論 文 の 内 容 の 要 旨

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論文題目

Structural analysis and functional modification of a novel dioxygenase from *Burkholderia ambifaria* AMMD in producing hydroxy amino acid

（水酸化アミノ酸生産に有用な *Burkholderia ambifaria* AMMD 由来新規ジオキシゲナーゼの構造解析と高機能化）

The hydroxy amino acids, which are components of glycopeptide antibiotics, cyclodepsipeptides and collagen, have many physiological activities. Some hydroxy amino acids can also be used as precursors in the asymmetric synthesis of pharmaceuticals. For example, (2*S*,3*R*,4*S*)-4-hydroxyisoleucine has insulinotropic and anti-obesity effects and seems to have potential for the treatment of diabetes.

The hydroxylation of amino acids is catalyzed by the ferrous [Fe(II)]- and α-ketoglutarate (α-KG)-dependent dioxygenases. These enzymes can also hydroxylate proteins, nucleic acids, lipids and small molecules. They participate in a vast array of protein side-chain modifications, repair of alkylated DNA/RNA, and biosynthesis of antibiotics and plant products. Dioxygenase-mediated hydroxylation requires dioxygen as well as Fe(II) and α-KG. One of the oxygen atoms is incorporated into the substrate to form hydroxy amino acid, while the other oxygen atom is used to oxidatively break down α-KG into succinate.

SadA is a member of the dioxygenase family from *Burkholderia ambifaria* AMMD. This enzyme is useful as a novel biocatalyst for the (*R*)-selective hydroxylation at the C-3 position of *N*-substituted L-amino acids, especially *N*-succinyl-L-leucine (NSLeu), to produce *N*-succinyl-(*R*)-3-hydroxy-L-leucine (NSHLeu) with >99% enantioselectivity (Fig. 1). (*R*)-3-hydroxy-L-leucine is a promising material for the preparation of certain

cyclic depsipeptides which function as platelet aggregation inhibitors and is also a component of the antibiotic lysobictin. In addition, SadA is the first characterized Fe(II)- and α-KG-dependent dioxygenase that catalyzes *N*-substituted aromatic L-amino acids. Thus, SadA has the potential for widely producing C3-hydroxylated amino acids with various types of branched chain or aromatic ring. Although a few Fe(II)/α-KG-dependent dioxygenases are known to hydroxylate free amino acids, their substrate specificities are restricted to hydrophilic amino acids such as L-arginine and L-asparagine. Therefore, the mechanism for the substrate specificity of SadA remains poorly understood.

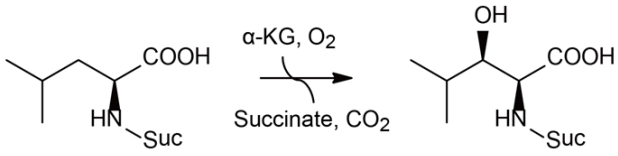

Fig. 1 Stereoselective hydroxylation of NSLeu into NSHLeu.

Here I report the structures of SadA.Zn(II) and SadA.Zn(II).α-KG. In addition, based on the structures and mutation analyses, I propose a substrate-binding model to elucidate the structural basis of the substrate specificity and enantioselective hydroxylation. The structural insights will be used for expanding the substrate specificities of SadA toward some derivatives of *N*-succinyl branched chain or aromatic amino acids by protein engineering approaches.

Overall structures of SadA complexes

The crystal structures of SadA.Zn(II) and SadA.Zn(II).α-KG were determined at 1.77 Å and 1.98 Å resolutions, respectively. The structure of SadA.Zn(II) contained 11 β-strands, 6 α-helices and one 3_{10} helix, and possesses the DSBH fold at the core of the structure (Fig. 2). The DSBH fold of SadA was comprised of seven β-strands, four of which (β3, β5, β8 and β10) formed a major β-sheet and other three β-strands (β6, β7 and β9) constituted a minor β-sheet. The β1, β2, β4 and β11 strands extended the major β-sheet. Six α-helices (α1-α6) packed along the major β-sheet of DSBH fold.

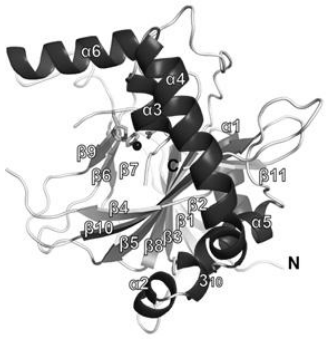

Fig. 2 Overall structure of SadA

SadA formed a dimer in the crystals as well as in solution. The dimeric contact area is mainly comprised of the residues of α4 and loop between α5 and β4. The dimer forms

an intermolecular disulfide bond, two salt bridges, the hydrophobic interactions and intermolecular hydrogen bonds. These interactions serve as key structural features in stabilizing the dimer formation.

