The structural basis of an NADP⁺-independent dithiol oxidase in FK228 biosynthesis

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The disulfide bond is unusual in natural products and critical for their bioactivity, thermal stability, cell permeability and bioactivity. DepH from *Chromobacterium violaceum* No. 968 is an FAD-dependent enzyme responsible for catalyzing the disulfide bond formation of FK228, an anticancer prodrug approved for the treatment of cutaneous T-cell lymphoma. Here we report the crystal structures of DepH and DepH complexed with a substrate analogue S,S'-dimethyl FK228 at 1.82 Å and 2.00 Å, respectively. Structural and biochemical analyses revealed that DepH, in contrast to the well characterized low molecular weight thioredoxin reductases (LMW TrxRs), is an NADP⁺-independent dithiol oxidase. DepH not only lacks a conserved GGGDXAXE motif necessary for NADP⁺ binding in the canonical LMW TrxRs, but also contains a 11-residue sequence which physically impedes the binding of NADP⁺. These observations explain the difference between NADP⁺-independent small molecule dithiol oxidases and NADP⁺-dependent thioredoxin reductases and provide insights for understanding the catalytic mechanism of dithiol oxidases involved in natural product biosynthesis.

The presence of a disulfide bond in natural products is rare but usually critical for their bioactivity, thermal stability and cell permeability. One classic example is the antitumorigen FK228 (also known as FR901228 or depsipeptide, registered as NSC 630176, romidepsin or Istodax; Fig. 1), a selective class I histone deacetylase (HDAC) inhibitor approved for the treatment of cutaneous T-cell lymphoma in 2009 by the US Food and Drug Administration1,2. FK228 is produced by the Gram-negative *Chromobacterium violaceum* No. 968, with a cage-shaped bicyclic depsipeptide structure and a rare disulfide linkage 3,4. The prodrug form of FK228 with a disulfide bond is more stable than its reduced form (red-FK228, Fig. 1) in medium or serum, and this disulfide bond facilitates compound diffusion across the cell membrane more efficiently. Once inside the cells, the prodrug FK228 is activated by glutathione reduction so that one of the free thiol groups of red-FK228 interacts strongly with an active-site zinc and thereby prevents substrate binding 1. Similar disulfide linkages are also present in a few other antibiotics or antitumor natural products, including holomycin5, gliotoxin6, triostin A and its derivatives 7, FR9013758, spiruchostatins 9, and thailandepsins 10–12.

The enzyme responsible for catalyzing the disulfide bond formation in FK228 biosynthesis is DepH13. A BLAST search for proteins homologous to DepH revealed several low molecular weight thioredoxin reductases (LMW TrxRs) from bacteria and fungi, and small molecule dithiol oxidoreductases including TdpH from *Burkholderia thailandensis* E26414 and Ecm17 from *Streptomyces lasaliensis*. TrxRs are homodimeric flavoproteins consisting of a cofactor FAD-binding site, an NADP⁺-binding site and a redox motif CXXC (two cysteine residues separated by two variable amino acids)15–16. They catalyze the oxidation or reduction of dithiol bonds through thiol-disulfide exchange between the enzymes and their substrate proteins. Extensive structural studies revealed that the NADP⁺- and FAD-binding sites of LMW TrxRs are located on opposite faces of the molecules and a conformational change is required for electron transfer from the reduced form of FAD to NADP⁺17.

**Results**

**Overall structure of DepH.** The full-length DepH (residues 1–319) from *C. violaceum* No. 968 was expressed in *E. coli* BL21(DE3) as an N-terminal 6 × His-tagged fusion protein and purified to homogeneity13. Decreasing the
expression temperature to 16 °C and maintaining the purification procedure at 4 °C were critical to prevent the recombinant protein from degradation. After extensive trials, the high diffraction-quality crystals were made by the micro-seeding method.

