Molecular insights into the endoperoxide formation by Fe(II)/α-KG-dependent oxygenase NvfI

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Endoperoxide-containing natural products are a group of compounds with structurally unique cyclized peroxide moieties. Although numerous endoperoxide-containing compounds have been isolated, the biosynthesis of the endoperoxides remains unclear. NvfI from Aspergillus novofumigatus IBT 16806 is an endoperoxidase that catalyzes the formation of fumigatonoid A in the biosynthesis of novofumigatonin. Here, we describe our structural and functional analyses of NvfI. The structural elucidation and mutagenesis studies indicate that NvfI does not utilize a tyrosyl radical in the reaction, in contrast to other characterized endoperoxidases. Further, the crystallographic analysis reveals significant conformational changes of two loops upon substrate binding, which suggests a dynamic movement of active site during the catalytic cycle. As a result, NvfI installs three oxygen atoms onto a substrate in a single enzyme turnover. Based on these results, we propose a mechanism for the NvfI-catalyzed, unique endoperoxide formation reaction to produce fumigatonoid A.
Endoperoxides are structurally unique heterocycles with a characteristic peroxide moiety. They are present in a variety of natural products, such as terpenoids, alkaloids, polyketides, and meroterpenoids, produced in plants, fungi, and other organisms. Due to the high reactivity of the endocyclic peroxide bond, these compounds display interesting biological properties, including antimalarial, antitrypanosomal, antibacterial, and antitumor activities.

Despite the promising biological properties of the endoperoxide-containing natural products, our knowledge regarding the enzymatic mechanism of endoperoxide formation remains limited. Presently, only two types of enzymes, the heme-dependent prostaglandin H synthase (also known as cyclooxygenase, COX) and the non-heme iron-dependent non-heme iron enzymes, remains enigmatic.

Here, we report structural and functional analyses of NvfI. The structural analysis and mutagenesis studies revealed that the reaction of NvfI is unlikely to involve tyrosyl radical, in contrast to the COX and FtmOx1 mechanisms. Instead, we propose that the dynamic conformational changes of NvfI enable the repositioning of the substrate radical intermediate in the active site, thus evading the biosynthetically undesired hydroxylation and resulting in the reaction with molecular oxygen.

### Results

#### In vitro characterizations of NvfI

The in vitro enzyme reaction of NvfI with asnovolin A (2) generated fumigatonoid A (1) and its isomer 3, which is produced via iron-mediated, non-enzymatic isomerization of 1, as dominant products using α-KG and O₂ as co-substrate (Fig. 2a). To confirm the stoichiometry of the co-substrates, in vitro enzyme reactions were performed using varied ratios of 2 and α-KG under aerobic conditions. As a result, 2 was almost fully consumed when the α-KG: 2 ratio reached ca. 1:1. (Fig. 2b and Supplementary Fig. 3a), which suggested that one equivalent of α-KG was consumed during the formation of 1. On the other hand, for measurement of oxygen stoichiometry, enzyme reactions were performed using different amount of O₂. As a result, the production of 1 increased with increasing O₂ concentrations, and reached plateau when the ratio was ca. 2 (Supplementary Fig. 3b, c), which indicated that two equivalents of O₂ were required for the installation of three oxygen atoms onto 2.

Next, to analyze the origin of the oxygen atoms in 1 generated by NvfI, we performed labeling experiments using 1⁸O₂ and H₂¹⁸O (Fig. 2c-g). When 2 was incubated with NvfI under ¹⁸O₂ (98 atom %¹⁸O), a major +4 peak (m/z 499) of 1 was observed in the production of 1 (m/z 501 (+6): m/z 499 (+4): m/z 495 (0) = 1.2%: 95.7%: 2.0%) (Fig. 2 and Supplementary Table 1). Interestingly, in the experiment with H₂¹⁸O (final concentration 78 atom %¹⁸O), a major +2 (m/z 497) peak was observed, along with a minor peak of the unlabeled 1 and +4 (m/z 499 (+4): m/z 497 (+2): m/z 495 (0) = 17.0%: 62.5%: 20.4%) (Fig. 2 and Supplementary Table 1). Furthermore, the double labeling with 78% H₂¹⁸O and 98% ¹⁸O₂ generated +4 (m/z 499), +6 (m/z 501) and +8 (m/z 503) products of 1 (m/z 503 (+8): m/z 501 (+6): m/z 499 (+4): m/z 497 (+2): m/z 495 (0) = 23.2%: 56.7%: 18.3%: 1.8%: 0.4%). Although three oxygen atoms should be newly installed in a reaction cycle, these results indicated that at most four oxygen atoms, two from O₂ and two from solvent water, were incorporated into 1.

