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An FAD-Dependent Pyridine Nucleotide-Disulfide Oxidoreductase Is Involved in Disulfide Bond Formation in FK228 Anticancer Depsipeptide

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SUMMARY

Disulfide bonds are rare in bacterial natural products, and the mechanism of disulfide bond formation in those products is unknown. Here we characterize a gene and its product critical for a disulfide bond formation in FK228 anticancer depsipeptide in *Chromobacterium violaceum*. Deletion of *depH* drastically reduced FK228 production, whereas complementation of the *depH*-deletion mutant with a copy of *depH* on a medium copy-number plasmid not only fully restored the FK228 production but also significantly increased the FK228 yield. Purified 6xHis-tagged DepH fusion protein in native form is a homodimer of 71.0 kDa, with each monomer containing one molecule of FAD. DepH efficiently converts an immediate FK228 precursor to FK228 in the presence of $NADP^+$. We conclude that DepH is an FAD-dependent pyridine nucleotide-disulfide oxidoreductase, specifically and efficiently catalyzing a disulfide bond formation in FK228.

INTRODUCTION

Disulfide bonds that link two nonadjacent cysteine residues often exist in ribosomally synthesized proteins and peptides as well as their derived products, such as lantibiotics, toxins, venoms, and hormones, to maintain proper folding configuration, to mediate redox cycling of enzyme activity, or to regulate a protein's activation and deactivation (Giles et al., 2003; Kadokura et al., 2003). Proteins that are capable of catalyzing protein/peptide disulfide bond formation are members of a large collection of thiol-disulfide oxidoreductases found in all living cells. Many of these enzymes belong to the thioredoxin superfamily, which is defined by an active site containing a CXXC redox motif (cysteines separated by two amino acids) and by a thioredoxin fold seen in three-dimensional structure of the prototypical thioredoxin 1 of *E. coli* (Lennon et al., 1999; Waksman et al., 1994). The most studied catalysts for disulfide bond formation are the Dsb-family of proteins (DsbA, DsbB, DsbC, and DsbD) of *E. coli* (Bardwell et al., 1991; Martin et al., 1993; Nakamoto and Bardwell, 2004). Other enzymes that are not members of the thioredoxin superfamily but use redox active cysteine residues in transferring electrons in oxidative and reductive pathways have entirely different three-dimensional structures from thioredoxin. They might use small molecule electron donors and acceptors, such as FAD, NAD^+/NADH, $NADP^+$/NADPH, quinone, or lipoic acid (Bryk et al., 2002).

Disulfide bonds are also found in small molecules (natural products) made nonribosomally by a serial of biochemical reactions catalyzed by enzymes other than the ribosomal machinery. Most of those natural products are produced by garlic plants or fungi (Jacob, 2006) and a few are produced by bacteria (Figure 1), but the enzymology of disulfide bond formation in those products is largely unknown. Based on a study of heterologous production of echinomycin in *E. coli*, Watanabe et al. proposed the Ecm17 protein (encoded by *ecm17* gene in the echinomycin biosynthetic gene cluster) as an oxidoreductase that catalyzes the formation of a disulfide bond in a triostin A precursor to afford triostin A (Watanabe et al., 2006), but there was no biochemical evidence to support this plausible notion. Disulfide bond in triostin A is not critical for bioactivities because it can be further modified by a SAM-dependent methyltransferase to form a thioacetal bond in the final product echinomycin. Triostin A is a member of the quinoxaline family of antibiotics that also include BE-22179, SW-163C, and thiocoraline (Dawson et al., 2007) (Figure 1). There is no molecular genetic study of the biosynthesis of BE-22179 or SW-163C. Surprisingly the thiocoraline biosynthetic gene cluster does not contain a gene encoding an Ecm17-like enzyme or, in a broad sense, a thioredoxin-like oxidoreductase (Lombo et al., 2006). It is unclear whether the disulfide bond in thiocoraline is formed by an unidentified enzyme encoded by a gene independent of the gene cluster or simply by chemical oxidation. Finally, the biogenesis of a disulfide bond in FR901,375 (Masakuni et al., 1991), spiruchostatins (Masuoka et al., 2001), somocystinamide A (Nogle and Gerwick, 2002), or a dithiolane bond in leinamycin (Tang et al., 2004), remains to be elucidated.

FK228 (depsipeptide; Figure 1) is a rare disulfide-containing natural product produced by Gram-negative *Chromobacterium violaceum* No. 968 as a prodrug (Shigematsu et al., 1994; Ueda et al., 1994). Prodrug FK228 can diffuse across the cell membrane and be readily activated by intracellular reduction of the disulfide bond inside the cytoplasmic environment of mammalian cells. Upon activation, the freed thiol group on the longer aliphatic tail of reduced FK228 fits inside the catalytic pocket of preferred class I histone deacetylases (HDACs),

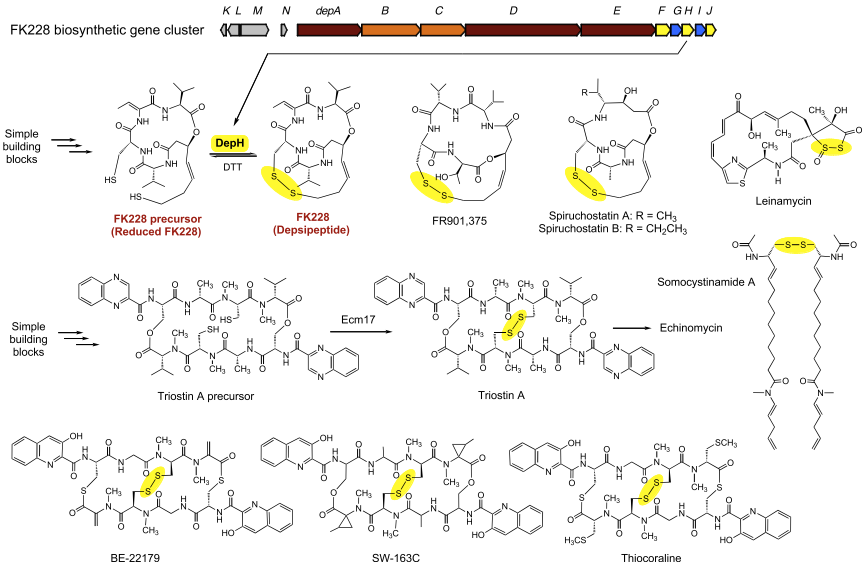

Figure 1. FK228 Biosynthetic Gene Cluster and Structures of Disulfide- or Dithiolane-Containing Natural Products Produced Nonribosomally by Bacteria

Gene *depH* is depicted as one of the three post-nonribosomal peptide synthetase (NRPS; dark red)/polyketide synthase (PKS; orange) tailoring genes (yellow). All natural products listed here have and are expected to have anticancer activities. Conceptual conversions from precursor (the immediate precursor of FK228 or triostin A) to product (FK228 or triostin A) by enzyme (DepH or Ecm17, respectively) are indicated. Disulfide and dithiolane bonds are highlighted by a yellow oval background shading.

chelating Zn^{2+}, and partially inhibits the enzyme activities (Nakajima et al., 1998). Selective but modest inhibition of HDACs leads to a cascade of chromatin remodeling, tumor suppressor gene reactivation, apoptosis, and regression of cancer (Bolden et al., 2006). FK228 has become one of the most promising anticancer agents specifically intervening cancer epigenetics (Yoo and Jones, 2006).

