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X-ray structure analysis of fructosyl peptide oxidases from *Phaeosphaeria nodorum* to elucidate the residues responsible for the oxidative half-reaction.

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1 Introduction

Fructosyl amine oxidases (FAOX) catalyze the oxidative deglycosylation of fructosyl amino acids such fructosyl valine and/or ε-fructosyl lysine, which are the degradation products of glycated hemoglobin (HbA1c) and glycated albumin, respectively. Instead of the measurement of HbA1c by HPLC or immunoassay, the enzyme assay systems based on the FAOX have been considered as a fast and simple monitoring method. As a FAOX family, fructosyl peptide oxidases (FPOX) are also known as candidates for diagnostic enzymes. In our previous study, we have reported on the novel FPOX derived from *Phaeosphaeria nodorum* (PnFPOX). Here we report the X-ray structures of the recombinant PnFPOX and a mutant form Asn56Ala (N56A). The mutant N56A showed decreased oxidase activity compared to the wild-type, while its dye-mediated dehydrogenase activity was higher than that of wild-type. The structure comparison reveals that Asn56 in PnFPOX is essential for maintaining an effective oxygen accession path, and support the role of Asp54 as a gate keeper that cooperates with Lys274 to enable oxygen to reach the active site properly [1].

2 Experiment

The recombinant wild-type PnFPOX and mutant N56A were expressed as His-tagged protein and purified by affinity chromatography [1]. For the initial crystal screening, the purified protein solutions of wild-type PnFPOX and N56A were concentrated to 9.8 and 7.1 mg/mL, respectively. Well-diffracting crystals were obtained by mixing the protein solution (0.8 μL) and the same volume of reservoir solution (8% (v/v) Tacsimate pH 5.0, 18–22% (w/v) PEG3350) against 50 μL of the reservoir solution by the sitting-drop method at 293 K.

X-ray diffraction data were collected on the NE3A in the PF-AR. Diffraction data were processed using the programs HKL2000 and the CCP4 program suite.

Initial phase was determined by molecular replacement using the structure of FPOX from *Eupenicillium terrenum* (PDB code: 4RSL) as a probe model.

3 Results and Discussion

The overall structure of wild-type PnFPOX (Fig.1) is equivalent to that of PnFPOX N56A. The structurally similar proteins were FPOX from *Eupenicillium terrenum* (EtFPOX, 4RSL) with 73% identity, 1.0 Å rmsd, amadoriase II without ligand (3DJD, 36% id, 1.8 Å rmsd), amadoriase II with bound inhibitor (FSA) (3DJE, 35% id, 1.9 Å rmsd), amadoriase I with bound substrate (fructosyl $^{\varepsilon}$lysine) (4XWZ, 33% id, 1.9 Å rmsd), amadoriase I without ligand (4WCT 33% id, 1.9 Å rmsd), and monomeric sarcosine oxidase (3ML3, 25% id, 2.4 Å rmsd).

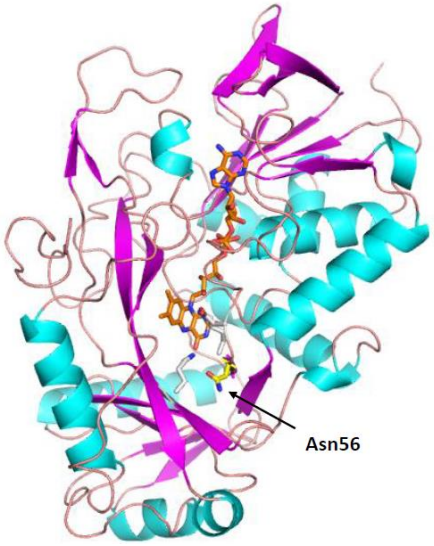

Fig. 1: Structure of fructosyl peptide oxidase from *Phaeosphaeria nodorum*.