The crystal structure of native DepH was determined by molecular replacement using the structure of a functionally unknown oxidoreductase from Agrobacterium tumefaciens (PDB code: 3FBS, sequence identity 52%, abbreviated as AtuOR in this work) as a search model. The final structure was refined to 1.82 Å resolution with an Rfactor of 16.8% and an Rfree of 20.0% (Table 1). There are two DepH molecules in the asymmetric units related by a non-crystallographic 2-fold axis (Fig. 2A). Similar to the structures of LMW TrxRs, each DepH polypeptide chain is arranged in two Rossmann-fold domains: an FAD-binding domain (residues 21–138 and 248–319) and a pseudo NADP+–binding domain (residues 139–247) (Fig. 2B). Two antiparallel strands, β7 (residues 135–138) and β16 (residues 248–251), connect the FAD-binding domain with the pseudo NADP+–binding domain. The redox motif CXXC is located on helix α3 within the pseudo NADP+–binding domain. Under native condition, residues Cys156 and Cys159 are close to each other and form a disulfide bond (2.07 Å) and the Pro157 adopts the trans conformation (Fig. 2C). Similar to LMW TrxRs, the S atom of Cys159 is in the range of electron transfer distance of 3.58 Å with the C4 atom of FAD, while the S′ atom of Cys156 is away from the FAD (Fig. 2C).

It is worth noting that the electron density of FAD is well defined (with B factors near 20 Å²) (Fig. 2D), but no electron density of NADP+ was found in the pseudo NADP+–binding pocket of DepH. The isoalloxazine ring of FAD is planar and stabilized by hydrogen bond interactions involving residues Ser36, Ala38, Ala58, Ala60, Arg62, His70, Val104, Asp290, and Val298, accompanied with non-polar interactions and hydrogen bond interactions are prevalent. The hydroxyl group of Tyr158 (through Wat174) (Fig. 3C) and the hydroxyl group of Ser252 (Supplementary Fig. 1). The conformation and relative location of FAD are similar to those observed in LMW TrxRs (Fig. 2A) and CXXC motifs indicate that the native DepH is in the oxidized state.

Molecular basis for FK228 binding. To investigate the molecular basis of substrate binding, we tried to co-crystallize DepH with FK228 in the presence of 5 mM DTT but only got the apo-DepH crystals. Later we obtained and refined the crystal structure of DepH complexed with its substrate analogue dm-FK228 at 2.00 Å resolution. The dm-FK228 molecule contained two methylated thiol groups and was prepared by full methylation of red-FK228 (Fig. 1). The overall complex structure resembles the structure of native DepH (with an r.m.s.d. of 0.7 Å for 294 Cx atoms) (Fig. 2A) and also contains a physiologically related homodimer in an asymmetric unit. Clear and ring-shaped density of dm-FK228 is well defined in the complex structure (Fig. 2E). Due to the crystal packing, one of the two dm-FK228 molecules is buried in the interior of the structures, whose B factor is 39.1 Å². The other dm-FK228 molecule is exposed to the solvent leading to a higher B factor of 74.0 Å².

Located in the same line as residues Cys156, Cys159 and the isoalloxazine rings of FAD, dm-FK228 is bound at the other side of the hydrophobic cleft between the FAD-binding domain and the pseudo NADP+–binding domain (Fig. 2A). The dm-FK228 molecule is located in a pocket surrounded by the β8–α3 loop, α4 and the β15–β16 loop from the pseudo NADP+–binding domain, the β17–β18 loop and the η4–α7 loop from the FAD-binding domain, and α7* from the other subunit (Fig. 3A). The two aliphatic chains containing sulfur atoms are in the same plane of the peptide ring of dm-FK228 (Fig. 3B). The sulfur atom from the longer aliphatic chain of dm-FK228 is near the CXXC redox motif, with a distance about 3.51 Å from the S atom of the residue Cys156. Although the isoalloxazine ring of FAD is still planar, the distance between the two S′ atoms of the residue Cys156 and Cys159 is 3.27 Å (Fig. 3C and Supplementary Fig. 2), indicating the breakage of disulfide bond and the existence of the sulfhydryl groups.