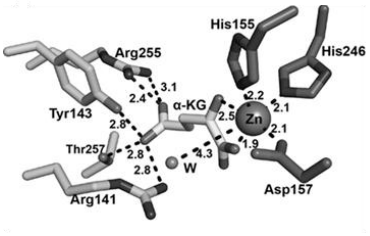

Fig. 3 Catalytic site of SadA

There is one α-KG molecule existing only in chain A (Fig. 3). The α-KG coordinates Zn(II) in a bidentate manner using its 2-oxo carbonyl and C-1 carboxylate groups, which form an octahedral coordination geometry complex. The 2-oxo oxygen of α-KG is located trans to Asp157 and the C-1 carboxylate is observed to be trans to His155 of the HXD/EXnH motif. The C-5 carboxylate forms two salt bridges with the side chains of Arg141 and Arg255, and two hydrogen bonds with the hydroxy group of Tyr143 and Thr257. A single water molecule is observed to be trans to His246 of the HXD/EX_nH motif. This water would be displaced by O_2 in the course of the catalytic reaction.

Structural basis of substrate recognition and specificity

I have performed cocrystallization and soaking experiments with *N*-oxalylglycine (NOG, an α-KG analogue) and some substrates under aerobic or anaerobic conditions, but failed to obtain the complex structure. Therefore, I attempted to build the docking model with NSLeu and NSPhe. Initially, the MOE suite was used to predict the locations of an NSLeu molecule in the active site, and I presumed the presence of several residues related to substrate-binding of SadA. The mutation analyses of the predicted residues were performed to evaluate whether the mutations affect the SadA activity toward NSLeu (Fig. 4).

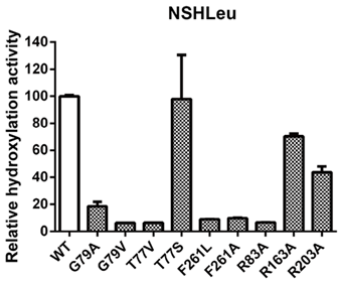

Fig. 4 Relative hydroxylation activities of SadA mutants were measured toward NSLeu.

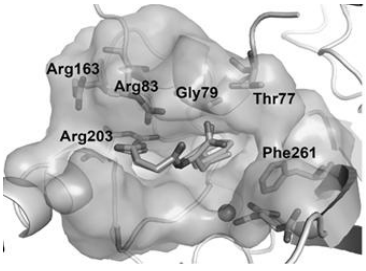

Fig. 5 Docking model of NSLeu and NSPhe into the SadA.Zn(II).α-KG structure.

The results were as follows: 1) The binding site of the *N*-succinyl group is located in

an electropositive-rich cavity by the formation of salt bridges with the side chains of Arg83, Arg163 and Arg203; 2) The hydrophobic interactions of the side chain of *N*-succinyl amino acid probably are formed with the main chain of Gly79 and the phenyl ring of Phe261; 3) the hydroxy group of Thr77 is predicted to bind the carboxyl group of the substrate. The substrate-binding models were refined according to the results of mutaion analyses (Fig. 5).

Functional modification of SadA targeting industrial manufacture

Based on the structures of SadA.Zn(II).α-KG and the proposed substrate-binding model, we used structure-based protein engineering approach to improve the efficiency of catalyzing C-3 hydroxylation of novel *N*-succinylated amino acids (NSX) and develop an effective industrial catalyst starting from an enzyme that owned little detectable α-KG turnover activity toward NSX. Notably, I have generated SadA mutants with increased α-KG turnover activity toward NSX.

Conclusions

The crystal structures of SadA.Zn(II) and SadA.Zn(II).α-KG were determined. The residues related to substrate recognition around the active site were predicted and verified by biochemical analyses. The structural and biochemical studies revealed the structural basis of the substrate specificity and enantioselective hydroxylation. Based on the proposed substrate-binding model, I have generated SadA mutants with increased α-KG turnover activity toward the novel *N*-succinylated amino acids compared with that of wild type. These results will serve as a model for commercial-scale manufacture in which an enzyme is desired as the target of an industrial biocatalyst.

References

1. H.-M. Qin *et al*. (2012) Expression, purification, crystallization and preliminary X-ray analysis of a branched-chain amino acid dioxygenase from *Burkholderia ambifaria* AMMD. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 68, 1067-1069.

2. H.-M. Qin *et al*. (submitted) Crystal structure of a novel *N*-substituted L-amino acid dioxygenase from *Burkholderia ambifaria* AMMD.