Aimed at diversification and optimization of FK228 through metabolic engineering, combinatorial biosynthesis, and chemoenzymatic synthesis, we previously cloned and partially characterized the FK228 biosynthetic gene cluster (designated as *dep* for depsipeptide). Based on the deduced protein functions of *dep* genes, we proposed an unusual hybrid nonribosomal peptide synthetase (NRPS)-polyketide synthase-NRPS pathway for FK228 biosynthesis in *C. violaceum* no. 968 (Cheng et al., 2007). This pathway would lead to the production of an immediate FK228 precursor (reduced FK228) with two free thiol groups from cysteine residues (Figure 1). Furthermore, we proposed DepH as a putative FAD-dependent pyridine nucleotide-disulfide oxidoreductase, encoded by *depH*, to catalyze a disulfide bond formation between two thiol groups as the final step in FK228 biosynthesis.

Here we report genetic and biochemical evidence in support of DepH as an FAD-dependent pyridine nucleotide-disulfide oxidoreductase, specifically and efficiently catalyzing a disulfide bond formation in FK228. To our best knowledge, this work represents the first biochemical characterization of an enzyme involved in the formation of a disulfide bond in a nonribosomally produced bacterial natural product.

RESULTS

Protein Sequence Analysis of DepH

Gene *depH* in the FK228 biosynthetic gene cluster has an open reading frame (ORF) of 960 bp and was predicted to encode a 319-amino-acid FAD-dependent pyridine nucleotide-disulfide oxidoreductase, DepH (GenBank accession number ABP57752), that converts two thiol groups from cysteines into a disulfide bond as the final step of FK228 biosynthesis (Cheng et al., 2007). Primary sequence of DepH shares a 72% identity/85% similarity to a hypothetical protein (GenBank accession number ABC38333; named TdpH in our ongoing work) of *Burkholderia thailandensis* E264 (Kim et al., 2005), and shares modest percentages of identity/similarity to a few hypothetical proteins of *Pseudomonas*, *Sinorhizobium*, or *Cellvibrio* species. Neither the DepH nor the TdpH sequence has more than 34% identity/46% similarity to the deduced Ecm17 sequence of the *ecm17* gene in the triostin A/echinomycin biosynthetic gene cluster in *S. lasaliensis* (Watanabe et al., 2006). A phylogenetic analysis of the sequences of DepH and its closest homologs, and other well-studied proteins involved in disulfide bond formation in proteogenic products, suggests that an active site containing a CXXC redox motif is conserved in all related proteins; but DepH, TdpH, Echm17 and a few hypothetical proteins constitute a distinctive clade (group 1) of proteins with a CPY/FC motif, which is clearly different from that (CAT/VC) of the thioredoxin reductase (TrxB/TrxR) family of enzymes (group 2) or that (CXXC; X represents any less conserved residue) of the Dsb-family of enzymes (group 3) (Figure 2A). Site-directed mutagenesis experiments have confirmed the catalytic essentiality of both cysteine residues in the CPYC redox motif of DepH (see Enzyme Activity and Kinetics). Furthermore, DepH can be dissected into an FAD-binding domain, an $NADP^+$/NADPH-binding domain and two terminal regions, according to its organizational similarity to the TrxR of *Mycobacterium tuberculosis* (Akif et al., 2005) (Figure 2B).

Genetic Confirmation of the Involvement of *depH* in FK228 Biosynthesis

We adopted and further improved an efficient, broad host-range genetic system for gene deletion, marker removal, and gene complementation in *C. violaceum* No. 968, and potentially in a wide range of other Gram-negative bacteria (Cheng et al., 2007; see Supplemental Data available online). Subsequently we created two lines of *C. violaceum depH*$^-$ mutant (Table S1; Figures S1 and S2). In mutant CvΔdepH::FRT, a 555 bp internal part of *depH* is replaced by an *FRT* cassette from pPS858 (Hoang et al., 1998). Due to a concern about a potential polar effect on the functioning of downstream genes (see the *dep*

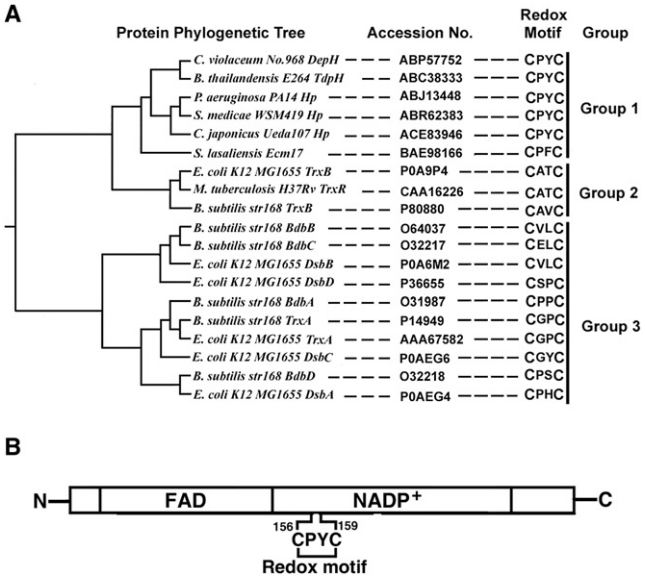

Figure 2. Sequence Analyses of DepH

(A) Phylogenetic analysis of DepH, its closest homologs, and representative bacterial proteins involved in protein/peptide disulfide bond formation. Amino acid sequences were obtained from the GenBank database, alignment was performed with ClustalW program (Chenna et al., 2003) and phylogenetic tree is presented as a rooted dendrogram. Each protein sequence is labeled by a strain name followed by a protein name where available, or by "Hp" (hypothetical protein). Database accession numbers are listed in the separate column. The redox motif (CXXC) of each protein was extracted from aligned sequences. Proteins are classified into three groups according to their phylogenetic relationships and their redox motif residues.

