nonenzymatic hydrolysis from the T domain (Figure S8, Supporting Information). Using LC–MS and the Ppant ejection assay to analyze intact DdaD, we also found that DdaD is capable of loading both diastereomers of  $N_\beta$ –trans–EpSmDAP (Figure S9, Supporting Information). We did not observe any loading of dapdiamide A (Figure S10, Supporting Information).

**DdaC Is an Fe(II)/ $\alpha$ -KG-Dependent Epoxidase.** When DdaC was added to reaction mixtures containing  $N_\beta$ FmmDAP–S–DdaD and  $\alpha$ -KG, a +16 Da mass shift in the intact mass of both the T domain active site tryptic peptide and small molecule Ppant ejection ion (to yield an ion at  $m/z$  460.2) was observed (Figure 4; Table S3, Supporting Information). This mass shift was observed following two types of enzymatic incubations: (1) reaction mixtures containing DdaC that had been incubated anaerobically with  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$  and  $\alpha$ -KG and then desalted (Figure 4) and (2) reaction mixtures containing aerobically purified DdaC to which excess  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$  was added (Figure S11, Supporting Information). No qualitative differences in the activities of the two enzyme preparations were observed, so the latter method was used for the remaining experiments. No +16 Da mass shift was observed in DdaC incubations that lacked  $\alpha$ -KG (Figure S12, Supporting Information). In contrast to the activity of DdaC on  $N_\beta$ FmmDAP–S–DdaD, we have observed no evidence for DdaC oxidation of free  $N_\beta$ FmmDAP by LC–MS or HPLC assay.

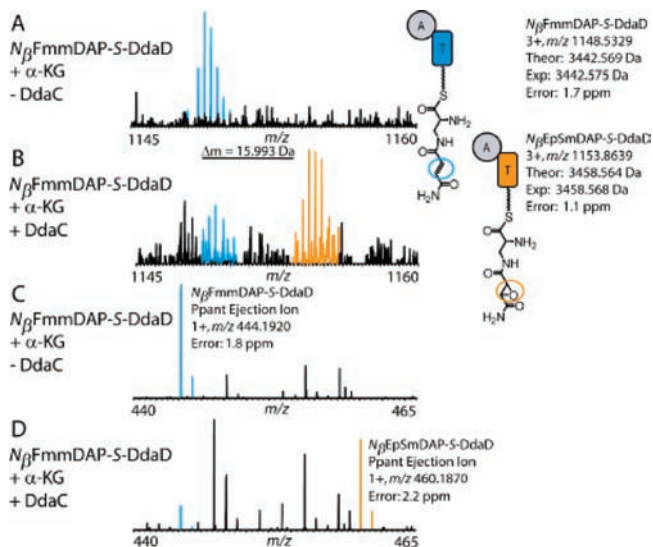
To confirm that the oxidation catalyzed by DdaC was indeed an epoxidation, we compared the MS<sup>n</sup> fragmentation patterns of the Ppant ejection ion generated from HS–DdaD loaded with synthetic  $N_\beta$ –trans–EpSmDAP (both diastereomers) and the Ppant ejection ion generated from  $N_\beta$ FmmDAP–S–DdaD incubated with DdaC. Fragment ions for MS<sup>n</sup> analysis were selected that reported a +16 Da shift when compared to similar analyses

(20) Dorrestein, P. C.; Bumpus, S. B.; Calderone, C. T.; Garneau-Tsodikova, S.; Aron, Z. D.; Straight, P. D.; Kolter, R.; Walsh, C. T.; Kelleher, N. L. *Biochemistry* **2006**, *45*, 12756–12766.

(21) Bumpus, S. B.; Kelleher, N. L. *Curr. Opin. Chem. Biol.* **2008**, *12*, 475–482.