Considering the structure of 1, the O₂ molecule is likely incorporated into the endoperoxide moiety in 1. An oxygen atom from water should be incorporated as the carbonyl oxygen at C-4.'
by non-enzymatic exchange via a hemiketal intermediate (Supplementary Fig. 4). Indeed, the $+2$ peak (m/z 497) was observed when 1 was incubated in H$_2^{18}$O without NvfI while the incorporation efficiency is relatively slow (Fig. 2g). The C-3’ hydroxyl group in 1 was the other possible site of oxygen incorporation from water. Surprisingly, however, only 1.2% of $+6$ peak (m/z 501) was observed in $^{18}$O$_2$-labeling experiment, which indicated that the oxygen atom of C3’ hydroxyl group of 1 is not from O$_2$ molecule, but mostly from H$_2$O. While the hydroxyl group introduced by the action of Fe(II)/α-KG-dependent hydroxylases is usually derived from O$_2$, the oxygen ligands in both the ferryl and ferric states can be exchanged with the solvent water during the catalysis40. In the case of NvfI catalytic cycle, it is remarkable that almost all of the ferryl and/or Fe(III)-OH species exhibited oxo/water exchange prior to the formation of 1. Alternatively, it would be also possible that water molecule is incorporated into the C-3’ position of 2 through formation of a cationic intermediate.

**Overall structure of NvfI.** Interestingly, the gel-filtration analysis of NvfI revealed that the apparent molecular weight of NvfI is 34 kDa, indicating that NvfI exists as a monomer in solution (Supplementary Fig. 5). The formation of the monomeric state is notable, because other reported fungal αKG-dependent oxygenases, involved in fungal meroterpenoid biosynthesis, normally exist as homodimers10,19,25,26. Since functions and oligomeric forms of the other NvfI homologs (Supplementary Fig. 2) have not been studied, we do not know how widespread this feature is. Notably, isopenicillin N synthases and deacetoxycephalosporin C synthases are also monomeric in solution34,41.

To understand the structural details of the NvfI-catalyzed endoperoxide-forming reaction, we solved the X-ray crystal structures of NvfI complexed with Fe, α-KG, and substrate 2 at 1.9 Å resolution. The overall structure of NvfI possesses a double-stranded β-helix core (DSBH) fold, which is highly conserved in the α-KG-dependent oxygenase family9,10,19,25,26 (Fig. 3 and Supplementary Fig. 6a–c). The major structural differences
between NvfI and other a-KG-dependent oxygenases lie in the N-terminal and C-terminal regions. The N-terminus of NvfI consists of two anti-parallel β-strands (β1 and β3) connected by a long loop. The β1 and β3 strands are involved in the formation of a β-like structure together with three anti-parallel β-sheets (β4, β5, and β7) in the DSBH fold, in remarkable contrast to other a-KG-dependent oxygenases\(^9,10,19,26,41\) (Supplementary Fig. 6c).

**The active site architecture of NvfI.** The active site of NvfI is located in the DSBH core and an Fe is coordinated by the His135-His137-Asp250 triad, which is conserved in a-KG-dependent oxygenases (Fig. 3d, e). Interestingly, a comparison of the binary complex structure with a-KG (monomer B, state I) and the ternary complex with a-KG (monomer A, state III) revealed that the residues Ser122-Gly128 of loop I and Trp199-Pro209 on loop II undergo significant conformational changes upon substrate binding (Figs. 3, 4). The Ser122-Gly128 residues on loop I flip toward the active site, and Phe127 on the loop closes the substrate binding site along with the movement of Trp199 on loop II (Figs. 3, 4 and Supplementary Fig. 7). Furthermore, the Arg201 side chain moves toward Tyr116 and forms hydrogen bond networks among Tyr116, Arg201, Asp206, and water molecules. In contrast, Glu208 moves away from the active site to avoid steric hindrance with the substrate, and hydrogen bonds with Lys205 to support the conformation of loop II.