(B) Scheme of domain organization and the position of the redox motif of DepH.

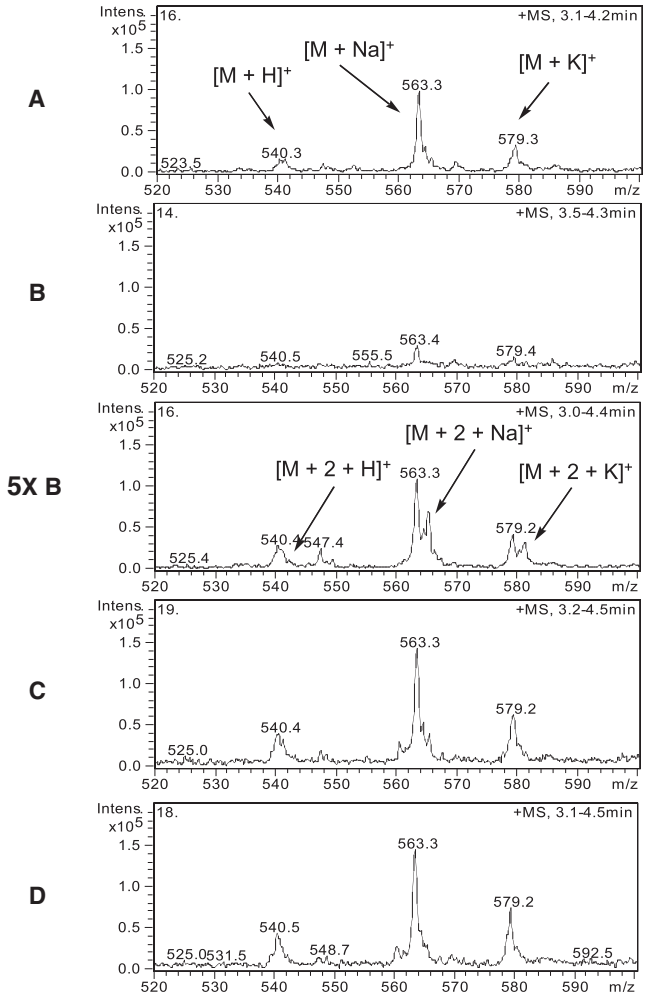

Figure 3. Detection and Quantification of FK228 Production by LC-MS Analysis

The relative abundance of FK228 production is estimated by the sum of ion signals from three signature peaks of each strain/sample.

(A) Sample from the wild-type strain (CvWT).

(B) 1X and 5X concentrated samples of the *depH*-deletion mutant strain CvΔdepH.

(C) Sample from the CvΔdepH/BMTL3-depH complementant strain.

(D) Sample from the CvWT/BMTL3-depH control strain.

gene cluster organization in Figure 1), we excised the *FRT* cassette from CvΔdepH::FRT by a site-specific Flp endonuclease encoded by vector pBMTL3-FLP2 and created a marker-free mutant CvΔdepH with only a 85 bp scar left at the site of gene deletion. Furthermore, we complemented this CvΔdepH mutant with a copy of *depH* on a medium copy-number expression vector (pBMTL3-depH), resulting in a complementant strain CvΔdepH/pBMTL3-depH.

Examination of FK228 production in the wild-type strain (CvWT), CvΔdepH mutant strain, CvΔdepH/pBMTL3-depH complementant strain, and a CvWT/pBMTL3-depH control strain of *C. violaceum* by liquid-chromatography mass spectrometry (LC-MS) analysis revealed very interesting results (Figure 3). First, consistent with a previous study (Cheng et al., 2007), the CvWT strain produced a signature profile of three FK228 ion adducts, $[M + H]^+$ = 540.3 *m/z*, $[M + Na]^+$ = 563.3 *m/z*, and $[M + K]^+$ = 579.3 *m/z*. Second, the CvΔdepH mutant strain produced much less FK228 (~20% of the wild-type level). When the sample extract of this strain was concentrated 5-fold and analyzed again, a shoulder ion signal peak right next to each main signal peak became noticeable. When we zoomed in, we found that those shoulder peaks had a +2 *m/z* value corresponding to each of the main signal peaks. Those signals, $[M + 2 + H]^+$ = 542.4 *m/z*, $[M + 2 + Na]^+$ = 565.4 *m/z*, and $[M + 2 + K]^+$ = 581.3 *m/z*, were apparently from an immediate FK228 precursor—the unoxidized (reduced) precursor with two free thiols (Figure 1). Furthermore, the CvΔdepH/pBMTL3-depH complementant strain not only regained FK228 production, but also produced about 30% more FK228 than the CvWT strain, indicating that the DepH-catalyzed disulfide bond formation is a rate-limiting step in FK228 biosynthesis inside the reducing environment of bacterial cells. Finally, the CvWT/pBMTL3-depH control strain indeed produced significantly (~34%) more FK228 than the CvWT strain, confirming that DepH-catalyzed disulfide bond formation is the rate-limiting step in FK228 biosynthesis.

Overexpression, Purification and Initial Characterization of DepH

DepH was overexpressed as an N-terminal 6xHis-tagged fusion protein and was purified using Ni-NTA agarose chromatography to 95% homogeneity. The yield was about 30 mg/L under the

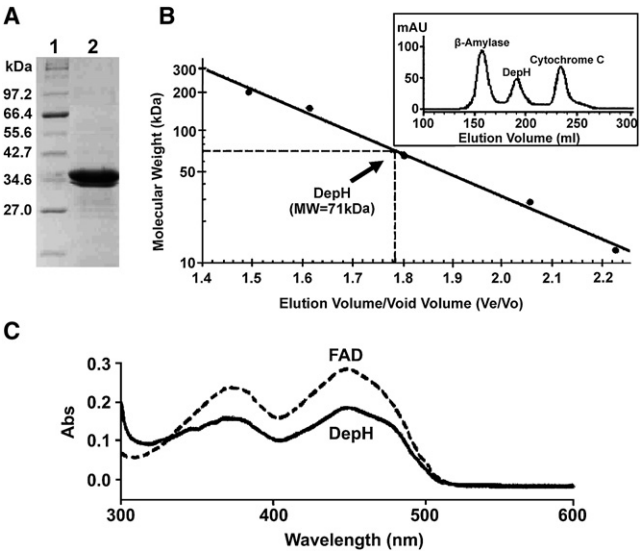

Figure 4. Purification and Characterization of DepH

(A) Ni-NTA affinity-purified DepH was visualized on a SDS-PAGE gel.

(B) The molecular mass of DepH was determined by size exclusion chromatography. Protein standards were used to generate a standard curve. The inset shows an elution profile of DepH bracketed by two protein standards.