**Figure 3.** Apo-DdaD is converted to holo-DdaD (HS-DdaD) in the presence of Sfp and CoA. In the presence of ATP and  $N_{\beta}$ FmmDAP, HS-DdaD is loaded with  $N_{\beta}$ FmmDAP to form  $N_{\beta}$ FmmDAP-S-DdaD. (A) In the presence of Sfp but the absence of CoA, only the active site peptide from apo-DdaD (green) is observed. (B) In the presence of Sfp and CoA, the active site peptide from HS-DdaD (red) is observed. This peptide shows a mass shift of +340.09 Da from the apo-DdaD active site peptide, the exact mass shift expected for PPTation. (C) The small molecule Ppant ejection ion (red) is observed from the HS-DdaD active site peptide when the peptide is subjected to MS/MS by NSD. (D) Both predicted Ppant ejection peptide marker ions are observed when the HS-DdaD active site peptide is subjected to MS/MS using CID. (E) In the presence of ATP but the absence of  $N_{\beta}$ FmmDAP, only the HS-DdaD active site peptide (red) is observed. (F) In the presence of both ATP and  $N_{\beta}$ FmmDAP, both the HS-DdaD (red) and  $N_{\beta}$ FmmDAP-S-DdaD (blue) active site peptides are observed. (G) When the HS-DdaD and  $N_{\beta}$ FmmDAP-S-DdaD active site peptides are subjected to MS/MS using NSD, both expected small molecule Ppant ejection ions are observed. The HS-DdaD Ppant ejection ion is shown in red, and the  $N_{\beta}$ FmmDAP-S-DdaD Ppant ejection is shown in blue. For the predicted structures of the Ppant ejection ions, see Figure S6 (Supporting Information).



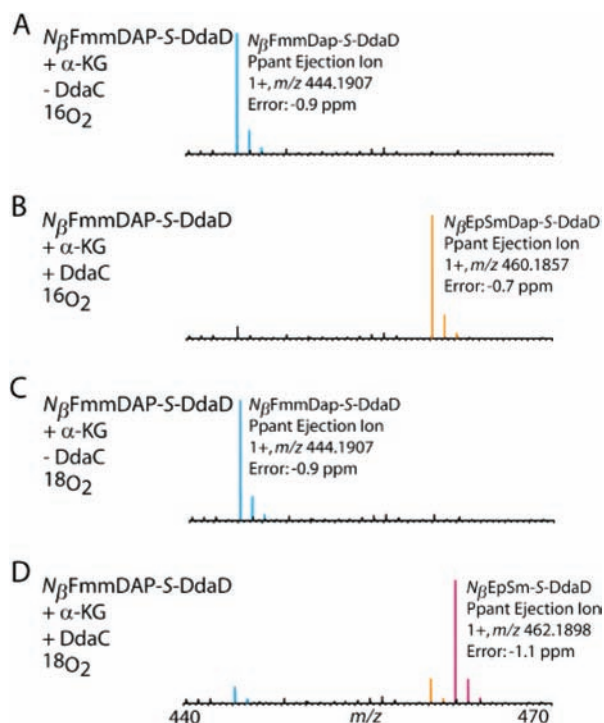
**Figure 4.** In the presence of both  $\alpha$ -KG and DdaC,  $N_{\beta}$ FmmDAP-S-DdaD is converted to  $N_{\beta}$ EpSmDAP-S-DdaD. (A) In the presence of  $\alpha$ -KG but the absence of DdaC, only the active site peptide from  $N_{\beta}$ FmmDAP-S-DdaD (blue) is observed. (B) In the presence of both  $\alpha$ -KG and DdaC (prepared anaerobically),  $N_{\beta}$ FmmDAP-S-DdaD is converted to  $N_{\beta}$ EpSmDAP-S-DdaD and the active site peptide from  $N_{\beta}$ EpSmDAP-S-DdaD (orange) is observed. The theoretical mass shift of this transformation is 15,995 Da. (C) In the presence of  $\alpha$ -KG and the absence of DdaC, only the small molecule Ppant ejection ion from  $N_{\beta}$ FmmDAP-S-DdaD (blue) is observed when the peptides are subjected to MS/MS using NSD. (D) In the presence of both  $\alpha$ -KG and DdaC (prepared anaerobically), the Ppant ejection ions from both  $N_{\beta}$ FmmDAP-S-DdaD and  $N_{\beta}$ EpSmDAP-S-DdaD (orange) are observed when the peptides are subjected to MS/MS using NSD. For the predicted structures of the Ppant ejection ions, see Figure S6 (Supporting Information).

conducted on the Ppant ejection ion from  $N_{\beta}$ FmmDAP-S-DdaD. In all cases, the fragmentation patterns produced were the same for all epoxide-containing species, confirming the DdaC-catalyzed oxidation as an epoxidation (Figure S13, Supporting Information).