A close view of the dm-FK228-binding pocket revealed that non-polar interactions and hydrogen bond interactions are prevalent. The residues involved in hydrophobic interactions are Leu177, Met246 and Pro267 from one subunit, and Ile310* and Phe318* from the other subunit. The binding of dm-FK228 triggered a torsion of the side chain in Arg314* to form van der Waals interactions. The dm-FK228 molecule also has a direct hydrogen bond interaction with the carbonyl oxygen atom of Gly295 and water-mediated hydrogen bond interactions with the NH1 atom of Arg294 (through Wat331) and the hydroxyl group of Tyr158 (through Wat174) (Fig. 3C and Supplementary Fig. 3).

To probe the role of these residues in catalysis, we generated DepH mutants by substitution with alanine. Mutations of the two key residues, Tyr158 and Arg294, disrupted the hydrogen bond interactions with the substrate and severely affected the catalytic activity of DepH, with approximate 40% residual activity for Y158A and 20% residual activity for R294A. Mutations of residues Pro267 and Met246 dedicated to hydrophobic interactions had negligible effect on enzyme activity. Although His293 has no direct interaction with the substrate, mutation of this residue to alanine caused a 50% decrease in the activity of DepH, indicating that the π–π stacking between His293 and Arg294 was necessary to maintain the conformation of Arg294. Double mutant H293A/R294A showed no synergistic effects (Fig. 3D).

DepH is an NADP+–independent enzyme. Although the pseudo NADP+–binding domain of DepH is similar to those of LMW
TrxBs and excess of NADP\textsuperscript{+} was added in the protein crystallization solutions, the density of NADP\textsuperscript{+} was not found in the electron density map of either the native or the complex structure (Fig. 2A). To seek for an interpretation, we compared the structure of DepH with those of LMW TrxBs from Escherichia coli (PDB code: 1TDE), Deinococcus radiodurans (PDB code: 2Q7V), Saccharomyces cerevisiae (PDB code: 3ITT), Helicobacter pylori (PDB code: 2Q0K), Mycobacterium tuberculosis (PDB code: 3ITJ), and Arabidopsis thaliana (PDB code: 2WHD) and M. tuberculosis (PDB code: 2Q0K). The counterparts in LMW TrxBs are three residues shorter and adopt a flexible loop conformation (Fig. 4A).

The lack of sequence fingerprint necessary for NADP\textsuperscript{+} binding and the missing of NADP\textsuperscript{+} in the native and complex DepH structures suggest that its catalysis might be NAD\textsuperscript{+}/NADP\textsuperscript{+} independent. To test this hypothesis, we measured the catalytic activity of DepH at various protein concentrations in the absence or presence of NAD\textsuperscript{+} or NADP\textsuperscript{+}. As shown in Fig. 4D, DepH alone exhibited high catalytic efficiency for converting red-FK228 to FK228, whereas addition of NAD\textsuperscript{+} or NADP\textsuperscript{+} actually hampered the catalytic reaction of DepH. All those evidences support DepH as an NADP\textsuperscript{+}-independent enzyme.

**Discussion**

Several small molecule di thiol oxidases have been identified as the thioredoxin reductases homologues from the biosynthetic gene clusters of disulfide containing natural products. Through structure
determination of DepH alone and in complex with the substrate analog dm-FK228, we disclosed the characteristics of DepH as an NADP$^+$-independent FAD-binding enzyme. Compared to LMW TrxRs, DepH not only lacks a conserved GGGDXAXE sequence required for NADP$^+$ binding but also contains a connecting $\beta$-turn in region II which protrudes into the pseudo NADP$^+$-binding site and prevents NADP$^+$ binding.