In addition, we also obtained a partially closed conformation of NvfI (monomer B, state II) from another data set of the wild type in complex with 2 and an a-KG analog N-oxalylglycine (NOG). The structural analysis revealed that the overall structure and the active site architecture of monomer A (complex structure with 2 and NOG) are almost identical to that of the ternary complex with 2 and a-KG (state III), while the loop I in monomer B (complex structure with NOG) takes a different conformation from that in the open state I and flips toward the active site (Figs. 3, 4 and Supplementary Fig. 7). In this state, loop II takes the same open conformation as that in state I but the electron density of the side chains of Ile126 and Phe127 on the loop I was not clearly observed, indicating that the conformations of these regions are still not fixed. These observations suggested that conformational changes of the active site occur during the enzyme reaction to create a tunnel for the substrate and close the substrate-bound active site, although the density of 2 was not clearly observed in state II (Fig. 4c, d).

**The substrate binding mode.** In the complex structure of NvfI with Fe, a-KG, and 2, the octahedral iron-center is coordinated by the bidentate a-KG and a water molecule, together with the catalytic triad (Fig. 3d, e). Substrate 2 interacts with Arg118 and His138 via hydrogen bonds at the C-4' ketone and C-3 ester carbonyl groups of 2, respectively. The C-9' carbonyl group of the methyl ester also interacts with Thr133 and Arg132 via water molecules.

Notably, the distance between C-7' of 2 and the iron-center (4.2 Å) is shorter than that between C-13 and the iron (6.5 Å), which may explain the formation of the previously characterized side product 4 (Fig. 5)\(^12\), even though the hydrogen atom at C-13 should be abstracted during the conversion to 1. This conformation may represent a different stage of the catalysis or a crystallographic artifact. The docking model with 2, based on the binary complex structure, suggested that the active site has enough space to bind the substrate in different conformations and that the C-13 position is located closer to the iron-center (Supplementary Fig. 8). Furthermore, a comparison of the substrate binding site in the binary and ternary complex structures revealed that the movement of the loop around Glu208 increases the active site volume around the A-ring moiety of 2. These analyses suggested that the initial binding site of 2 is closer to loop I due to the steric hindrance between Glu208 and 2. This conforma...
the substrate (Fig. 4c–e), and the radical formation at C-13 and subsequent conformational changes of the active site move the substrate radical deeper inside the tunnel, as observed in the ternary complex structure.

**Mutagenesis experiments of NvfI.** Phe127 and Trp199 in loops I and II, which undergo dramatic conformational changes, may function as gatekeeping residues to load the substrate into the active site. Moreover, the flipping of Glu208 on loop II creates additional space for binding the A-ring moiety of 2 (Fig. 3). To investigate the importance of these conformational changes, variants of Phe127, Trp199, and Glu208 were constructed (Figs. 5c and 6). The substitution of Phe127 and Trp199 with Ala completely abolished the enzyme activity, while the E208A variant increased the activity to 144%. Furthermore, the substitution of Ser114 and Arg201 with Ala and Phe still maintained 44% activity (Supplementary Fig. 11). The slight decrease in the $k_{cat}$ values toward M and K of the Y116A variant was not significantly altered (1.2-fold lower than the wild-type), while the $K_M$ and $k_{cat}$ values toward 2 were decreased by 1.3-fold and by 1.7-fold, respectively, as compared to the wild-type enzyme (Supplementary Fig. 11). The slight decrease in the $k_{cat}$ value would be caused by the decrease in the contacts between the enzyme and product. These results indicated that the Tyr116 residue does not participate in the catalytic mechanism of NvfI, which is clearly distinct from those of COX and FtmOx1.

