

## Structure-Guided Redesign of Fungal Non-Heme Iron Dependent Dioxygenases

AusE from *Aspergillus nidulans* and PrhA from *Penicillium brasilianum* are the non-heme iron and  $\alpha$ -ketoglutarate dioxygenases in fungal meroterpenoid biosynthesis. Both AusE and PrhA accept preaustinoid A1 as a common substrate to catalyze divergent rearrangement reactions to form the spiro-lactone in austinol and cycloheptadiene moiety in paraherquonin, respectively. The crystal structures of AusE and PrhA as well as complex structures with substrates uncovered three key active-site residues that control their reactivity. Structure-guided redesign of key residues afforded engineered PrhA, which catalyzes AusE-type ring modification reaction. Furthermore, PrhA double and triple mutants expanded the catalytic repertoire to generate non-natural novel meroterpenoids compounds.

Non-heme iron and  $\alpha$ -ketoglutarate ( $\alpha$ KG)-dependent dioxygenases play an important role in diversifying the molecular scaffold of natural products [1]. Biosynthetic studies of fungal meroterpenoid compounds revealed that many of the Fe(II)/ $\alpha$ KG-dependent dioxygenases catalyze multi-step modification reactions including simple hydroxylations to remarkable skeletal rearrangements [2]. The  $\alpha$ KG dioxygenases AusE from austinol biosynthesis in *Aspergillus nidulans* and PrhA from paraherquonin biosynthesis in *Penicillium brasilianum* are multi-functional enzymes, sharing ~78% amino acid sequence identity with each other. Although both AusE and PrhA accept preaustinoid A1 as a substrate, these enzymes catalyze different types of oxidation reaction [3, 4]. In the first step, while AusE catalyzes the desaturation of C1–C2 of preaustinoid A1 to form preaustinoid A2, PrhA catalyzes the desaturation of C5–C6 of preaustinoid A1 to produce berkeleyone B. Furthermore, in the following step, AusE catalyzes the dynamic rearrangement reaction of preaustinoid A2 to generate the spiro-lactone moiety in preaustinoid A3 [Fig. 1(A)]. On the other hand, PrhA rearranges the B-ring to form the cycloheptadiene moiety in berkeleydione. Further, the binding mode of  $\alpha$ KG was also conserved.  $\alpha$ KG interacts with Arg72, Gln127, Thr167, and Arg226 of the enzymes with salt bridge and hydrogen bonding networks. On the other hand, a detailed comparison of the active

To clarify the structural basis of such functional differences between AusE and PrhA, and to engineer the catalytic potential of Fe(II)/ $\alpha$ KG dioxygenases, we first determined the crystal structures of AusE and PrhA. Two crystal structures of AusE complexed with Mn(II) and  $\alpha$ KG (AusE-Mn/ $\alpha$ KG), and structures of PrhA in apo-form, in complex with Fe(II),  $\alpha$ KG, and the substrate preaustinoid A1 (PrhA-Fe/ $\alpha$ KG/ preaustinoid A1) were solved at 2.1–2.8 Å resolutions [Fig. 2(A, B)]. The overall structures of AusE and PrhA have a double-stranded  $\beta$ -helix fold and form a funnel-like reaction chamber conserved in the jelly-roll barrel, which is found in typical non-heme iron dioxygenases.

The active sites of AusE and PrhA are almost identical with conserved 2-His-1-Asp catalytic triad to coordinate metal ions in the  $\alpha$ KG dioxygenase family. The metal ions Mn and Fe were coordinated His130-Asp132-His214 residues and  $\alpha$ KG in the structures of AusE-Mn and PrhA-Fe, respectively. Further, the binding mode of  $\alpha$ KG was also conserved.  $\alpha$ KG interacts with Arg72, Gln127, Thr167, and Arg226 of the enzymes with salt bridge and hydrogen bonding networks. On the other hand, a detailed comparison of the active