We observed that there is a disulfide bond in the native DepH structure whereas the corresponding disulfide bond is broken in the complex structure. As dm-FK228 is a pseudo substrate with two sulfydryl groups protected by methylation, no reaction should have occurred between the enzyme and the substrate analog. The molecular dynamics simulations suggested that there were little conformational changes of the CXXC motif in DepH upon dm-FK228 binding (data not shown). Given the fact that the same level of DTT were applied for protein crystallization and the same beamline was used for data collection, we speculated that unusual breakage of disulfide bond in the complex structure might have been the effect of radiation damages or other unexpected factors.

Recently, two FAD-dependent, homodimeric enzymes, GliT from Aspergillus fumigatus and HlmI from Streptomyces clavuligerus, were identified as dithiol oxidases and to catalyze the oxidation of...
in intramolecular dithiol groups to disulfide during the biosynthesis of gliotoxin and holomycin. Although GliT and HlmI are analogs to the LMW TrxRs superfamily, the electron transfer during the dithiol-disulfide exchange involving the CXXC motif is independent of NADP$^+$ and utilizes molecular oxygen as the terminal electron acceptor. To compare DepH with other small molecule dithiol oxidoreductases, we conducted a sequence alignment among GliT, HlmI and DepH as well as other structurally unknown but functionally homologous enzymes, such as TdpH, PA14_09950, CJA_3737 and Smed_3568. As shown in Fig. 4A and Supplementary Fig. 4, all of these enzymes have almost the same redox motif CP/ly/FC, which is totally different from those of the canonical LMW TrxRs, whose redox motifs are CAT/VC. Moreover, these enzymes involved in the oxidation of diethiols into disulfide lack the signature sequence GG/DAXE necessary for NADP$^+$ binding. In addition, the aforementioned four highly conserved basic residues absent in DepH are accordingly missing in these homologous proteins as well as GliT and HlmI. Based on the sequence alignment and the information that NADP$^+$ was not necessary for the catalytic activity of DepH, we concluded that DepH is a dithiol oxidase which uses molecular oxygen as the terminal electron acceptor to catalyze intramolecular disulfide formation in FK228.

The crystal structures of DepH also shed new light on understanding the catalytic mechanism of dithiol oxidases. We found that there might be an oxygen channel of DepH to the vicinity of the N5 nitrogen of FAD. This channel is mainly formed by residues C156, Y158, W232, W174 and W331, which mediate hydrogen bonds between dm-FK228 and DepH.