We solved the ternary complex structure of the NvfI W199F variant with 2 and NOG at 2.3 Å. Although the overall structure is almost identical to that of the wild type enzyme (rmsd value of 0.8 Å for Ca-atom), a comparison of the two structures revealed that the space around the carboxyl ester of D-ring and the active site entrance is significantly increased due to the substitution of the large indole ring to a smaller benzene ring (Supplementary Fig. 10). As a result, the D ring of substrate moves ~1 Å toward the 199 position, and the substrate is not suitably accommodated within the deeper part of the tunnel, while the C-13 position is still close to the iron-center to generate compound 5. In contrast, the increased activity of the E208A variant would be due to the decreased steric hindrance between the carboxylic acid of Glu and the C-14-C-15 dimethyl moiety and the clash during the conformational change of loop II. These observations suggested that after hydrogen abstraction from the C-13 methyl group, the bulky aromatic rings of Phe127 and Trp199 shift the substrate radical toward Glu208, thereby blocking the hydroxylation at the C-13 position.

A Tyr residue plays a crucial catalytic role in the reactions of COX and FtmOx1. In NvfI, only one Tyr116 is located in the active site (Fig. 4b). This residue forms part of the active site around the B- and C-rings of the substrate. To test whether Tyr116 is involved in the NvfI catalysis, it was substituted with Ala and Phe residues (Figs. 5c and 6). Interestingly, the substitution of Tyr116 with Ala and Phe still maintained 44% and 101% activities, respectively. The kinetic analysis revealed that the catalytic specificity ($k_{cat}/K_M$) of the Y116A variant was not significantly altered (1.2-fold lower than the wild-type), while the $K_M$ and $k_{cat}$ values toward 2 were decreased by 1.3-fold and by 1.7-fold, respectively, as compared to the wild-type enzyme (Supplementary Fig. 11). The slight decrease in the $k_{cat}$ value would be caused by the decrease in the contacts between the enzyme and product. These results indicated that the Tyr116 residue does not participate in the catalytic mechanism of NvfI, which is clearly distinct from those of COX and FtmOx1.

To identify the catalytic residues, other candidates such as Ser114, His138, and Arg201, located close to 2, were substituted with Ala. As a result, the substitution of Ser114 and Arg201 with
Ala modestly reduced the 1-forming activity (66% and 78%, respectively) (Figs. 5c and 6). Interestingly, the substitution of His138 with Ala reduced endoperoxide-forming activity, and generated the previously identified 4\textsuperscript{12} and a product with m/z = 463 as major products. NMR analyses identified the product as the C-2\textsuperscript{'-}C-3\textsuperscript{'-} epoxide \textsuperscript{6} with the (2\textsuperscript{'-S},3\textsuperscript{'-R}) configuration (Supplementary Fig. 9b and Supplementary Table 2). In contrast, the product profile of the H138F variant reaction was similar to that of the wild-type, while the production of 4 was increased (total activities were 184%) (Figs. 5 and 6). The initial binding mode of the substrate would be altered, due to the enlargement of the active site by the large-to-small substitution of His138 with Ala (Supplementary Fig. 12). The substrate may bind in the deeper part of the active site toward α\textsubscript{2}, and the C-2\textsuperscript{'-}C-3\textsuperscript{'-} double bond is closer to the iron-center, resulting in the formation of 6. Thus, the interaction between His138 and the C-3 ester group of 2 is critical for the hydrogen atom abstraction from C-13, to initiate the endoperoxide formation leading to 1. Taken together, these results suggested that NvfI does not utilize any active site residues to mediate the abstraction or donation of a hydrogen atom in the catalytic mechanism, in sharp contrast to the well-studied endoperoxidases COX and FtmOx1\textsuperscript{6–10}.

**Fig. 5** The binding modes of 2 and α-KG in the active site of NvfI and enzyme reactions of NvfI variants. a Close-up view of the active site of NvfI in complex with 2 and α-KG. b The mutated residues in the active site of NvfI. The substrates 2 and α-KG are depicted by magenta stick models. The catalytic triad is represented by a green stick model. Dashed blue lines represent hydrogen bonds. The dashed yellow line shows the distance between C7\textsuperscript{-}Fe and C13-Fe. Water molecules are represented by red nb\_spheres. c LC-MS charts of the enzyme reactions of NvfI variants. d Structures of shunt products.