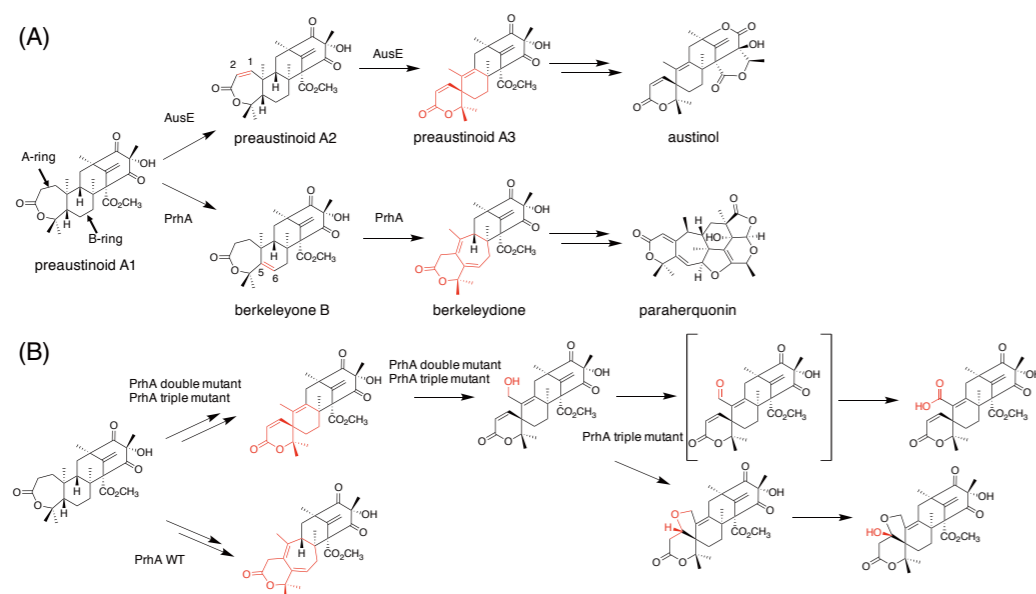


Figure 1: Reactions catalyzed by (A) AusE and PrhA, and (B) PrhA mutant dioxygenases from preaustinoid A1.

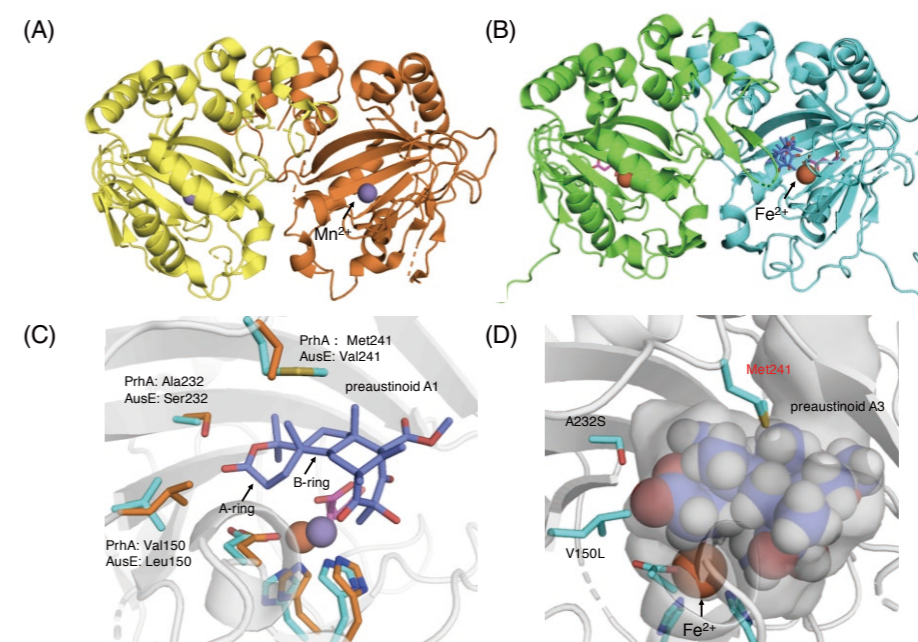


Figure 2: Structures of AusE and PrhA. (A, B) Overall structures of AusE (A) and PrhA (B). (C) The active sites of AusE-Mn/ $\alpha$ KG and PrhA-Fe/ $\alpha$ KG/ preaustinoid A1. (D) The active sites of PrhA-V150L/A232S variant-Fe/ $\alpha$ KG/ preaustinoid A3.

