

# 博士論文（要約）

## **Structure and Function Analysis of A Novel Endoperoxide-Forming Non-heme Fe(II)- and $\alpha$ KG- dependent Dioxygenase from Filamentous Fungi**

（糸状菌由来新規 $\alpha$ -ケトグルタル酸依存性ジオキシゲナーゼの構造機能解析）

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## Introduction

Many peroxy-containing secondary metabolites have been isolated and shown to provide beneficial effects to human health.<sup>[1]</sup> However, the reaction mechanism of most endoperoxide-forming enzymes is not well understood. To date, fumitremorgin B endoperoxidase (FtmOx1) from *Aspergillus fumigatus* is the first reported non-heme Fe(II)- and  $\alpha$ -ketoglutarate ( $\alpha$ KG)-dependent dioxygenase (DIOs) that catalyzes an endoperoxide-formation reaction<sup>[2]</sup>. In this reaction, a Tyr residue is important to form a radical intermediate to perform incorporation of a second oxygen molecule.

Previously in our group, an Fe(II)/ $\alpha$ KG-dependent enzyme Nvfl was characterized to perform endoperoxide-forming reaction to produce the key intermediate fumigatonoid A in novofumigatonin biosynthesis in *Aspergillus novofumigatus*.<sup>[3]</sup> To the best of our knowledge, Nvfl is the first enzyme that can introduce three O atoms (including one endoperoxide bridge) into product in one step. Sequence alignment indicates that Nvfl shares low sequence similarity to other Fe(II)/ $\alpha$ KG-dependent dioxygenases (10-15% of amino acid identity). This sequence uniqueness as well as the unusual catalytic function attracts our great interest to study the structure of this enzyme by X-ray crystallography to shed light on the mechanism.

## Methods

X-ray crystallography is one of the essential techniques to study the structure of a biological macromolecule. The principle for crystallization is the supersaturation of protein in the solution state. When protein exceeds the solubility limit in the supersaturation state, some of them began to be pushed out of the solution to undergo the nucleation transition state where crystal grows gradually. There are several methods to help the protein to reach a supersaturation state in solution. Usually, chemicals known as precipitants are used to achieve the supersaturation state by reducing the protein's solubility in solution. In this study, sitting-drop vapor diffusion was utilized for crystal growth and optimization. The crystallization condition was optimized by adjustment of pH, the precipitant's concentration, the temperature of the environment, and the screening for proper additives. As a result,  $\alpha$ KG was found to be crucial for crystallization, and NaSCN and DTT were essential for the crystal to grow bigger and thicker.

## Result

### 1. Overall structure of Nvfl

The His-tagged recombinant proteins of Nvfl was expressed in *Escherichia coli* and purified with Ni-affinity and gel filtration chromatographies. Its initial phasing was solved by SAD method using Zn atom, and the crystal structure of this novel Fe(II)/ $\alpha$ KG-dependent enzyme was successfully solved at 2.0 Å resolution.

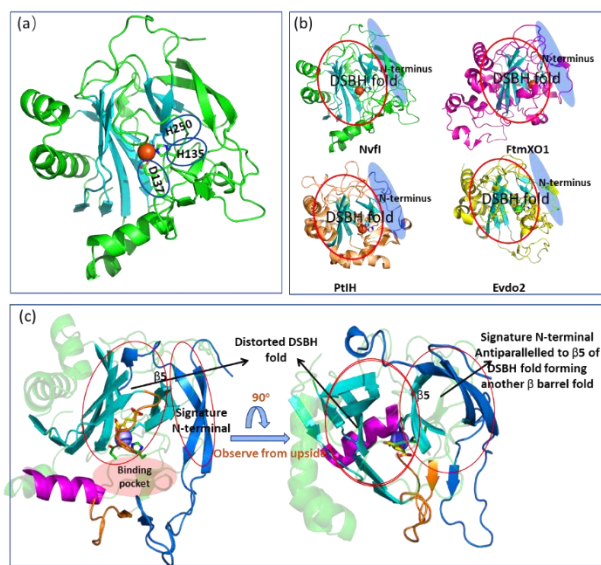
The molecular weight judged by size exclusive gel-filtration is 34 kDa, indicating that it exists as a monomer in solution state. Different from other Fe(II)/ $\alpha$ KG-dependent DIOs whose substrate binding sites lie between surfaces of their two homodimers, Nvfl itself can form a closed binding pocket composed of two loops and an  $\alpha$  helix. The overall structure of Nvfl consists of a signature N-terminus followed by a distorted double-stranded  $\beta$ -helix (DSBH) core fold made up of 8  $\beta$ -strands. The signature N-terminus of Nvfl contains two  $\beta$ -strands anti-parallel to the  $\beta$ 5 of the conserved DSBH fold and forms another  $\beta$ -barrel fold, which causes the distortion of DSBH fold (**Fig. 1c**) and is verified to be important for the activity by truncation experiment.