**Fig. 6** Graph representing the relative activities of the NvfI variants. The bars are means of n = 3 independent experiments and error bars indicate standard deviations. Data are presented as mean values ± SD. All experiments were repeated independently three times with similar results. The relative activities were calculated from the total turnover number.
Discussion

Despite the many synthetic and biosynthetic approaches for the production of structurally unique and biologically active endoperoxide-containing compounds, the detailed biosynthetic mechanisms for the endoperoxide-formation reactions still remain to be elucidated. In some cases, non-enzymatic reactions with singlet oxygen (\(1^\text{O}_2\)), generated through light absorption by photosensitizers, have been proposed (e.g., for the production of the antimalarial drug artemisinin in plants). On the other hand, in the previously proposed mechanisms for the enzymatic formation of endoperoxides by COX and FtmOx, the active site Tyr residues play critical roles as a hydrogen atom transfer intermediary in COX and a hydrogen atom donor in FtmOx, respectively. In contrast, our present mechanistic and structural characterizations of NvfI indicated that NvfI does not utilize any active site residues to generate amino acid-based radical intermediates in the endoperoxide-forming reaction. Thus, NvfI employs a different mechanism from those of the COX and FtmOx enzymes.

Several pathways can be proposed for the NvfI-catalyzed conversion of 2 to 1 (Supplementary Fig. 13). The ferryl species may abstract a hydrogen atom at C-13 of 2 to generate the primary radical, which then reacts with \(\text{O}_2\) to form a peroxy radical. The O-centered radical may undergo radical addition at C-2’ and the resulting radical may receive the hydroxyl group from the iron(III)-hydroxyl species to yield 1 (Path 1). It is also possible that hydrogen atom abstraction or donation is mediated by an amino acid residue of NvfI as the COX-like or FtmOx-like mechanisms. Alternatively, C may be reduced by a reductant (such as ascorbate under the in vitro condition) to generate 1 via D (Path 2). Other possible routes are generation via epoxide intermediates (Supplementary Fig. 13b). In path 2-4, NvfI thus requires two equivalents of \(\alpha\)-KG. Our stoichiometric analysis of \(\alpha\)-KG strongly suggested that only one \(\alpha\)-KG is required for the production of 1, which is consistent with the path 1. Consequently, any reaction pathways involving two equivalents of \(\alpha\)-KG per 1 formation appear to be unlikely.

In the X-ray crystallographic analysis of NvfI, we obtained three different conformations of active site (state I–III). These conformational changes of the loops between Ser122-Gly128 and Trp199-Pro209 in the crystal structures suggested dynamic movement of active site during the enzyme reaction. Based on our structural and functional analyses, we propose a unique mechanism for the NvfI-catalyzed endoperoxide-forming reaction, as follows (Fig. 7). The enzyme reaction is initiated by loading substrate 2 into the active site of NvfI in the state I. Then,
the conformation of active site changes to state II via movement of loop I upon substrate binding. In this binding mode, C-13 of 2 is located close to the iron-center due to steric hindrance between the A-ring of substrate and Glu208, and then the direct hydrogen abstraction from the C-13 position by the ferryl species occurs. Before the hydroxyl rebound at C-13 with the ferric-hydroxy species, further conformational changes of the residues on loop II, especially flipping of Glu208, together with the movement of Phe127 and Trp199 could relocate the radical intermediate toward the inside of the tunnel to prevent the reaction between the C-13-radical and the ferric-hydroxy species. Instead, the C-13-radical reacts with O₂, which may enter the tunnel in the proximity of the A-ring moiety of the substrate. The resulting peroxyl radical attacks C-2′ to form an endoperoxide-containing C3′-radical intermediate. Since the ferric-hydroxy and C-3′ become closer by the relocation of the intermediate, the radical is finally quenched via the usual hydroxyl-rebound from the ferric-hydroxy species to produce 1. Alternatively, it would be also possible that one electron is directly transferred to the ferric iron to generate the C-3′ carbocation intermediate, which is then hydrolyzed to yield the ferric-hydroxy species to produce 1. The stereochemistry of the hydroxylation at C-3′ is also restricted to the R-configuration by the binding mode of the substrate in the active site. Further studies are required to completely conclude the dynamic conformational changes of active site and repositioning of the radical intermediates. The Fe(II)/α-KG-dependent enzymes catalyze various types of reactions, including desaturation, epimerization, C-C bond formation, halogenation, ring expansion, and other skeletal rearrangements. In these reactions, the enzymes control the outcome by suppressing the direct oxygen rebound between the ferric radical and the ferric-hydroxy species. A similar mechanistic scenario with a long-lived ferric state may transpire in the NvfI-catalyzed endoperoxide formation, consistent with our 18O labeling experiments in which the solvent-derived oxygen atom is also incorporated into the hydroxyl group at C-3′. In the case of NvfI, the elusion of the radical intermediate from the ferric-hydroxy intermediate, facilitated by the large conformational changes in the active site, would extend the lifetime of the intermediate to prevent direct oxygen rebound, thus facilitating the reaction with molecular oxygen to generate the endoperoxide. In conclusion, our structure-function analyses of NvfI have suggested its unique mechanism for endoperoxide formation, which is distinct from the previously proposed mechanisms for the COX- and FtmOx1-catalyzed reactions. Future discoveries of endoperoxide-forming enzymes will provide further insights into the mechanistic details of rare endoperoxide-formation reactions in nature.