To determine the origin of the putative incorporated oxygen, DdaC incubations were carried out either in  $H_2^{18}O$  or under an  $^{18}O_2$  atmosphere. When the reaction was conducted in  $H_2^{18}O$  and then analyzed by trypsin digest followed by LC-MS, a +16 Da mass shift was observed in the DdaD tryptic active site peptide and the corresponding Ppant ejection ion. If the oxygen atom were derived from water, a +18 Da mass shift would be observed (Figure S14, Supporting Information). In contrast, incubations under  $^{18}O_2$  followed by LC-MS and Ppant ejection analysis of intact DdaD resulted in a +18 Da (as opposed to a +16 Da mass shift in a  $^{16}O_2$  atmosphere) mass shift in the observed  $N_{\beta}$ EpSmDAP-S-DdaD Ppant ejection ion, indicating that DdaC uses  $O_2$  as a cosubstrate (Figure 5; Table S3, Supporting Information).

## Discussion

The dapdiamides comprise a family of acylated dipeptide natural antibiotics that likely inhibit the glucosamine-6-phosphate synthase enzyme of susceptible organisms and consequently disrupt cell wall assembly and integrity.<sup>1</sup> The putative target has an active site Cys that can be covalently captured by the warheads of the dapdiamides and related scaffolds.<sup>2</sup> Most commonly, the electrophilic warhead is the enamide moiety provided by the fumaramoyl group of this antibiotic class (e.g., dapdiamides A–D), which can be a Michael acceptor for the Cys thiolate. An alternative electrophile is found in the  $\alpha$ -epoxysuccinamoyl-containing dapdiamide E and in the corresponding epoxysuccinamoyl



**Figure 5.** In an atmosphere of  $^{18}\text{O}_2$ ,  $^{18}\text{O}$  is incorporated into the substrate loaded onto DdaD and  $N_\beta\text{FmmDAP}$  is converted to  $N_\beta\text{-trans-EpSmDAP}$ . (A) Under normal reaction conditions (an atmosphere of  $^{16}\text{O}_2$ ), in the presence of  $\alpha\text{-KG}$  and the absence of DdaC, the only Ppant ejection ion observed (blue) during Ppant ejection analysis (using NSD) of  $N_\beta\text{FmmDAP-S-DdaD}$  corresponds to the unoxidized starting substrate. (B) Under normal reaction conditions (an atmosphere of  $^{16}\text{O}_2$ ), in the presence of both  $\alpha\text{-KG}$  and DdaC, Ppant ejection ions corresponding to both  $N_\beta\text{FmmDAP-S-DdaD}$  and  $N_\beta\text{EpSmDAP-S-DdaD}$  (orange) are observed. (C) Under an atmosphere of  $^{18}\text{O}_2$ , in the presence of  $\alpha\text{-KG}$  but the absence of DdaC, the only Ppant ejection ion observed corresponds to  $N_\beta\text{FmmDAP-S-DdaD}$ . (D) Under an atmosphere of  $^{18}\text{O}_2$ , in the presence of both  $\alpha\text{-KG}$  and DdaC, Ppant ejection ions are observed for both  $N_\beta\text{FmmDAP-S-DdaD}$  and  $N_\beta\text{EpSmDAP-S-DdaD}$ . A +2 Da mass shift (from the  $N_\beta\text{EpSmDAP-S-DdaD}$  Ppant ejection ion in (B)) is observed in the Ppant ejection ion corresponding to  $N_\beta\text{EpSmDAP-S-DdaD}$  (purple), indicating that the oxygen in the epoxide originates from molecular oxygen. For the predicted structures of the Ppant ejection ions see Figure S6, Supporting Information.

$\beta$  regioisomer  $N_\beta\text{EpSmDAP-Val}$  (Figure 1C), in which the olefin of the fumaroyl moiety is replaced with an epoxide that is proposed to be opened by the active site Cys residue. The presumption has been that the fumar(am)oyl group gets epoxidized to the epoxysuccin(am)oyl group to create this second potential electrophile; the timing and the identity of the catalysts responsible for this conversion are the subject of this study.

Regiochemical variation is observed among the dapdiamides with regard to which amino group of 2( $\alpha$ ),3( $\beta$ )-DAP the fumaroyl/epoxysuccinamoyl moieties are attached in amide linkage. Dapdiamides A–C have an  $N_\beta\text{FmmDAP}$  attachment, while dapdiamide D has an  $N_\alpha\text{FmmDAP}$  linkage. Dapdiamide E, with an epoxysuccinamoyl group in  $\alpha$  amide linkage, could in principle arise from epoxidation of dapdiamide D or its likely precursor amino acid  $N_\alpha\text{Fm(m)DAP}$ . The natural product  $N_\beta\text{EpSmDAP-Val}$  could arise from comparable epoxidation of dapdiamide A or  $N_\beta\text{Fm(m)DAP}$ . In this work we demonstrate that it is  $N_\beta\text{FmmDAP}$  that is the substrate for T domain loading and then epoxidation.

