論文の内容の要旨

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論文題目

Mechanism of electron transfer between ferredoxin and $\alpha_3\beta_3$-type oxygenase components of Rieske non-heme iron oxygenase

（Rieske non-heme iron oxygenase におけるフェレドキシンとオキシゲナーゼ間電子伝達機構の解明）

Introduction

Aromatic compounds are utilized by microorganisms as carbon and energy sources and are transformed into non- or less-hazardous substances. Microorganisms overcome the resonance stabilization energy of aromatic ring systems using enzymes called oxygenases, which are oxidoreductases that can incorporate one (monooxygenases, also called hydroxylases) or both (dioxygenases) atoms of molecular oxygen into the molecule to cleave aromatic ring. The monooxygenases can be classified as P450-dependent systems, flavin-dependent system, non-P450 hydroxylases and non-heme-dependent oxygenases. The dioxygenases include two major classes, viz., heme-dependent iron-sulfur plant dioxygenases and Rieske non-heme iron dioxygenases (ROs).

ROs often play a key role in the initial step of bacterial degradation of various aromatic compounds, such as naphthalene, biphenyl, isopropylbenzene (cumene) and carbazole. ROs are composed of one or two electron transfer component(s) and a catalytic component (terminal oxygenase, Oxy). The reaction is triggered by electrons from NAD(P)H, which are transferred from reductase (Red) to the Oxy protein subunits directly or via a ferredoxin (Fd).

Oxy of ROs usually contains a large α-subunit having a Rieske cluster domain and a mononuclear iron-containing catalytic domain. The Rieske cluster receives electrons from electron transfer component(s) and transfers them to the mononuclear iron of the neighboring α-subunit. Apart from the large α-subunit, many Oxys contain an additional small β-subunit which contains no prosthetic group, and the function of the β-subunit is suggested to provide structural support to the overall enzyme. To date, all the structures of Oxy demonstrate that Oxy are either α_3 doughnut-shaped or $\alpha_3\beta_3$ mushroom-shaped quaternary structures.

Electron transfer is essential for triggering the degradation in ROs and it is also related with catalytic activity. In three-component RO systems, Fd shuttles electrons through non-covalent bindings between Red and Oxy. The binding manner between Fd and Oxy has been determined previously with Fd-Oxy complex structure of carbazole 1,9a-dioxygenase (CARDO) which is a α_3-type Oxy by Ashikawa *et al*., 2006. The study showed that Fd of CARDO (CARDO-F) binds to

the hydrophobic groove surrounded with charged residues at the interface of the α-subunits of Oxy of CARDO (CARDO-O).

Cumene dioxygenase (CDO; EC: 1.14.12.-) from *Pseudomonas fluorescens* IP01, which is a three-component RO system, catalyzes the initial reaction in cumene degradation pathway. Electrons are transferred from NADH via iron-sulfur flavoprotein Red of CDO (CDO-R) and Rieske-type Fd of CDO (CDO-F) to $\alpha_3\beta_3$-type Oxy of CDO (CDO-O). Of the three components, only the structure of CDO-O has been determined until now and the structure revealed a $\alpha_3\beta_3$ mushroom-shaped quaternary structure. CDO is classified as a class IIB RO according to the Batie's classification, which is the same class as the well-studied ROs, biphenyl dioxygenase (BDO) and toluene dioxygenase (TDO), both of which are $\alpha_3\beta_3$-type Oxy and are associated with Rieske-type Fd. CDO-O is possessed of 64-74%, 50-59%, 50-77% and 48-73% identities with α-, β-subunits, Fds, and Reds, respectively, of BDO from *Paraburkholderia xenovorans* LB400, BDO from *Rhodococcus jostii* RHA1, and TDO from *P. putida* F1. The structures of CDO-Oα and CDO-Oβ have RMSD values of 0.94-1.45 Å and 0.89-1.43 Å compared to α- and β-subunits of above BDO and TDO.

The current study aimed to determine the mechanism of electron transfer between Rieske-type CDO-F and $\alpha_3\beta_3$-type CDO-O by two different approaches. In approach I, I performed docking simulations with homology-modelled CDO-F and wild type (WT) CDO-O and electron transfer efficiency analyses to determine the residues on CDO-O involved in their interaction. Moreover, the conservation of the interaction related residues in $\alpha_3\beta_3$-type CDO-O has also been proposed. In approach II, I tried to determine the complex structure by X-ray crystallography. The predicted interactions were further confirmed using chemical and photo cross linking followed by mass spectrometry.