Method

General remarks. Oligonucleotide primers were purchased from Eurofin Genomics. Other chemicals were purchased from Wako Chemical Ltd. (Tokyo, Japan). Analytical grade solvents were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The HRMS data were obtained by using a compact microTOF-MS (Bruker) attached to an LC-20AD UHPLC system (Shimadzu) with a COSMOSIL 25C18- MS-II column (2 mm i.d. × 75 mm; Nacalai Tesque, Inc.). Analytical and semi-preparative HPLC runs were performed on a Shimadzu LC20-AD HPLC system, using a COSMOSIL C18-MS-II analytical column (4.6 × 250 mm, 5 μm) and a COSMOSIL 2.5C18 semi-preparative column (10.0 × 250 mm, 5 μm).

Expression and purification of recombinant NvfI and variants. The pET28a vectors containing the NvfI gene were prepared previously. The plasmid was transformed into Esherichia coli RosettaTM 2(DE3) pLysS and used for the expression of NvfI and its variants. The cells harboring the plasmids were cultured at 37 °C to an OD₆₀₀ of 0.6 in Luria-Bertani medium, containing 50 mg ml⁻¹ kanamycin and 34 mg ml⁻¹ chloramphenicol. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to 0.2 mM (final concentration) for target protein expression, and the cultures were continued for 20 h at 16 °C. All purification steps were performed at 4 °C. The cultured cells were resuspended in 50 mM Tris–HCl buffer, pH 7.5, containing 5% (v/v) glycerol, 100 mM NaCl, and 5 mM imidazole (buffer A). The cells were lysed by sonication and the insoluble debris was removed by centrifugation at 12,000 g for 40 min. The supernatant was loaded onto a Ni-NTA Resin (Thermo Fisher Scientific) column. The resin was washed with 50 column volumes of buffer A containing 20 mM imidazole, and then the NvfI was eluted with buffer A containing 300 mM imidazole. The protein solution was concentrated with Amicon Ultra-15 centrifugal filter devices (30 K MWCO, Millipore). The ultrafiltration of the NvfI solution was run on a TSK-gel (8.8 mL, GE Healthcare). The protein was eluted with a linear gradient of 50–1000 mM NaCl in 50 mM HEPES buffer (pH 7.6). The NvfI protein was further purified on a Superdex 200 pg column (GE Healthcare). The target protein was eluted with 20 mM Tris (pH 7.6) containing 50 mM NaCl, and concentrated to 21 mg ml⁻¹ with an Amicon Ultra-4 filter at 4 °C. The purity of the enzymes was monitored by SDS-PAGE. The protein concentrations were calculated by measuring the ultraviolet absorption at 280 nm.