In our prior study of the biosynthetic pathway for this antibiotic family, we demonstrated that DdaG and DdaF are

the two ATP-cleaving amide-forming ligases that are responsible for assembling the Fmm-dipeptide scaffold. DdaG uses ATP to activate fumarate to fumaroyl-AMP on the way to making  $N_\beta\text{FmDAP}$ . DdaF then makes the second amide bond, but only after the acid of FmDAP has been converted enzymatically to the amide in FmmDAP. DdaF is a member of the ATP grasp family,<sup>22</sup> and as such cleaves ATP to ADP and  $\text{P}_i$ , presumably making the FmmDAP-phosphate mixed anhydride as an activated intermediate. To date only dapdiamide enzymes that form and utilize  $N_\beta$ -acyl-DAP species have been characterized; the enzyme creating the  $N_\alpha\text{FmmDAP}$  linkage has not yet been identified.

In this study, we assayed both synthetic  $N_\beta\text{-trans-EpSmDAP}$  diastereomers and validated that they are substrates for DdaF in the presence of Val and ATP to produce the  $N_\beta\text{EpSmDAP-Val}$  dipeptide.  $N_\beta\text{EpSmDAP-Val}$  is the natural product (of as yet unassigned epoxide stereochemistry) recently identified in *P. agglomerans* strains (48b/90, 39b/90, and C9-1) as well as a strain of *Serratia plymuthica*.<sup>5,6</sup> We observed selectivity of DdaF for  $N_\beta\text{RREpSmDAP}$  over the (*S,S*) diastereomer, suggesting that the dapdiamide pathway produces a natural product with (*R,R*)-epoxide stereochemistry. The precedence of (*R,R*)-epoxide stereochemistry in the related natural product Sch37137 is in accord with this hypothesis.<sup>8</sup>

Sequence analysis of the Dda gene cluster suggested that, in addition to DdaG and DdaF, one additional ORF should be capable of using ATP to activate an acid cosubstrate, namely, DdaD. This protein is predicted to be a member of a third family of ATP-cleaving enzymes and contains two domains which comprise a minimal NRPS module. The first domain of approximately 50 kDa is predicted to be an A domain, while the second of 10 kDa should be a T domain that can be post-translationally primed with a Ppant group. The adjacent ORF DdaE, encoding a predicted thioesterase, is the only other NRPS-related ORF in the dapdiamide biosynthetic gene cluster, suggesting the adenylation–thiolation (A–T) didomain DdaD acts as a stand-alone module and not as part of a classical NRPS assembly line.

We have shown in several other contexts that stand-alone A–T didomains (or isolated T domains) are used in bacterial metabolism to sequester some fraction of an amino acid pool, tethered as the aminoacyl-*S*-pantetheinyl T domain thioester. The tethered aminoacyl-*S*-T domain is then subjected to covalent modification, which is often oxidative. Thus, in the biosynthesis of the vancomycin class of glycopeptides<sup>23,24</sup> and the nikkomycin<sup>25</sup> and aminocoumarin<sup>26</sup> classes of antibiotics,  $\text{C}_\beta$  hydroxylation of the sequestered aminoacyl thioester moiety is effected by either heme iron or nonheme iron oxygenases. In the biogenesis of syringomycin, chlorination occurs at  $\text{C}_4$  of a tethered threonyl moiety,<sup>27</sup> while in the assembly of the jasmonate phytohormone mimic coronatine, cryptic chlorination occurs at  $\text{C}_\gamma$  of the *allo*-Ile moiety prior to subsequent ring closure to the cyclopropane.<sup>28,29</sup>

- (22) Galperin, M. Y.; Koonin, E. V. *Protein Sci.* **1997**, *6*, 2639–2643.  
 (23) Chen, H.; Thomas, M. G.; O'Connor, S. E.; Hubbard, B. K.; Burkart, M. D.; Walsh, C. T. *Biochemistry* **2001**, *40*, 11651–11659.  
 (24) Hubbard, B. K.; Walsh, C. T. *Angew. Chem., Int. Ed.* **2003**, *42*, 730–765.  
 (25) Chen, H.; Hubbard, B. K.; O'Connor, S. E.; Walsh, C. T. *Chem. Biol.* **2002**, *9*, 103–112.  
 (26) Chen, H.; Walsh, C. T. *Chem. Biol.* **2001**, *8*, 301–312.  
 (27) Vaillancourt, F. H.; Yin, J.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 10111–10116.