(C) UV/Vis absorbance spectra of DepH and an FAD standard. The concentrations of FAD standard and DepH were 25 μM and 36 μM, respectively, both in 20 mM Tris-HCl (pH 7.0) and 100 mM NaCl buffer.

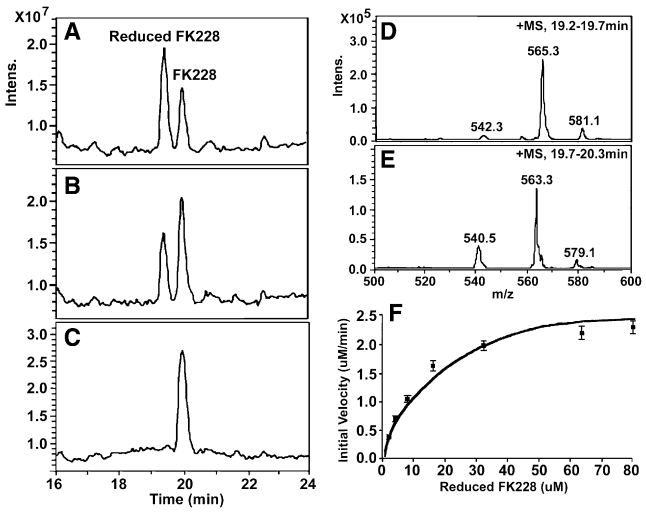

Figure 5. In Vitro Assays of DepH in Converting an Immediate FK228 Precursor to FK228

(A) A control reaction without DepH and kept at −80°C had ~40% reduced FK228 converted to FK228 due to spontaneous oxidation in prior steps.

(B) Another control reaction without DepH but kept at room temperature had ~60% reduced FK228 converted to FK228 due to spontaneous oxidation.

(C) A complete assay had 100% reduced FK228 converted to FK228 in 5 min or less at room temperature.

(D) Mass spectrum of reduced FK228.

(E) Mass spectrum of FK228.

(F) Michaelis-Menten plot for DepH-catalyzed reactions with varying concentrations of reduced FK228 substrate. Error bars refer to standard deviation.

conditions specified in Experimental Procedures. On SDS-PAGE gel, denatured 6xHis-tagged DepH appeared to have a molecular mass of approximately 36.5 kDa (including a 2.1 kDa 6xHis tag) (Figure 4A). The minor protein band migrating slightly in front of the major band might result from weak protein cleavage at a specific point, even though a protease inhibitor cocktail was added to buffers during purification steps. Without the presence of a reducing agent in our purification scheme, DepH is expected to exist in an oxidized form with an intact disulfide bond formed at the CPYC redox motif/catalytic site (Figure 2B). By size exclusion chromatography, native 6xHis-tagged DepH was determined to have an apparent molecular mass of 71.0 kDa (Figure 4B). These results suggest that DepH exists as a homodimer under native conditions.

Identification of FAD as a Cofactor of DepH

Several lines of evidence suggested the presence of FAD cofactor in DepH. First, purified 6xHis-tagged DepH has a distinctive yellow color, indicating the presence of a flavin prosthetic group. Second, the major absorption wavelengths of DepH were at 377 nm and 455 nm, which are comparable with those of an FAD standard (Figure 4C). Third, to confirm FAD as the cofactor, DepH was denatured and the denatured protein was cleared by centrifugation. The supernatant retained the distinctive yellow color, suggesting that the suspected cofactor is not covalently bound to the protein. Subsequently, the supernatant was subjected to high-performance liquid chromatography (HPLC) analysis, along with FAD as a control. The supernatant generated a HPLC profile that is essentially identical to that of FAD standard in terms of retention time and peak shape (Figure S3). Finally, the suspected cofactor peak was collected from HPLC and analyzed by LC-MS. The sample yielded a signal of 785.3 *m/z*, which is in agreement with the ion signal of FAD standard (Figure S3 inserts).

The DepH-FAD stoichiometry was approximately 1.1 to 1, suggesting that each monomeric DepH contains one molecule of FAD cofactor. This is consistent with a generalized observation regarding the flavoprotein disulfide reductase-family of enzymes (Argyrou and Blanchard, 2004).

Enzyme Activity and Kinetics

To assay the enzyme activity of DepH in catalyzing the conversion of an immediate FK228 precursor (reduced FK228) to FK228 (Figure 1), reduced FK228 was freshly prepared by reducing FK228 with an excess amount of DTT in acetonitrile/water (20% v/v), purified with preparative HPLC, dried in vacuo, and kept airtight at −20°C. Prior to assays, an appropriate amount of reduced FK228 in amorphous state was resuspended in acetonitrile to approximately 0.54 mg/ml (1.0 mM).

For qualitative assays performed in duplicate, a fixed amount of substrate (reduced FK228) was mixed with a fixed amount of enzyme (DepH) in an appropriate buffer for 5 min at room temperature in a nitrogen environment. The reaction mixture was then quenched and subjected to LC-MS analysis. Due to unavoidable spontaneous chemical oxidation during prior purification, storage, and resuspension steps, a control assay that was without enzyme and kept at −80°C had approximately 40% substrate oxidized (Figure 5A); a second control assay that was

Table 1. Relative Activities of the Wild-Type and Mutants of DepH in Converting Reduced FK228 to FK228

Enzyme	Substrates	Products	Relative Activity (%)
Blank (buffer)	Reduced FK228, $NADP^+$	–, –	0
DepH	Reduced FK228, $NADP^+$	FK228, NADPH	100
DepH-C156S	Reduced FK228, $NADP^+$	FK228, NADPH	7.5
DepH-C159S	Reduced FK228, $NADP^+$	FK228, NADPH	3.8
DepH-C156S/C159S	Reduced FK228, $NADP^+$	FK228, NADPH	3.6

without enzyme but kept at room temperature in a nitrogen gas-filled chamber had approximately 60% substrate oxidized (Figure 5B). In a complete assay, 100% of substrate was oxidized in 5 min or less (Figure 5C). The identity of reduced FK228 and FK228 (oxidized) was confirmed by subsequent LC-MS analysis (Figures 5D and 5E). Reduced FK228 with two free thiol groups is more polar, and was eluted slightly earlier than FK228.

Single point mutants (C156S or C159S) and a double mutant (C156S/C159S) of the redox motif (CPYC) of DepH were obtained by site-directed mutagenesis, and the mutant proteins were purified to homogeneity accordingly (Figure S4). The relative activities of three mutant forms of DepH compared with that of wild-type DepH were determined (Table 1). It was found that mutation of either or both cysteine residues in the redox motif resulted in a drastic reduction of activity, suggesting that both cysteine residues are critical for enzyme activity.