Crystalization and Structure Determination. The NvfI crystals were obtained at 10 °C in 50 mM Tris–HCl (pH 7.0), containing 20% (v/v) PEG3350, 20 mM α-KG, 200 mM NaSCN, and 2 mM DTT, with 15 mg ml⁻¹ of purified NvfI, by the sitting-drop vapor-diffusion method. The initial model of NvfI was solved by single-wavelength anomalous diffraction method using anomalous signal of Zn. The NvfI crystals were incubated in the crystallization buffer containing 1 mM ZnCl₂ for 5 min at 20 °C to obtain the complex structure. To obtain ternary complex structure with 2 and α-KG, the crystals were moved into anaerobic Coy chamber and then the NvfI crystals were incubated in the crystallization buffer containing 1 mM 2 and 50 mM α-KG. NvfI W199F and NvfI wild type in complex with 2 and N-oxyalylglycine (NOG) were obtained at 10 °C in 50 mM HEPES (pH 7.5), containing 20% (v/v) PEG3350, 20 mM α-KG, 100 mM NaSCN with 20 mg ml⁻¹ of purified enzyme and seed solution (10% volume of drop). The crystal drops were 10 times diluted with crystallization buffer (without 20 mM α-KG) containing 20 μM NOG for overnight at 10 °C. Then, the crystals were transferred into the soaking buffer containing 50 mM HEPES (pH 7.5), 20% (v/v) PEG3350, 20 mM NOG, 100 mM NaSCN, 1 mM FeSO₄, and 20 mM 2. The crystals were transferred into the cryoprotectant solution (reservoir solution with 25% (v/v) glycerol), and then flash cooled at −173 °C in a nitrogen-gas stream. The X-ray diffraction data were collected at BL-1A (Photon Factory, Tsukuba, Japan), using a beam wavelength of 1.1 Å. The diffraction data sets for NvfI were processed and scaled using the XDS program package and Aimless. The determination of Zn sites and the generation of the initial model were performed with Crank2 in CCP4i. The initial phase of the NvfI complex structure was determined by molecular replacement, using NvfI-Zn as the search model. Molecular replacement was performed with Phaser in PHENIX. The initial phase was further calculated with AutoBuild in PHENIX. The NvfI complex structure was modified manually with COOT and refined with PHENIX. The cif parameters of 2 for the energy minimization calculations were optimized by using the PRODRG server. The final crystallographic statistics are summarized in Supplementary Table 1. The Ramachandran statistics are as follows: 97.1% favored, 2.9% allowed for NvfI complexed with 2 and α-KG, 96.7% favored, 3.3% allowed for NvfI complexed with 2 and NOG, and 97.0% allowed for NvfI W199F complexed with 2 and NOG. A structural similarity search was performed, using the DALI program. All crystallographic figures were prepared with PyMOL (DeLano Scientific). The structural analysis of NvfI complexed with 2 and NOG revealed that the overall structure and active site architecture of monomer A (complex structure with 2 and NOG) are almost identical to those of NvfI complexed with 2 and α-KG (state III) while the loop I in monomer B (complex structure with NOG) is different from that in state I. For the docking model, 2 was manually added in the active site, with COOT. The structures were then calculated by PHENIX.refine with simulated annealing.

Preparation of NvfI substrates. Compound 2 was obtained from the crude extract of the Aspergillus novofumigatus JBT 16806 νnfI strain, grown on PDA plates for four weeks at room temperature. The metabolites were extracted with acetonitrile, and the solvent was evaporated by lyophilization. The crude product was purified by silica gel chromatography (Wakogel C-200, 100% chloroform). The fraction containing 2 was collected and further purified with a Shimadzu HPLC system, using a TSK-gel ODS-80Tm column (Tosoh Co. Ltd., 7.8 mm i.d. × 300 mm, 5 μm). The UV absorbance was monitored at 270 nm.

Enzyme reaction of NvfI. The standard enzyme reaction of NvfI was performed in 50 mM Tris–HCl buffer (pH 7.5), containing 100 μM 2, 2 mM α-KG, 2 mM ascorbate, 200 μM FeSO₄, and 10 μM NvfI, for 8 h at 30 °C. The reaction was quenched by adding an equivalent volume of methanol. The samples were...
centrifuged and clarified with a 0.22 μm filter, and the reaction products were analyzed by a compact microTOF-MS (Bruker) attached to an LC-20AD UHPLC system (Shimadzu). Isocitric elution was performed with 58% of CH₃CN/H₂O solution both containing 0.1% formic acid. All measurements were conducted in triplicate.