Approach I: Computational study and electron transfer efficiency analyses

To determine the mechanism of electron transfer between CDO-F and CDO-O by computational studies, structures of both CDO-F and CDO-O were needed. The structures of 4 modeled CDO-Fs were generated by homology modeling using SWISS-MODEL. Then, docking simulations were performed between CDO-O and the 4 homology-modelled CDO-Fs, independently. The results showed that there were two potential docking sites on CDO-O, one was at the interface of two α-subunit at top-wise site of CDO-O molecule (on the cap of the mushroom-shaped structure), the other was at the interface of α and β-subunit at the side-wise site of CDO-O molecule (at the stem of the mushroom-shaped structure). The putative binding manner at top-wise site corresponded to that in CARDO. Both potential binding sites had similar features, which were found in the binding sites between CARDO-F and CARDO-O, and that was a hydrophobic groove surrounded by several charged residues.

Alanine-scanning mutagenesis was performed on surface-exposed residues of CARDO-O, which may be involved in the binding with CDO-F. Based on results of docking simulations, 16 charged or hydrophobic residues were chosen for alanine substitution, individually. Eight residues,

viz. Lys33, Leu35, Arg39 and Arg407 on α1-subunit and Asp158, Trp159, Leu162, Glu180 on α2-subunit corresponding to the top-wise site. Eight residues, viz. Lys117 and Lys141 on α1-subunit, Leu241, Asp253 and Lys258 on α2-subunit, Arg65 on β1-subunit, and Leu98, Trp100 on β2-subunit corresponding to the side-wise site.

Electron transfer efficiency were measured by the reduction of the Rieske cluster on CDO-O. The efficiencies were measured between the wild type electron transfer components and each of 16 single alanine-substituted, respectively (Fig. 1A). The mutants with alanine substitution at top-wise site showed no or negligible decrement in reduction efficiency compared with that of WT CDO-O. On the other hand, the reduction efficiency of alanine-substituents at the side-wise site significantly decreased, especially with αK117 and βR65 mutations. The efficiencies of αK117A and βR65A decreased to 21% and 46%, respectively, compared to that of the WT CDO-O. In addition, the efficiencies of αK141A and βW100A seemed to decrease slightly to 71-75%, although statistical significance was not significant. Electron transfer efficiency were also measured with 9 double-alanine-substituted Oxys (Fig. 1B). The efficiencies of mutants which included either αK117A or βR65A, decreased to less than 20%, while that of αK141A;βW100A decreased to 69%. These results indicated that CDO-F binds at the side-wise site of CDO-O and transfers an electron to the Rieske cluster of CDO-O.