A Dali Server search indicates that the closest analogues of Nvfl are PtIH (PDB.ID:2rdn), Evdo2 (PDB.ID:4xac), and FtmOX1 (PDB.ID:4zon). However, the overall identity is low (15% or 17% of amino acid identity) and the R.M.S.D is higher than 3, which indicates a great difference in the structure (**Fig. 1b**). The phylogenetic study reveals that Nvfl belongs to a new subfamily of Fe(II)/ $\alpha$ KG-dependent DIOs that is not closely related to other DIOs involved in the fungal meroterpenoid biosynthetic pathwa.

## 2. Active site architecture of Nvfl and mutagenesis study

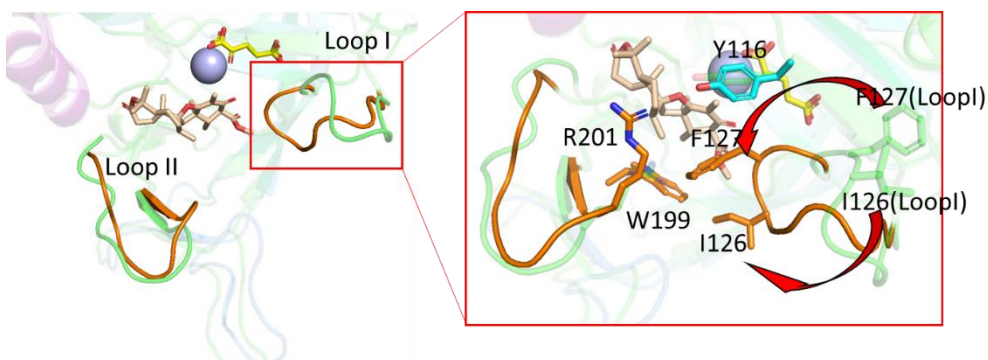
The catalytic triads of Nvfl are H135-H137-D250, responsible for chelating to an iron (**Fig. 1a**). By soaking asnovolon A into the buffer, a complex structure of Nvfl-Fe/ $\alpha$ KG and asnovolin A was obtained.

Two loops (loop I and loop II) are involved in the formation of substrate binding pocket. W199 (on loop II), F127 (on loop II), R201 (on loop II), Y116 and the backbone of T207 (on loop II) and D206 (on loop II) create a closed cavity upon substrate binding. By comparison of the apo structure and complex structure, a significant movement of the loop121-128 (loop I) and a delicate movement of loop198-208 (loop II) is observed, where F127 moves inward upon substrate binding and forms a closed cavity together with W199 and R201 (**Fig. 2**). W199 moves slightly upward and R201 extends further upward to form hydrogen bonding network with Y116, T207, and D206 through a water.



**Fig. 1** (a) Overall structure of Nvfl with H135-H250-D137 as catalytic triads (b) Best homologues given by Dali server (c) Signature N-terminal antiparalleled to DSBH fold

Asnovolin A is recognized by H138 and R118 via hydrogen bond interactions on both side of the substrate. Interestingly, H138R, R118A, R118H, and R118K variant abolished the activity, indicating that these residues are required for correct substrate recognition.



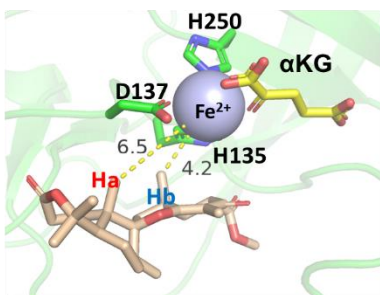
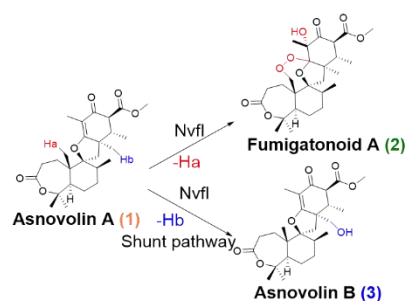
Mutagenesis studies further verified

**Fig.2** Loop movement upon substrate binding: Movement of Loop I is framed.

the importance of the active site residues for proper substrate conformation upon binding. W199A, F127A and R201A abolished the activity. F127 and W199 were responsible for major interaction with the ring system of substrate, as mutation of F127 and W199 to other bulky aliphatic or aromatic residues led to dramatic loss of endoperoxide-forming activity and formation of some other new products.