For steady-state kinetic assays, variable concentrations of substrate (reduced FK228) were assayed with a fixed amount of enzyme (DepH) in an appropriate buffer for variable durations of time at room temperature in a nitrogen environment. Assay reactions were stopped by mixing with iodoacetamide, which reacts with free thiol groups to form a stable adduct (Mieyal et al., 1991). This adduct of reduced FK228 was quantified by LC-MS analysis. The DepH catalytic parameters toward reduced FK228 in the presence of $NADP^+$ were determined to be V_{max} = 2.6 ± 0.2 μM min^{-1}, K_m = 11.7 ± 0.5 μM, k_{cat} = 1.4 × 10^2 ± 0.2 min^{-1}, and k_{cat}/K_m = 12.0 $\mu M^{-1} \cdot min^{-1}$ (Figure 5F). When $NADP^+$ was replaced by NAD^+, the K_m was measured to be 19.4 ± 0.9 μM. Therefore, $NADP^+$ is a preferred electron acceptor of DepH for the reaction.

DISCUSSION

Disulfide bonds are common in proteogenic biomolecules but rare in natural products produced nonribosomally by bacteria. In proteogenic biomolecules such as proteins and peptides, disulfide bonds often serve to maintain structural integrity or to mediate redox cycling of enzyme activity (Giles et al., 2003; Kadokura et al., 2003). In natural products there are two scenarios (Figure 1). First, as seen in the quinoxaline family of antibiotics, disulfide bonds exist either as a transit stage of biosynthesis or as a part of the final static structure; they might contribute to the stability of molecules but are not critical for bioactivity. Second, as seen in FK228 and likely in spiruchostatins as well, disulfide bonds serve to not only stabilize the molecules in the form of prodrug, but also mediate the mechanism of bioactivity. In the last case, once the disulfide bond is opened by cellular reduction, a freed thiol group selectively chelates the Zn^{2+} ion of class I HDACs, thus inhibiting the enzyme activities (Nakajima et al., 1998). Selective but modest inhibition of HDAC activities leads to a cascade of epigenetic consequences including chromatin remodeling and cancer regression (Bolden et al., 2006).

There have been extensive studies about disulfide bond formation in proteogenic biomolecules catalyzed by the thiol-disulfide oxidoreductase family of enzymes (Kadokura et al., 2003). In contrast, little is known about disulfide bond formation in natural products, particularly those of bacterial origin. In this paper, we have characterized DepH as an FAD-dependent pyridine nucleotide-disulfide oxidoreductase, specifically and efficiently catalyzing a disulfide bond formation in FK228, an epigenetically acting anticancer natural product.

Phylogenetic analysis of protein sequences classified DepH, TdpH, Ecm17, and a few hypothetical proteins into a unique group of proteins that share a conserved CPY/FC redox motif, which is different from that (CAT/VC) of the TrxB/TrxR family of thioredoxin reducatses or that (CXXC) of the Dsb family of enzymes (Figure 2). Site-directed mutagenesis also confirmed the essentiality of both cysteine residues in the CPYC motif of DepH (Table 1). Therefore, the DepH-family of disulfide bond formation enzymes might represent a new class of oxidoreductases specifically involved in natural product biosynthesis. We are in the process of crystallizing DepH, solving its structure, and further elucidating the mechanism of DepH-catalyzed disulfide bond formation in FK228.

Inspired by prior knowledge about reactions catalyzed by thioredoxin reductase and thioredoxin (Lennon et al., 1999), here we propose a cascade of reactions that lead to the oxidation of the immediate FK228 precursor to afford FK228 (Figure 6). In this model, DepH is produced by bacterial cells in an oxidized form, which grabs electrons from the immediate FK228 precursor (reduced FK228) and passes the electrons through cofactor FAD on to $NADP^+$/NADPH-mediated oxidative cellular processes. As a net consequence, the reduced FK228 is oxidized with the formation of a disulfide bond to become FK228. Interesting questions to be answered include how DepH is produced and maintained in the first place in an oxidized form in the reducing environment of bacterial cytoplasm, and whether mature FK228 would be reversibly reduced inside the bacterial cytoplasm and thus would require constant enzymatic maintenance.

Deletion of *depH* led to a drastic decrease of FK228 production by the CvΔdepH mutant strain, which confirmed the critical role of *depH* in FK228 biosynthesis (Figure 3). The notably increased accumulation of an immediate FK228 precursor with positive ion signals of $[M + 2 + H/Na/K]^+$ *m/z* in the extract of this mutant strain suggests that the immediate FK228 precursor contains two free thiol groups and that the oxidative conversion from this precursor to FK228 is indeed

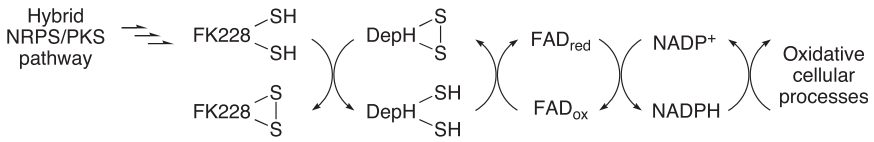

Figure 6. Proposed Sequence of Oxidation-Reduction Reactions in the Final Step of FK228 Biosynthesis

Abbreviations or symbols: FK228=$(SH)_2$, the reduced form of FK228 (immediate FK228 precursor); FK228=(S-S), the oxidized (natural) form of FK228; DepH=$(SH)_2$, the reduced form of DepH; DepH=(S-S), the oxidized form of DepH; FAD_{ox}, the oxidized form of FAD; FAD_{red}, the reduced form of FAD.

the final step in FK228 biosynthesis. Apparently it takes a dedicated DepH catalyst to efficiently convert the precursor into FK228 inside the reducing environment of bacterial cells. Nevertheless, the mutant still produced about 20% as much FK228 as the wild-type strain. A plausible explanation is that spontaneous chemical oxidation could slowly convert the precursor into FK228, despite the reducing microenvironment of bacterial cells.

However, why is the immediate FK228 precursor in the mutant strain not accumulated to a level comparable to the FK228 level in the wild-type strain? We speculate that the precursor with free thiol groups might be liable to hydrolysis inside or outside of bacterial cells, or the precursor might be toxic to bacterial cells by allowing the free thiol groups to interact with some of the hundreds of Zn^{2+}-containing enzymes (Blencowe and Morby, 2003). If the later speculation were proven true, it could explain why the *C. violaceum* no. 968 strain produces FK228 in the form of a prodrug, because prodrug FK228 with an intact disulfide bond is stable, inert, and nontoxic. Prodrug strategy has become a privileged scheme in modern drug development for improving physicochemical, biopharmaceutical, or pharmacokinetic properties of pharmacologically active agents (Rautio et al., 2008).