A covalent bond is formed between FAD and Cys343 of PnFPOX as observed in amadoriase I (Cys342), amadoriase II (Cys335), and EtFPOX (Cys347). The residues (Thr18, Asp41, Ser47 and Ser50 (Ala49 in amadoriase I), Ala51 (main chain), Lys57 and Ile58 (main chain), and Lys376 (Met375 in amadoriase I), which form hydrogen bonds with FAD are almost conserved in FPOX and FAOX. In addition, Lys57 and Lys274 which approach the isoalloxazine ring of FAD at the *si*-face, are also conserved in FPOX and FAOX. Since the substrates of the FAOX family enzymes bind at the *re*-face of the isoalloxazine ring of FAD, the FAD binding mode and environment at the *si*-face of the isoalloxazine ring do not affect substrate specificity and are conserved among the FAOX family members.

In wild-type PnFPOX, Asn56 forms a hydrogen bond with Lys274; Lys274 forms water (W1)-mediated hydrogen bonds with N5 of FAD and hydrogen bonds with Asp54 and Lys57 and O2 of FAD via a water molecule (W2) (Fig.2 (a)). In PnFPOX N56A, Lys274, and Asp54 approach each other, and the water molecule is displaced from the W2 position such that Lys274 forms salt bridges with Asp54 at 2.92–3.35 Å (Fig.2 (b)). The direct distance between Lys274 and Asp54 is 5.48-6.03 Å in wild-type PnFPOX. The relocated Asp54 also forms water (W3 and W4)-mediated hydrogen bond with His239 and is stabilized in this position. Considering that PnFPOX N56A has lower oxidase activity and a concomitant increase in dehydrogenase activity [2], the role of Asn56 is likely to keep Lys274 free, and to prevent the formation of salt bridges with Asp54, thereby creating a space for accepting a significant water molecule at the W2 position.

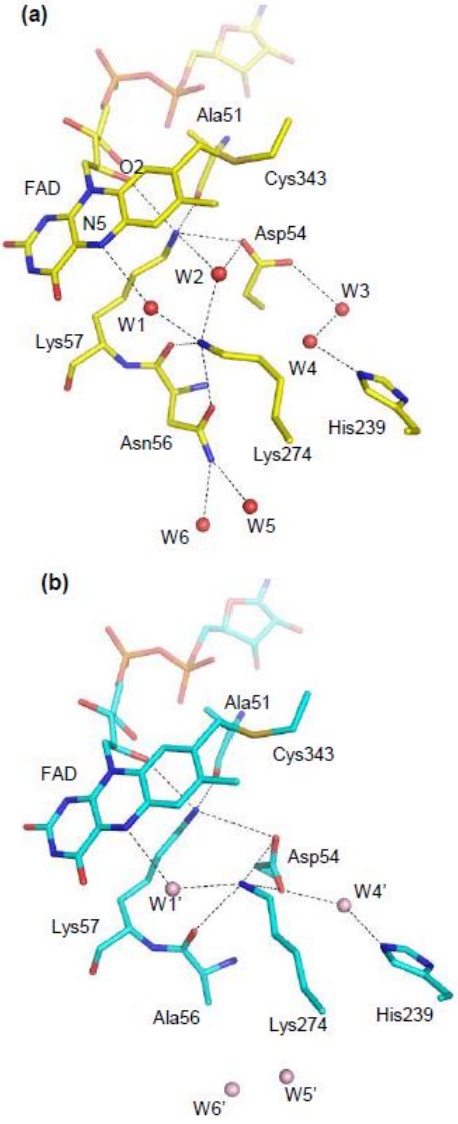

Fig. 2: Comparison of active site structures between PnFPOX wild-type (a) and mutant N56A (b).

Since PnFPOX N56A exhibited decreased oxidase activity with a concomitant increase in dehydrogenase activity in our previous mutagenesis study [2], the role of Asn56 appeared to maintain the freedom of Lys274, and did not form salt bridges with Asp54, which maintains a space for accepting oxygen at the W2 position. The observed increase in dye-mediated dehydrogenase activity in PnFPOX N56A can be explained by the decrease in competition between the dye mediator and oxygen. Our structural comparison revealed that Asn56 in PnFPOX has a crucial role in assisting and stabilizing the oxygen path involving Asp54, Lys57, and Lys274 in terms of oxidase activity. This comparison also suggests that Asn56 in PnFPOX is essential for properly maintaining a plausible main oxygen channel and oxidase activity involved in the movement of Lys274. In addition, the results of the mutagenesis study provide direct evidence of decreased oxygen activity in PnFPOX N56A (data not shown here) [1].

Acknowledgement

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References

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