**Stoichiometric analysis of NvfI.** To measure 2 concentration, the enzyme reaction of NvfI was performed in 50 mM HEpES buffer (pH 7.5), containing 100 mM 2, 1 mM ascorbate, 20 mM FeSO₄, and 20 mM NvfI at various concentration of 2 (0, 25, 50, 75, 100, 125, and 200 mM), for 30 min at 30 °C. The reaction was quenched by adding an equivalent amount of acetonitrile. The samples were centrifuged and clarified with a 0.22 μm filter, and the reaction products were analyzed by Primrose UHPLC system (Shimadzu). Isocitric elution was performed with 54% of CH₃CN/H₂O solution both containing 0.1% formic acid.

To measure α-KG consumption, the enzyme reaction of NvfI was performed in 50 mM HEpES buffer (pH 7.5), containing 200 mM α-KG, 1 mM ascorbate, 20 mM FeSO₄, and 20 mM NvfI, and various concentration of 2 (0, 100, 150, 200, and 300 mM), for 30 min at 30 °C. The incubation mixture was filtered through a NANOSPE 10 K Omega centrifugal device to remove protein. The samples were diluted 40 times with water and subjected to next α-KG detection reaction. The concentration of α-KG was quantified using a α-KG detection kit (Dojindo) by following their protocol. Briefly, α-KG was converted into pyruvate using glutamic acid-pyruvic acid transaminase, and then the pyruvate was converted to H₂O₂ and following their protocol. Brie

**O₂ stoichiometry analysis.** The concentration of oxygen-saturated buffer was determined to be 1.3 mM according to published method9. Oxygen-saturated buffer (5 ml) was transferred into TERUMO syringe (10 ml) preloaded with 2 and NOG. As a result, when I added 10.4 ml of 2.5 mM Na₂S₂O₃ solution used for titration. As a result, when I added 10.4 ml of 2.5 mM Na₂S₂O₃ solution, the solution became completely colorless. Therefore, the Na₂S₂O₃ solution used for titration. As a result, when I added 10.4 ml of 2.5 mM Na₂S₂O₃ solution (2.1 M KI and saturated KOH dissolve in oxygen-free water) and 2.1 M 50 mM HEPES buffer (pH 7.5), containing 200 mM α-KG, 1 mM ascorbate, 20 mM FeSO₄, and 50 μM of enzyme, for overnight at 30 °C in 200 of Eppendorf tubes. The reaction products were extracted by ethyl acetate and evaporated to get rid of the solvent. The crude extract was redissolved by methanol and isolated by semi-preparative HPLC using TSK-gel ODS-80TM column (Toosoh Co. Ltd., 7.8 i.d. x 300 mm, 43% acetonitril, isocratic 3.0 ml min⁻¹). 5: HR-ESI-MS m/z [M + H⁺] = 461.2532 (calc. 461.2534, C₁₂H₁₀O₂+). 6: HR-ESI-MS m/z [M + H⁺] = 463.2658 (calc. 463.2690, C₁₂H₁₀O₂+).

**Steady-state enzyme kinetics.** To determine the time point for analyzing kinetic values, the consumption of substrate was measured at different time points. 0.1 mM of NvfI wild type or Y116A was incubated with 1.25 mM (for wild type) or 2.5 mM (for Y116A) of 2 in the buffer containing 50 mM HEpES (pH 7.5), 1 mM FeSO₄, 500 mM ascorbate, and 500 μM of α-KG at 30 °C for 1, 3, 5, and 10 min for wild type and 1, 3, 5, and 10 min for Y116A. The reactions were quenched by addition of equal amount of acetonitrile and the aggregated protein was removed by centrifugation at 20,400 g for 10 min. The reaction products were analyzed by primrose UHPLC system (Shimadzu). Isoacetic elution was performed with 54% of CH₃CN/H₂O solution both containing 0.1% formic acid.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data generated in this study are provided in the Supplementary Information/Source Data file. The crystallographic data for the apo structures of NvfI wild type in complex with 2 and α-KG, NvfI wild type in complex with 2 and NOG, and W199F variant in complex with 2 and NOG have been deposited in the Protein Data Bank (PDB) under accession codes 7DE297, 7ENB58, and 7EMZ59, respectively. All other relevant data are available from the corresponding author upon request. Source data are provided with this paper.

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