The promising anticancer activities of FK228 and the unique role of DepH in FK228 biosynthesis entitle the use of the DepH sequence as bait for genome mining of new biosynthetic gene clusters that might produce additional FK228-like natural products. In fact, we have identified a strong homolog of DepH that we named TdpH (GenBank accession number ABC38333), which is putatively encoded by a gene (*BTH_I2359*) in the published genome of *B. thailandensis* E264 (Kim et al., 2005). DepH and TdpH sequences share a 72% identity/85% similarity. The TdpH-encoding gene is located within a cryptic biosynthetic gene cluster that shares striking similarity to the FK228 biosynthetic gene cluster, and our laboratory is currently exploring this finding for the discovery of FK228-like compounds from *B. thailandensis* E264.

Furthermore, DepH, TdpH, and Ecm17 are the only known or proposed enzymes involved in disulfide bond formation in natural products; therefore, their genes can be exploited in biosynthetic pathway engineering schemes for the formation of disulfide bonds in new drug molecules. Similarly, the DepH, TdpH, and Ecm17 proteins can be exploited in chemoenzymatic synthesis schemes for in vitro conversion of synthetic precursors containing two free thiol groups into final products with a disulfide bond.

Finally, disulfide bond formation in FK228 biosynthesis appeared to be a rate-limiting step; overexpression of *depH* has led to an increased yield of FK228 in the CvΔdepH/pBMTL3-depH complementant strain as well as in the wild-type control strain CvWT/pBMTL3-depH (Figure 3). This finding provides opportunities for improving the yield of FK228 or related natural products by genetic engineering.

SIGNIFICANCE

The biochemistry of disulfide bond formation in nonribosomally produced natural products, including some important anticancer agents, is largely unknown. In this work, we genetically confirmed the involvement of *depH* in the biosynthesis of FK228, an epigenetically acting anticancer natural product produced by *Chromobacterium violaceum* no. 968, and we biochemically characterized DepH as an FAD-dependent pyridine nucleotide-disulfide oxidoreductase, specifically and efficiently catalyzing a disulfide bond formation in FK228. Sequence analysis suggested that DepH contains a signature redox motif CPYC, a conserved FAD-binding domain, and an $NADP^+$/NADPH-binding domain. When either or both cysteine residues in the redox motif were mutated, the protein lost most of its catalytic activity. We speculate that this motif might represent a subfamily of disulfide oxidoreductases specifically involved in secondary metabolism. We further showed that deletion of *depH* led to a drastic decrease of FK228 production, while the relative abundance of the immediate FK228 precursor increased notably. Interestingly, complementation of the *depH*-deletion mutant with *depH* restored FK228 production to a level 30% higher than that of the wild-type strain. Additionally, we showed that purified 6xHis-tagged DepH protein in native form is a homodimer of 71.0 kDa, with each monomer contains one molecule of FAD cofactor. Finally we showed that DepH can efficiently convert the immediate FK228 precursor into FK228 in the presence of an $NADP^+$ electron acceptor, and the catalytic parameters are V_{max} = 2.6 ± 0.2 μM min^{-1}, K_m = 11.7 ± 0.5 μM, k_{cat} = 1.4 × 10^2 ± 0.2 min^{-1}, and k_{cat}/K_m = 12.0 $\mu M^{-1} \cdot min^{-1}$. To the best of our knowledge, this work represents the first biochemical characterization of an enzyme involved in disulfide bond formation in a nonribosomally produced bacterial natural product.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Growth Conditions

Bacterial strains and plasmids used in this study are summarized in Table S1. All chemicals, biochemicals, and media components were purchased from Fisher Scientific (Pittsburgh, PA), unless otherwise indicated. *C. violaceum* no. 968 and *E. coli* strains were maintained, cultivated, and genetically manipulated as described elsewhere (Cheng et al., 2007), except that only 1% (w/v)

Diaion HP-20 (Supelco, Bellefonte, PA) resin was added to fermentation medium to absorb bacterial byproducts.

DNA Manipulation, PCR, Cloning, and Sequencing

General DNA manipulations were performed according to standard protocols (Sambrook and Russell, 2000). Bacterial genomic DNA was extracted with an UltraClean microbial DNA isolation kit from MO BIO Labs (Carlsbad, CA). DNA modification enzymes and restriction enzymes were purchased from New England BioLabs (Ipswich, MA). Oligonucleotide primers were ordered from Operon (Huntsville, AL). QIAprep plasmid purification kit and QIAEX II gel extraction kit were from QIAGEN (Valencia, CA). Polymerase chain reaction (PCR) was performed on a DNA Engine Dyad thermocycler (Bio-Rad, Hercules, CA). DNA sequencing was performed with an ABI 3730 automated DNA sequencer (Applied BioSystems, Forster City, CA) at the University of Wisconsin-Madison Biotechnology Center.

Gene Deletion, *FRT* Cassette Removal, and Gene Complementation

The general scheme of using a broad host-range Flp-*FRT* recombination system for site-specific gene replacement/deletion and marker removal has been described elsewhere (Choi and Schweizer, 2005; Hoang et al., 1998), but was modified in this study (Figures S1 and S2).

To construct a *depH*-gene replacement vector, two DNA fragments (amplicons) were first amplified by PCR from total DNA of the wild-type *C. violaceum* no. 968 strain: a 470 bp 5′-end of *depH* gene fragment (amplicon 1) was amplified with primer set KpnI-depH-UpF (5′-AGGTACCGGGATTCGTCGCTGTTGC-3′) and FRT-F-depH-UpR (5′- TCAGAGCGCTTTTGAAGCTAATTCGATCACCAGCACGCGGC-3′), a 460 bp 3′-end of *depH* gene fragment (amplicon 2) was amplified with primer set FRT-R-depH-DnF (5′- AGGAACTTCAAGATCCCCAATTCGACGGGCTGTTCACGATG-3′) and BamHI-depH-DnR (5′- AGGATCCGCGCGGCGGCTTTGC-3′), and a 1.8 kb *FRT* cassette containing a *Gm*^{r} marker gene and a *GFP* reporter gene flanked by two *FRT* recognition sequences (amplicon 3) was amplified from pPS858 with primer set FRT-F (5′-CGAATTAGCTTCAAAAGCGCTCTGA-3′) and FRT-R (5′- CGAATTGGGGATCTTGAAGTTCCT-3′). Amplicons 1–3 were assembled into a 2.7 kb amplicon 4 by multiplex PCR using Long Amp DNA polymerase. Amplicon 4 was digested with KpnI/BamHI and the insert was subsequently cloned into suicide vector pEX10Tc to make a *depH*-gene replacement vector pYC04-18.