Figure 3 | The interactions between DepH and its substrate analog dm-FK228. (A) The dm-FK228 binding site is located on the dimer interface of DepH. The surfaces of two subunits are shown in gray and pink, respectively. The secondary structures involved in the substrate binding pocket are labeled. (B) Comparison of the structures of the native DepH and its complex with dm-FK228 in the substrate-binding pocket. Cofactor FAD and residues from the native DepH were colored white, while those from complex were depicted cyan and salmon individually. Essential water molecules were depicted as magenta spheres. (C) A close view of the substrate binding pocket. Residues interacting with dm-FK228 are depicted by sticks in, magenta and green, respectively, indicating the residues belong to different monomers. Two water molecules (W174 and W331) mediating the hydrogen bonds between dm-FK228 and DepH are shown by red spheres. (D) Relative enzyme activity of the wild type and mutants of DepH.
Figure 4 | (A) Sequence alignment and structural superimposition of regions I and II of DepH with other homologous proteins, including TdpH from *B. thailandensis* E264, PA14_09950, CJA_3737, Smdd_3568, AttoOR, GlIT, HlmI and structure-solved LMW TrxRs from *E. coli* (1TDE), *M. tuberculosis* (2A87), *D. radiodurans* (2Q7V), *H. pylori* (2Q0K), *S. aureus* ssp. (4GCM), *H. vulgare* (2WHD), *A. thaliana* (1VDC) and *S. cerevisiae* (3ITJ). Details are described in Supplementary Figure 4. The sequences with known structures were aligned using STRAP43. The sequences lacking of three dimensional structures were aligned using ClustalW44. The alignment results were merged with ESPript45. (B) Comparison of the solvent-accessible surface of the NADP$^+$-binding pocket in TrxR from *M. tuberculosis* (2A87) (left panel) and that of the pseudo NADP$^+$-binding pocket in the DepH/dm-FK228 complex (right panel). The bound NADP$^+$ in TrxR and dm-FK228 in DepH complex are depicted using stick model and colored in magenta and green, respectively. Four basic residues anchoring the ribosyl and phosphate groups of NADP$^+$ in TrxR were labeled. (C) Superposition of the DepH complex structure (colored in violet) with the structure of the NADP$^+$-dependent LMW TrxR from *M. tuberculosis* (2A87, colored in teal). The cofactor NADP$^+$ from TrxR and dm-FK228 from DepH are shown in sticks. The presence of NADP$^+$ in DepH would impede substrate binding. (D) Relative activities of DepH in the presence or absence of NAD$^+$ or NADP$^+$: A, no additives; B, with NAD$^+$; C, with NADP$^+$. 
Arg62, His155 and Cys156, which are strictly conserved in GliT, HlmI and other dithiol oxidases (Supplementary Fig. 6 and Table S4). The initial half-stage was improved using OASIS® and PARROT®, and then used to rebuild the main chain of DepH by ARP/WARP®. Refinement was performed with PHENIX® and a random selection of 5% reflections was set aside for cross-validation. The model was extended and rebuilt manually with Coot®.

The structure of the DepH/dm-FK228 complex was obtained and refined by molecular replacement using the program Phaser® and the DepH coordinates as the search model. The atomic model was refined using the same protocol as mentioned. The model of dm-FK228 was generated by the PRODRG® server. MolProbity® and PROCHECK® were used to assess the overall quality of the structural models. The dimer interface of DepH was analyzed using the PISA server®. All graphic presentations of structures were generated using PyMOL® and CCP4MG®. All simulated-annealing omit maps were calculated using CNS®.

**Enzymatic activity assay.** Dithiobis(5-nitrophenyl) disulphide (DTNP, Sigma-Aldrich) was dissolved in acetonitrile (4 μmol) and used as a color development substrate. The reaction buffer was 20 mM Tris-HCl, pH 7.0 and 100 mM NaCl, and 96-well cell culture plate (Corning Incorporated) were used for the reactions. The measurements were performed on BioTek microplate reader (Winooski, VT, USA) and the A405nm was measured after 15 min.

Methods

**Preparation of reduced FK228 (red-FK228) and dimethylated FK228 (dm-FK228).** FK228 was obtained from C. violaceum (ATCC No. 968 in one of our laboratories. Preparation of red-FK228 was accomplished by using tris(carboxylethyl)phosphine hydrochloride (TCEP, Sigma-Aldrich) as the reducing agent. The reaction was performed at room temperature by mixing 20 mg (36 μmol) of FK228 and 100 μmol of TCEP in 1 ml of acetonitrile for 20 min. The red-FK228 was purified by preparative HPLC, confirmed by liquid chromatography mass spectrometry (LC-MS; [M + H]+ = 542 m/z) and lyophilized. To prepare dm-FK228, 10 mg red-FK228 (18 μmol) was dissolved in 1 ml of 50% acetonitrile and 3 μl of CH3OH (50 μmol) was added. Then 14 mg of K2CO3 (100 μmol) was added into the reaction mixture and the reaction was performed at 60 °C for 15 min. The final product dm-FK228 was purified by preparative HPLC, confirmed by LC-MS ([M + H]+ = 571 m/z), and lyophilized.