site architectures between AusE and PrhA revealed that almost all of the active site residues are conserved, while the residues of 150, 232, and 241 are substituted. AusE has Leu 150, Ser232, and Val241 at these moieties, while PrhA has Val150, Ala232, and Met241. Especially, the complex structure of PrhA-Fe/ $\alpha$ KG/ preaustinoid A1 suggested that two residues of PrhA (Val150 and Ala232) and AusE (Leu150 and Ser232) interact with the A-ring of preaustinoid A1 [Fig. 2(C)]. Therefore, we hypothesized that these substitutions are important for the functional difference between AusE and PrhA. To test this hypothesis, these residues in AusE and PrhA were replaced with respective residues in each other.

Surprisingly, a single mutant AusE-S232A showed both activities of “spiro-lactone formation” and PrhA-type “B-ring expansion” activity. Moreover, the double mutant of AusE-L150V/S232A was functionally converted to PrhA-type enzyme, which catalyzes only the “B-ring expansion” reaction instead of the AusE-type “spiro-lactone formation” reaction. Similarly, PrhA-V150L/A232S double mutant generated preaustinoid A2 through AusE-type oxidation and rearrangement reactions. Thus, we succeeded in complete interconversion of the function of the two dioxygenases by replacing only a couple of active site residues. Interestingly, careful analysis of enzyme reaction products further revealed that PrhA-V150L/A232S variant yielded two new oxidized products of preaustinoid A2 [Fig. 1(B)].

To understand the structural basis for the expanded catalytic functions of the PrhA mutants, we also solved the crystal structures of PrhA-V150L/A232S mutant in

complex with Fe(II),  $\alpha$ KG, and its substrates. The crystal structure of PrhA-V150L/A232S variant complexed with preaustinoid A3 suggested a potential for further engineering of the PrhA active site. The Met241 residue protrudes toward preaustinoid A3 and decreases the size of the active site [Fig. 2(D)]. Therefore, Met241 was substituted with valine residue to increase the volume of the active site. As a result, triple mutant catalyzes AusE-type “spiro-lactone formation” and further three rounds of oxidation reactions from preaustinoid A3.

In summary, we have succeeded in the structure-guided engineering of meroterpenoid dioxygenases AusE and PrhA [5]. These findings provide not only insight into the catalytic mechanisms but also strategies for expanding the catalytic potential of the enzymes to generate structurally divergent and biologically active novel meroterpenoids for drug discovery.

### REFERENCES

- [1] R. P. Hausinger, *Crit. Rev. Biochem. Mol. Biol.* **39**, 21 (2004).
- [2] Y. Matsuda and I. Abe, *Nat. Prod. Rep.* **33**, 26 (2016).
- [3] Y. Matsuda, T. Awakawa, T. Wakimoto and I. Abe, *J. Am. Chem. Soc.* **135**, 10962 (2013).
- [4] Y. Matsuda, T. Iwabuchi, T. Fujimoto, T. Awakawa, Y. Nakashima, T. Mori, H. Zhang, F. Hayashi, and I. Abe, *J. Am. Chem. Soc.* **138**, 12671 (2016).
- [5] Y. Nakashima, T. Mori, H. Nakamura, T. Awakawa, S. Hoshino, M. Senda, T. Senda and I. Abe, *Nat. Commun.* **9**, 104 (2018).

### BEAMLINES

BL-1A and BL-17A

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