To create a *depH*-gene replacement mutant of *C. violaceum*, pYC04-18 was first transformed into *E. coli* S17-1 cells, which subsequently passed the vector to *C. violaceum* cells via interspecies conjugation. Mutant strains of *C. violaceum* with *depH* partially replaced by the *FRT* cassette were selected on LB agar supplemented with 200 μg/ml ampicillin (Ap; *C. violaceum* is naturally resistant to Ap up to 500 μg/ml), 50 μg/ml gentamicin, and 5% (w/v) sucrose at 30°C. The genotype of independent mutants was verified by colony PCR using the primer set depH-F (5′-CGACGTCATCGTGATCGGCGGC-3′) and depH-R (5′- CATTTCCTGAGCGGTCAGGC-3′) (Figure S2A). One representative mutant strain was saved and named CvΔdepH::FRT.

To create a marker-free mutant by removing the *FRT* cassette from CvΔdepH::FRT, a broad host-range Flp-expression vector was first constructed. A 5.16 kb SacI/SphI fragment containing the *cI857* < P_{ts}-P_{λ} > *flp*-P_{sac} > *sacB* genetic determinants was excised from pFLP2, blunt ended, and cloned into the EcoRV site of pBMTL-3 to make pBMTL3-FLP2. This vector replicates in a broad range of bacterial hosts at or above 37°C and expresses a site-specific Flp endonuclease. Vector pBMTL3-FLP2 was introduced into CvΔdepH::FRT by electroporation and marker-free mutants were selected on LB agar supplemented with 200 μg/ml Ap and 25 μg/ml chloramphenicol (Cm) at 37°C. Vector pBMTL3-FLP2 was subsequently cured from the mutants by steaking for two rounds on LB agar supplemented with 200 μg/ml Ap and 5% (w/v) sucrose at 30°C. The genotype of independent gene-deletion mutants was verified by colony PCR using the primer set depH-F and depH-R (Figure S2B). One final representative marker-free mutant strain was saved and named CvΔdepH.

To complement the CvΔdepH mutant and CvWT strain (as control) with a functional *depH* gene on a vector, the entire *depH* ORF (960 bp) was amplified from genomic DNA with primer set depH-exp-FP (5′-GTCTAGACATATGAAGGCCGCCCGCGCG-3′) and depH-exp-RP (5′-GCAAGCTTTCACCCGAACACCAACTTGCG-3′). This product was digested with NdeI/HindIII and the insert was cloned into pET29a to make pET29a-depH intermediate construct. The *depH* ORF along with an upstream ribosomal binding site from the vector was excised by XbaI/HindIII digestion from pET29a-depH and cloned into pBMTL-3 to make the final *depH* complementation vector pBMTL3-depH. The gene fidelity was verified by resequencing and the vector was introduced by conjugation into CvΔdepH mutant to create a complementation strain CvΔdepH/pBMTL3-depH and into CvWT to create a control strain CvWT/pBMTL3-depH.

Bacterial Fermentation, Extraction, Identification, and Quantification of FK228, and Preparation of Reduced FK228

Fermentation of the CvWT strain, CvΔdepH mutant strain, CvΔdepH/pBMTL3-depH complementant strain, and CvWT/pBMTL3-depH control strain of *C. violaceum* in 50 ml nutrient broth supplemented with 200 μg/ml Ap, 1% (w/v) of Diaion HP-20 resins, and with 0.5% (v/v) lactose (lactose induces the expression of *depH* on the pBMTL-3 vector) for 4 days at 30°C, was performed similarly as described elsewhere (Cheng et al., 2007). After fermentation resins and cell debris of each strain were collected by centrifugation and lypholized to dryness. Ten ml of ethyl acetate was used to extract the dried mass and 20 μl of such organic extract was analyzed with an Agilent 1100 series LC/MSD Trap mass spectrometer (Agilent, Santa Clara, CA) for the detection and quantification of FK228 production by relating the peak area of ion signals to that of FK228 standard, as described elsewhere (Cheng et al., 2007; Hwang et al., 2004).

A larger quantity of FK228 was purified from 15 L fermentation culture of wild-type *C. violaceum* according to a previously published procedure (Ueda et al., 1994), and saved as amorphous powder at −20°C until use. To prepare for the immediate FK228 precursor (reduced FK228 with two free thiols), a proper amount of FK228 was redissolved in acetonitrile/water (20% v/v) and mixed overnight at room temperature with 50 mM DTT. Reduced FK228 (almost 100% reduction) was purified from reaction mixture by preparative HPLC on a ProStar HPLC system (Varian, Walnut Creek, CA) with a 10 μm particle size, 21.2 × 250 mm Prep-C18 column (Agilent). A gradient from 100% buffer A (20% acetonitrile) to 100% buffer B (100% acetonitrile) was achieved in 30 min. The fraction containing the reduced FK228 was lypholized and stored airtight at −20°C until use. Reduced FK228 was readily redissolved in acetonitrile to make suitable concentrations of substrate solution for enzymatic assays.

DepH Overexpression and Purification, and Site-Directed Mutagenesis

The previously amplified *depH* ORF (960 bp) was cloned into the NdeI/HindIII sites of pET28a to make pCW01-1212 in *E. coli* DH5α cells. The DNA fidelity was verified by sequencing and the vector was introduced into *E. coli* BL21(DE3) cells for protein overexpression and purification.

E. coli BL21(DE3)/pCW01-1212 was cultured in 4 L LB medium supplemented with 50 μg/ml kanamycin at 37°C to reach an OD_{600} of 0.6. Then IPTG was added to a final concentration of 0.05 mM to induce gene expression and the cells were further cultured at 28°C for 12 hr. Cells were harvested by centrifugation and resuspended in 50 ml lysis buffer (50 mM phosphate [pH 7.0], 300 mM NaCl, 0.1% [v/v] Tween-20, and two complete EDTA free protease inhibitor cocktail tablets [Roche, Indianapolis, IN]). Cells were broken by passing twice through a French Press (Sim-Aminco) and cell lysate was clarified by centrifugation at 48,400 g for 30 min at 4°C. The resulting supernatant was loaded on a Ni-NTA agarose column (QIAGEN) that was equilibrated with a wash buffer (50 mM phosphate [pH 7.0], 300 mM NaCl, and 20 mM imidazole) and the column was washed extensively with the same wash buffer. DepH was eluted with an elution buffer (50 mM phosphate [pH 7.0], 300 mM NaCl, and 250 mM imidazole). The purified protein was dialyzed using a 6–8 kDa Spectra/Pro membrane tubing (Spectrum; Gardena, GA) at 4°C against a storage buffer (20 mM Tris-HCl [pH 7.0], and 100 mM NaCl) overnight. Because DepH is expected to exist naturally in an oxidized form with an intact disulfide bond, no reducing agent (e.g., DTT or 2-mercaptoethanol) was added to the purification steps. The purity of DepH was assessed by SDS-PAGE and the protein concentration was determined by Bradford assay (Bradford, 1976), using bovine serum albumin as a standard. Finally aliquots of DepH were flash frozen and stored at −80°C until use.