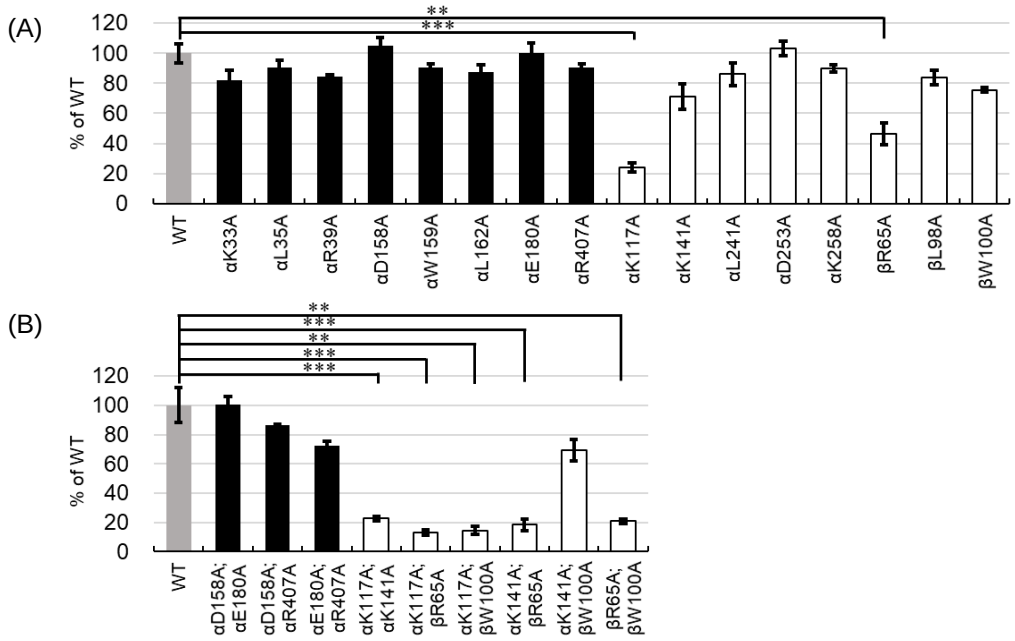

Fig. 1. Reduction efficiencies of alanine substituted CDO-O
Panels (A) and (B) show the reduction efficiencies of single alanine-substituted CDO-Os and double alanine-substituted CDO-Os, respectively. That of WT CDO-O was also shown in both panels as gray bars, which were set to be 100%. The black and white bars were the reduction efficiencies of CDO-O derivatives which had amino acid-substitution at the top-wise and side-wise potential docking site, respectively. Error bars indicate the standard deviation from three independent experiments. n = 3, ** $p < 0.005$, *** $p < 0.001$

Above results showed that the electron transfer was disrupted by of alanine-substitutions. To determine whether the effect by alanine substitution was disruption of the electron transfer pathway or components-binding, isothermal titration calorimetry was performed. Unfortunately, I cannot

optimize the buffer condition to measure experimental heat of CDO-F and CDO-O interaction.

Ten structures of $\alpha_3\beta_3$-type Oxy were superimposed and the position of positive charged residues at the side-wise site were compared. The positions of αK117 and βR65 were conserved in all the Oxys. Furthermore, sequence alignment also showed that αK117 and βR65 were conserved in 73 different α- and β-subunits of ROs. It suggested a possibility that the common mechanism of electron transfer is shared by most Rieske-type Fd and $\alpha_3\beta_3$-type Oxy combinations.

Approach II: X-ray crystallography, chemical- and photo-cross linking

X-ray crystallography was performed with mixture of WT CDO-F and WT CDO-O or its derivatives (αK117A and αR39A). Crystals were obtained within few days with following conditions, 0.7% (v/v) Jeffamine® ED-2001, 1.4-1.6 M sodium malonate and 0.1 M MES (pH 6.4 or 6.5) or 0.7% (v/v) Jeffamine® ED-2001, 1.4-1.6 M sodium malonate and 0.1 M HEPES (pH 6.6, 6.7 or 6.8). X-ray diffraction data were collected with 19 crystals and structure refinement was performed. Although the immunoblotting results showed that most of crystals contained both CDO-F and CDO-O, only CDO-O structure could be built with all X-ray diffraction data. It might be because that CDO-F was coating on the surface of crystals or not obtained in all asymmetric unit.

Chemical cross linking and photo cross linking were performed with CDO-F and CDO-O. In chemical cross linking, WT CDO-F and WT CDO-O were mixed with chemical cross linker, sulfo-SMPB or DMS for 60 min, then the mixture was separated by electrophoresis. The novel band was detected at less than 98 kDa when sulfo-SMPB was used. The band was cut off and analyzed by liquid chromatography/mass spectrometry (LC/MS). However, the binding site of CDO-F could not be identified with the mapping result of LC/MS.

On the other hand, a genetically encoded photoreactive amino acid, ρ-benzoyl-L-phenylalanine (ρBPA), was used to survey the binding site of CDO-F. Six amino acid residues of CDO-F (Q19X, S49X, L65X, A80X, P81X and P85X) was replaced by ρBPA, individually. Among them, only Q19X and L65X could be expressed and purified successfully. Photoreactive CDO-F and WT CDO-O were mixed in 3:1 ratio and treated with 365 nm UV light for 30 to 120 minutes. The cross linked CDO-F Q19X and CDO-O was detected by electrophoresis.

Conclusion and future prospects

The current study demonstrated that CDO-F binds at the interface of α- and β-subunit of CDO-O and that the residues, αK117 and βR65, plays critical roles in the interaction. The residues are conserved in most $\alpha_3\beta_3$-type Oxy, suggesting a similar mode of binding in most Rieske-type Fd and $\alpha_3\beta_3$-type Oxy. The study also revealed that the function of β-subunit of $\alpha_3\beta_3$-type Oxy is not only structural but it also stabilizes the interaction with Rieske-type Fd. The reproducibility of photo cross linking results will be confirmed and followed by LC/MS analyses. To determine the mechanism of interaction between Rieske-type Fd and $\alpha_3\beta_3$-type Oxy in atomic level, more detailed structural studies of complex are necessary.