With such precedents, we sought an analogous role for modification of an aminoacyl thioester covalently attached to DdaD. The adjacent ORF DdaC has homology to the Fe(II)/ $\alpha$ -KG-dependent dioxygenase family of enzymes, which typically catalyze O<sub>2</sub>-dependent substrate hydroxylations.<sup>12</sup> Examples of members of this family include the syringomycin biosynthetic enzyme SyrP<sup>30</sup> and the kutzneride pathway enzymes KtzO and P,<sup>31</sup> which carry out  $\beta$ -hydroxylations of T domain-tethered aspartate and glutamate, respectively. In addition to hydroxylations, members of this family have been shown to carry out a range of other oxidative transformations.<sup>12</sup> Evidence from bioconversion and cell extract studies has implicated Fe(II)/ $\alpha$ -KG enzymes in epoxidation reactions,<sup>32,33</sup> but to our knowledge no in vitro characterization of a purified epoxidase in this class has been reported previously.

In the context of the known dapdiamide family members (Figure 1A) it seemed likely that DdaC could be an epoxidase that acts on the fumaramoyl/fumaramoyl moiety of an intermediate tethered in thioester linkage to the T domain of DdaD. In addition, DdaE is a predicted thioesterase; thus, the tandem action of DdaD, C, and E could be a branch pathway for selection and activation of an olefin-containing pathway intermediate, epoxidation, and then hydrolysis to produce an epoxysuccin(am)oyl building block for condensation with another monomer via DdaG and/or DdaF. (We have not been able to heterologously express DdaE in a soluble form in *E. coli* to establish such a thioesterase role.)

Validation of the proposed roles for DdaD and DdaC started with determination of the selectivity of the A domain of DdaD. Using the classical ATP-[<sup>32</sup>P]PP<sub>i</sub> exchange assay, diagnostic for reversible formation of tightly held (amino)acyl-AMPs in enzyme active sites, DdaD showed clear preference for N $\beta$ FmmDAP. The K<sub>m</sub> value for DdaD with respect to N $\beta$ FmmDAP was found to be 420  $\mu$ M, comparable to K<sub>m</sub> values reported for other NRPS A domains.<sup>28,34,35</sup> These results provided a key early insight: DdaD is indeed selecting an olefin-containing pathway intermediate for activation as the AMP mixed anhydride. This was strongly suggestive that the fumaramoyl moiety of thioester-tethered FmmDAP would be the species epoxidized.

To validate the second step of A-T didomain function, the predicted covalent loading of N $\beta$ FmmDAP-AMP onto the Ppant arm of the T domain of DdaD, we turned to mass spectrometry. We found that apo-DdaD could be post-translationally converted to the Ppant-containing holo-DdaD by action of purified Sfp. Incubation of holo-DdaD with ATP and N $\beta$ FmmDAP allowed detection of the N $\beta$ FmmDAP-S-Ppant adduct in the T domain by peptide mass analysis and by the release of the N $\beta$ FmmDAP-S-Ppant thioester fragment ion. Thus, the second step of A-T

didomain function, the covalent tethering of the substrate activated by the A domain, was operant.

When DdaC was incubated with the covalent N $\beta$ FmmDAP-S-DdaD enzyme intermediate, a mass increase of +16 Da was observed for both the T domain active site tryptic peptide containing the tethered acyl-DAP thioester and the ejected Ppant ion. We found that, as anticipated for a member of the Fe(II)/ $\alpha$ -KG family, the activity of DdaC is dependent on  $\alpha$ -KG. Additionally, incubation under <sup>18</sup>O<sub>2</sub>(g) resulted in a +18 Da mass shift, demonstrating that DdaC uses molecular oxygen as a cosubstrate.

The ejected pantetheinyl fragment from DdaC/D experiments had the M + 16 Da mass increase anticipated for the epoxide product. However, it was formally possible that the introduction of one oxygen atom into the FmmDAP moiety arose not by epoxidation of the double bond but by C- or N-hydroxylation of the DAP residue. MS<sup>n</sup> fragmentation of Ppant ejection ions from both HS-DdaD loaded with authentic N $\beta$ -trans-EpSmDAP and from N $\beta$ FmmDAP-S-DdaD incubated with DdaC resulted in the same fragmentation pattern, suggesting that DdaC indeed acts as an epoxidase.