### 3. Labelling experiment indicating a long-lived Fe(III)-OH/R• state

In the  $^{18}\text{O}_2$  labelling experiment, a +4 peak was observed for the production of fumigatonoid A, indicating the endoperoxide bridge shall derive from  $\text{O}_2$  molecule. The reason that +6 peak was not observed may be due to a fast solvent exchange than hydroxyl rebound in the ferryl-oxo and Fe(III)-OH state. To verify the hypothesis, a  $\text{H}_2^{18}\text{O}$  labelling experiment was also carried out and +2 peak was successfully observed, revealing fast solvent exchange with  $\text{H}_2\text{O}$ . According to Pan *et. al*, the suppression of hydroxyl rebound is generally important for non-



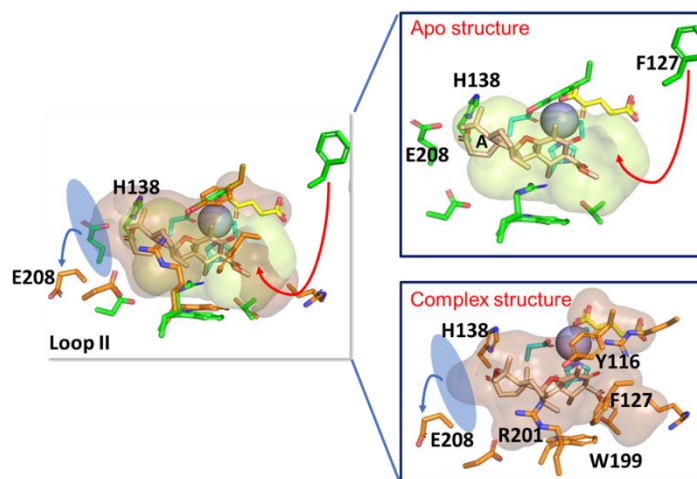
**Fig.3** Initial hydrogen abstraction lead to different result

hydroxylation outcomes by Fe(II)/ $\alpha\text{KG}$ -dependent DIOs.<sup>[4]</sup> This long-lived Fe(III)-OH/R• state increases the chance of conformation change of substrate radical in the binding pocket.

### 4. Discussion

A close comparison of the binding-pocket of the apo and complex structure shows that upon substrate binding, the pocket is enlarged by the movement of E208 away from the substrate. This movement avoids steric hindrance, and thus leaves space to accommodate A ring of the substrate. The substrate goes through conformation change by the fine-tuning of F127, W199, and H138. Finally it reaches the most stable conformation state (observed in the complex structure), where  $\text{O}_2$  molecules are anchored by Y116 and trapped by the radical, and endoperoxide formation reaction proceeds.

The *in-vitro* result of the H138 mutant also provided evidence supporting the importance of the space that accommodates the A ring of the substrate: H138A produced more shunt-pathway product Asnovolin B than endoperoxide Fumigatonoid A, indicating the substrate tent to adopt the conformation observed in complex structure to form the shunt-pathway product. Mutating to a bulkier but also basic residue of arginine led to the abolishment of activity, showing the size of the residue was also very important in substrate recognition.



**Fig.4** Movement of E208 leaves space upon substrate binding to accommodate A ring of substrate

Based on these results, we proposed a mechanism as followed: In the initial conformation where Ha is closer to iron center (**Fig.3**), the substrate binding mode is at a high energy level, and the radical intermediate generated via abstraction of Ha goes through delicate conformational change in the binding pocket to reach more stable state, where it is trapped by another O<sub>2</sub> molecule and the endoperoxide-forming reaction is successfully proceeded.

## Conclusion

The structure of Nvfl was successfully solved with X-ray crystallographic analysis. Except for the conserved DSBH fold, it showed a great structure difference with other Fe(II)/ $\alpha$ KG-dependent DIOs, in accordance with the phylogenetic study. The mutagenesis studies revealed the importance of F127, W199, H138, and T133 for the endoperoxide-formation via adjusting the proper conformation of the substrate. In addition, the labelling experiment indicated that a long-live Fe(III)-OH/R• after initial hydrogen abstraction went through delicate conformational change and the radical intermediate reacted with an O<sub>2</sub> molecule to form the characteristic endoperoxide structure.

## Reference

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- [4] J. Pan., *et al., J. Am. Chem. Soc.*, **2019**, 141, 15153.