**Expression and purification of DepH proteins.** The full-length DepH (residues 1-319) from G. stearothermophilus was expressed in a BL2(DE3) N-term His-tagged fusion protein and purified to homogeneity as previously reported. Decreasing the expression temperature to 16 °C and maintaining the purification procedure at 4 °C were critical to prevent the recombinant protein from degradation. The DepH protein was further purified by gel filtration chromatography using a Superdex 75 10/300 column (GE Healthcare), which showed that it exists as a homodimer in solution. Peak fractions were dialyzed against buffer containing 50 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 8.0 and then concentrated in an Amicon 10 (10 K MWCO) spin concentrator to approximately 9.7 mg/ml. All DepH mutants were expressed and purified similarly as the wild-type protein.

**Crystallization and data collection.** Initial crystallization trials were performed using the sitting-drop vapor-diffusion method at 20 °C with commercially available kits. Micro-crystals of DepH were produced in 2 μl drops containing 1:1 mixture of the protein solution and a reservoir solution consisting of 15% PEG4000, 0.2 M (NH4)2SO4, 0.1 M citric acid, pH 5.0. The best diffraction-quality crystals were obtained by the micro-seeding method3 at an optimized reservoir condition with 0.1 M sodium acetate, 15% PEG4000, 0.2 M (NH4)2SO4, 0.1 M citric acid, pH 5.0 and 100 μl paraffin oil. We did not try the cocystalization of DepH with the substrate red-FK228 because red-FK228 is sensitive to oxidation and will be catalyzed to build FK228 by DepH during the cocystalization process. Cocystalization of DepH with FK228 was performed using the same method in the presence of 5 mM DTT but only yielded the apo-DepH crystals. Fortunately, cocystalization of DepH with dm-FK228 turned out to be successful. The protein drops in which seeds were transferred contained 1.8 μl of the protein solution and 0.2 μl of 10 mM dm-FK228 dissolved in a mixture of dimethyl sulphoxide and PEG 200 (1: 1 ratio). The molar ratio of protein to ligand was close to 1:4. We attempted to cocystalize DepH with NADP+ and to get the tertiary structure of DepH/dm-FK228/NADP+ by controlling the ratio of protein to NADP+ from 1:4 to 1:10, but failed to find any NADP+ densities in the obtained crystal structures. All crystals were mounted in nylon loops and flash-frozen in liquid nitrogen. The cryoprotectant for both native and complex crystals consisted of the reservoir solution plus 15% ethylene glycol. Diffraction data were collected on the SSRF BL17U1 beamline (Shanghai, China). Intensity data were integrated and scaled by using the program HKL2000®. The statistics for data collection are listed in Table 1.

**Structure determination and refinement.** The structure of DepH was determined by molecular replacement method using the program Phaser® in the CCP4 suite4 and the monomer structure of an oxidoreductase from A. tumefaciens (PDB code: 3FBS) as the search model. The initial half-stage was improved using OASIS® and PARROT®, and then used to rebuild the main chain of DepH by ARP/WARP®. Refinement was performed with PHENIX® and a random selection of 5% reflections was set aside for cross-validation. The model was extended and rebuilt manually with Coot®.

The structure of the DepH/dm-FK228 complex was obtained and refined by molecular replacement using the program Phaser® and the DepH coordinates as the search model. The atomic model was refined using the same protocol as mentioned. The model of dm-FK228 was generated by the PRODRG® server. MolProbity® and PROCHECK® were used to assess the overall quality of the structural models. The dimer interface of DepH was analyzed using the PISA server®. All graphic presentations of structures were generated using PyMOL® and CCP4MG®. All simulated-annealing omit maps were calculated using CNS®.
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Author contributions
C.W. purified the protein samples and performed activity assays. J.L. obtained the crystals
and structures, Z.-M.Z. assisted in protein purification and crystallization, Y.-Q.C. and J.Z.
designed and supervised the project, J.L., C.W., Y.-Q.C. and J.Z. analyzed the data and wrote
the manuscript. All authors provided feedback on the manuscript.

Additional information
Accession Coordinates. Atomic coordinates and structure factors for the apo- and
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