Site-directed mutagenesis to create single mutants (C156S or C159S) and a double mutant (C156S/C159S) of DepH were accomplished using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the following primer pairs (where underlined letters indicate base

change): C156S-F (5′-GGAAAGCGTGTTCCACTCCCCTTACTGCCACG-3′) and C156S-R (5′-CGTGGCAGTAAGGGGAGTGGAACACGCTTTCC-3′), C159S-F (5′-CACTGCCCTTACTCCCACGGCTACGAACTG-3′) and C159S-R (5′-CAGTTCGTAGCCGTGGGAGTAAGGGCAGTGG-3′), and C156S/C159S-F (5′-GCGTGTTCCACTCCCCTTACTCCCACGGCTACG-3′) and C156S/C159S-R (5′-CGTAGCCGTGGGAGTAAGGGGAGTGGAACACGC-3′). Mutated *depH* genes based on pCW01-1212 were verified by sequencing, and mutant proteins were purified with the same procedure as for the wild-type DepH.

Determination of Physical and Biochemical Properties of DepH

The oligomeric status of DepH was determined by size exclusion chromatography at 4°C using the ÄKTA Prime FPLC equipped with a HiPrep Sephacryl 26/60 S300 high-resolution column (GE Life Sciences, Piscataway, NJ). Running buffer used was 20 mM Tris-HCl (pH 7.0) and 100 mM NaCl, and the flow rate was 0.5 ml/min. Protein elution was monitored at UV_{280}. To establish a reference curve, gel-filtration molecular weight markers (Sigma-Aldrich) including blue dextran (2000 kDa), amylase from sweet potato (200 kDa), alcohol dehydrogenase from yeast (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase from bovine (29 kDa), and cytochrome *c* from horse heart (12.4 kDa) were used. DepH was analyzed separately or in combination with some markers under the same conditions.

Ultraviolet and visible (UV/Vis) spectra of DepH (36 μM in 20 mM Tris-HCl [pH 7.0] and 100 mM NaCl buffer) and FAD standard (25 μM in the same buffer) from 200 to 800 nm were obtained sequentially with Cary 100 Bio spectrophotometer (Varian, Walnut Creek, CA) at room temperature.

The presence of FAD cofactor in DepH was determined by HPLC and LC-MS analysis. Separation of sample was performed on a ProStar HPLC system from Varian, equipped with the Eclipse XBD C18 column (5 μm particle size, 4.6 × 250 mm) from Agilent. Flow rate was maintained at 1 ml/min and the column was equilibrated with a buffer mixture of 85% solvent A (5 mM ammonium acetate [pH 6.5]/15% solvent B [100% methanol]). DepH (36 μM) was denatured by boiling at 100°C for 5 min to free the cofactor. Denatured protein was removed by centrifugation and 20 μl supernatant was analyzed by HPLC. A linear gradient from the equilibration stage to a final buffer composition of 25% solvent A/75% solvent B was achieved in 20 min. The ultraviolet irradiation absorbance of cofactor was monitored at 264 nm. An HPLC fraction containing the cofactor was dried in vacuo, resuspended in 50 μl acetonitrile, and examined by LC-MS analysis. FAD standard (25 μM) was used as a reference.

The stoichiometry of DepH-FAD was determined in duplicate as follows. An aliquot of DepH (36 μM) was saturated with an excess amount of FAD (1 mM) at 4°C overnight and was then dialyzed against the storage buffer (20 mM Tris-HCl [pH 7.0], and 100 mM NaCl) to remove unbound FAD. DepH concentration was redetermined using Bradford assay. DepH was subsequently denatured by boiling for 5 min to release bound FAD. FAD concentration was determined from the absorption at 450 nm, measured on a Cary 100 Bio spectrophotometer (Varian) at room temperature, using a molar extinction coefficient of $A_{450} = 11.0\ mM^{-1} \cdot cm^{-1}$. Buffer was used as a blank for background subtraction.

Determination of Enzyme Activity and Enzyme Kinetics

Enzyme Activity of the Wild-Type and Mutated Forms of DepH

First, the reaction buffer (20 mM Tris-HCl [pH 7.0], 100 mM NaCl, with 200 μM $NADP^+$) used in assays was purged extensively with bubbling of nitrogen gas. For a complete assay, 5 μl reduced FK228 (~100 μM) and 1 μl enzyme solution (36 μM) were added into 95 μl reaction buffer and the reaction was allowed to proceed for 5 min at room temperature in a nitrogen-gas-filled chamber. Each reaction was quenched with 400 μl ice-cold acetone for 5 min on ice. Acetone was then removed by a flow of nitrogen gas and the remaining solution was analyzed with LC-MS. Enzyme was omitted from control reactions, which were kept either in a −80°C freezer or at room temperature, and received the same treatment as the complete reaction until LC-MS analysis. The total time needed from the start of reaction to LC-MS analysis was about 20 min. All reactions were assayed in duplicate.

Enzyme Kinetics of DepH

Due to a high rate of reaction and unavoidable spontaneous chemical oxidation, the assay conditions were optimized by numerous tests (data not shown). The reactions were performed in 30 μl buffer (20 mM Tris-HCl [pH 7.0], 100 mM NaCl, 200 μM $NADP^+$ or NAD^+) at room temperature in a nitrogen-gas-filled chamber. Variable concentration of substrate (reduced FK228, from 2 to 80 μM final concentration) and fixed amount of enzyme DepH (0.02 μg; 18 nM final concentration) was added into each reaction. Five microliters of 500 mM iodoacetamide was used to stop reactions and to block free thiol groups at different time points (0, 0.5, 1, 2, and 4 min). Afterwards, 20 μl of each reaction mixture was analyzed with LC-MS. All reactions were assayed in duplicate. Values from the 0 min time point were used for background subtraction.

ACCESSION NUMBERS

The sequence reported in this article has been deposited into GenBank under accession number EF210776 (Cheng et al., 2007).

SUPPLEMENTAL DATA

Supplemental Data include one table and four figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00152-5.

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