Biosynthetic study and production of novel fungal meroterpenoids

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Report

Non-heme Fe(II)/α-ketoglutarate (α-KG)-dependent dioxygenases are widely distributed in nature and catalyze a variety of enzymatic transformations in both primary and secondary metabolism. In this study, we performed biochemical and structural studies on the unique meroterpenoid dioxygenase NvfE, which catalyzes the isomerization reaction without utilizing α-KG as a co-factor. Based on these results, we proposed a detailed mechanism for the NvfE-catalyzed orthoester formation reaction.

Research aims

Non-heme Fe(II)/α-ketoglutarate (α-KG)-dependent dioxygenases catalyze a wide range of oxidative reactions including hydroxylation, desaturation, and ring formation[1]. Recent studies on the biosynthesis of meroterpenoids from fungi have revealed that non-heme Fe(II)/α-KG-dependent dioxygenases are one of the key enzymes involved in the structural diversification and complications of many meroterpenoids[2]. This class of enzymes utilizes α-KG and O_2 as co-substrates to carry out a series of oxidative transformations.

Novofumigatonin is a heavily oxygenated fungal meroterpenoid from *Aspergillus novofumigatus* IBT 16806 (CBS117520), and possesses a unique orthoester group[3]. A biosynthetic study on novofumigatonin revealed that two α-KG-dependent dioxygenases, NvfI and NvfE, are involved in the formation of the orthoester group. Functional analysis of these enzymes revealed that NvfI catalyzes the endoperoxidation of asnovolin A to generate fumigatonoid A and NvfE catalyzes the endoperoxide isomerization of fumigatonoid A to produce an orthoester group containing fumigatonoid C. Interestingly, although NvfE shares sequence similarity with Fe(II)/α-KG-dependent dioxygenases, it catalyzes an unusual isomerization reaction in an α-KG-independent manner. However, the detailed mechanism behind this special enzyme is still unclear. A previous study suggested that NvfE might belong to a new class of enzymes that gained isomerization activity by losing α-KG binding ability.

To better understand the mechanism underlying the NvfE enzyme activity and the late-stage biosynthesis of novofumigatonin, we performed biochemical characterization and crystallization of NvfE. Structure-based enzymatic analysis and mutagenesis studies were then conducted based on the crystal structure and sequence comparison of NvfE with other characterized Fe (II)/α-KG dioxygenases.

Methods

NvfE was expressed in *Escherichia coli,* and the purified enzyme was used for enzyme reaction

and crystallization. A cubic crystal was obtained in 400 mM potassium phosphate dibasic, 1600 mM sodium phosphate monobasic, 100 mM sodium phosphate dibasic/citric acid pH4.2, and 8% Jeffamine®-M600 at 20°C, with 10 mg/mL of enzyme. From this condition, the crystal structure of apo-type NvfE with Fe(II) was successfully obtained at 1.85 Å resolution.

Results

First, to investigate the metal dependency of the enzyme, *in vitro* enzymatic reactions of wild-type NvfE were performed with fumigatonoid A and various metal ions, including Fe^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+}. The results indicated that Fe is essential for NvfE activity and cannot be replaced by other metal ions.

To understand the reaction mechanism, X-ray crystallography of NvfE was conducted. The overall crystal structure of NvfE possesses a double-stranded β-helix fold and forms a funnel-like reaction chamber conserved in a jelly roll barrel, as in the case of other Fe(II)/α-KG-dependent dioxygenases such as AndA (PDB ID 5ZM3), which catalyzes two-step desaturation and isomerization in the biosynthesis of anditomins[4]. NvfE exists as a homodimer and its dimer structure is constructed mainly through hydrogen-bonding interactions between the residues from each monomer. Comparison of the active site between NvfE and AndA revealed that the interaction of the conserved 2-His-1-Asp facial triad with iron in the active site is nearly identical in both enzymes.

The main differences between the apo-type NvfE crystal structure and the AndA in complex with α-KG and the substrate lie in the three loop regions. The length of loop A, between the Ser70-Cys83 region, is shorter in NvfE compared to that of AndA. Loop A is highly flexible and encapsulates the substrates in the active site of AndA. From the crystal structure of AndA, the density of loop A can only be clearly observed in the complex structure, which represents the closed conformation of AndA. In contrast, the density of loop A can be clearly observed in the apo-type crystal structure of NvfE, even without substrate binding. Interaction between Y78 and E149 fixes the conformation of loop A to form a closed conformation in the apo-type NvfE structure. Furthermore, loop B, between Pro288-Gly294 from the adjacent monomer is known to participate in interaction with the substrate. Loop B in NvfE is also shorter than that in AndA and other dioxygenases. In addition, there is a long loop C from the adjacent monomer located close to the active site in the NvfE crystal structure. Thus, these structural differences, especially the closed conformation of NvfE without the substrate, could be the reasons for the unique function of NvfE.

To further understand why NvfE does not require α-KG as a cofactor, we performed mutagenesis experiments. First, Y78A, Y78F, E149Q, and a double mutant, Y78F/E149Q, were constructed and analyzed using *in vitro* assays. Unexpectedly, these mutants showed only slightly reduced activity in comparison with the wild-type enzyme. Further, no new product or oxidation product was found in these mutants with or without α-KG. These results suggest that the interaction between Y78 and E149 as well as other interactions could be important for forming a closed conformation. Therefore, we also

constructed mutants of hydrophilic active site residues as D76A, D135A, S136A, S140A, S177A, Y226A, and E261A. All mutants demonstrated activities comparable to that of the wild-type enzyme. These results suggest that a single mutation is not significant for displaying the functional difference of NvfE, and that these active site residues are not essential for catalytic activity. Therefore, the function of the enzyme should be fixing the endoperoxide moiety of the substrate close to the iron center for cleavage and rearrangement of the endoperoxide linkage.

Based on these observations, we propose that NvfE catalyzes the orthoester formation reaction, which starts from the cleavage of endoperoxide that is mediated by iron. At the beginning, Fe(II) binds to the C13 oxygen, which leads to the cleavage of the peroxide bond, generating an oxygen-centered radical, which subsequently undergoes β-scission leading to the formation of a carbon-centered radical. Finally, this intermediate is oxidized by Fe(III) to generate carbocation, which is quenched by two rounds of heterocyclization to form fumigatonoid C.

Conclusion

Our biochemical and structural studies of the unique meroterpenoid isomerase NvfE have unveiled the detailed reaction mechanism of the orthoester formation reaction. To further understand the function of active site residues, we are now investigating double and triple mutants of NvfE as well as the loop engineering of this enzyme. We are also working on the random mutagenesis of NvfE to alter its substrate specificity, reactivity, and co-factor dependence. The engineered enzymes will be heterologously expressed in fungi harboring the novofumigatonin gene cluster enzymes, to construct a platform for the production of novel unnatural compounds.

References

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