Our studies of DdaC have generated a number of questions to be answered in future investigations. We have not attempted to determine single-turnover kinetics of the enzyme because of the difficulty of quantifying its substrate, the covalent N-acylaminoacyl thioester adduct of DdaD. We have also been unable to obtain sufficient N $\beta$ EpSmDAP from DdaC/D incubations to directly determine the stereochemistry of the epoxide carbons in the product, nor have we yet evaluated the epoxidation mechanism. In analogy to proposed mechanisms for Fe(II)/ $\alpha$ -KG hydroxylases, an Fe(IV)-oxo intermediate is the likely oxygen transfer species. However, whether C-O bond formations are stepwise and ionic or radical, as suggested in Scheme S2 (Supporting Information), is yet to be probed.

Additionally, the question arises of why *P. agglomerans* makes both the enamide electrophile (fumaramoyl) and the epoxide electrophile (epoxysuccinamoyl) as parallel N-acyl warheads in this antibiotic family. Two future studies will compare the epoxysuccinamoyl versus the fumaramoyl groups. First, minimum inhibitory concentration (MIC) determinations of the N $\beta$  molecules dapdiamide A and N $\beta$ EpSmDAP-Val will test for any differences in uptake by susceptible bacteria and fungi. Once taken up by the oligopeptide permease systems, intracellular proteases are thought to liberate the N $\beta$ -acyl-DAPs as the proximal inactivators for glucosamine synthase. Thus, it will be useful to compare FmmDAP and EpSmDAP side by side against the target enzyme to determine inactivation efficiencies. It is possible that the epoxide warhead is more selective than the enamide: the epoxide may require acid catalysis in the enzyme active site for covalent capture, whereas Michael addition to the fumaramoyl moiety may not. In that context a proteomics<sup>36</sup> study to evaluate how many proteins in a susceptible cell are targeted covalently would offer a global comparison of "off-target" labeling by the two types of electrophilic N-acyl warheads.

**Acknowledgment.** We thank Emily Balskus, Christopher Neumann, Elizabeth Sattely, and Albert Bowers for helpful discussions. We thank John Heemstra for providing synthetic BODIPY-CoA,

(28) Couch, R.; O'Connor, S. E.; Seidle, H.; Walsh, C. T.; Parry, R. J. *Bacteriol.* **2004**, *186*, 35–42.

(29) Vaillancourt, F. H.; Yeh, E.; Vosburg, D. A.; O'Connor, S. E.; Walsh, C. T. *Nature* **2005**, *436*, 1191–1194.

(30) Singh, G. M.; Fortin, P.; Koglin, A.; Walsh, C. T. *Biochemistry* **2008**, *47*, 11310–11320.

(31) Strieker, M.; Nolan, E. M.; Walsh, C. T.; Marahiel, M. A. *J. Am. Chem. Soc.* **2009**, *131*, 13523–13530.

(32) Watanabe, M.; Sumida, N.; Murakami, S.; Anzai, H.; Thompson, C. J.; Tateno, Y.; Murakami, T. *Appl. Environ. Microbiol.* **1999**, *65*, 1036–1044.

(33) Hashimoto, T.; Matsuda, J.; Yamada, Y. *FEBS Lett.* **1993**, *329*, 35–39.

(34) Mootz, H. D.; Marahiel, M. A. *J. Bacteriol.* **1997**, *179*, 6843–6850.

(35) Ehmann, D. E.; Shaw-Reid, C. A.; Losey, H. C.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2509–2514.

(36) Han, X.; Aslanian, A.; Yates, J. R., III. *Curr. Opin. Chem. Biol.* **2008**, *12*, 483–490.



Elizabeth Sattely for providing Sfp, and Jessica Dawlaty for providing synthetic dapdiamide A and the pUC19 A10A plasmid. This work was supported in part by NIH Grant GM 20011 (C.T.W.), NIH Medical Scientist Training Program GM 07753 (M.A.H.), NIH Grant GM 067725-08 (N.L.K.), NSF Grant MCB-642058 (J.M.B.), and a fellowship from the American Chemical Society Division of Analytical Chemistry (S.B.B.).

**Supporting Information Available:** Additional materials and methods, Tables S1–S5, Figures S1–S14, and Schemes S1–S2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

JA1072367