Understanding the catalytic mechanism of flavin-containing dehydrogenases and their bioindustrial applications

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Research objective

Octopine and nopaline are synthesized in crown gall tumor tissues by NAD(P)H-dependent soluble dehydrogenases from pathogenic *Agrobacterium tumefaciens*, which catalyze the reductive condensation of pyruvate (for octopine) or α-ketoglutarate (for nopaline) with L-arginine. This bacterium possesses membrane-associated dehydrogenase(s), by which opines are utilized as selective growth substrates that favor their propagation. Therefore, it is possible that the characterization of this enzyme will lead to the generation of agricultural chemicals. Previously, I biochemically characterized homologous proteins from the non-pathogenic bacteria *Pseudomonas putida* KT2440 and *Bradyrhizobium japonicum* USDA110 at the first time. The novel opine dehydrogenase (Odh) belongs to a group of so-called "dye-linked dehydrogenases" that catalyze the oxidation of various organic acids, amino acids, and alcohols in the presence of an artificial electron acceptor, such as 2,6-dichloroindophenol (Cl2Ind), in which FAD and/or FMN are commonly contained as a prosthetic group(s). This enzyme has high potential for utilization as a specific element for electrochemical processing (e.g., in enzyme sensors and batteries) because electrons from the substrate can be introduced to an electrode using an artificial dye as the mediator. Based on these insights, I aimed to determine the crystal structure of putative flavin-dependent Odh from *Aureimonas altamirensis* by elucidating the catalytic mechanism at the atomic level.

Methods

1. Expression and purification of AaOdbB3 gene

The putative opine dehydrogenase gene from *A. altamirensis* (AUL31_RS11475; AaOdhB3) was cloned into a pACYC-Duet vector. Recombinant $(His)_6$-tagged AaOdhB3 protein was overexpressed by isopropyl-β-D-thiogalactopyranoside (IPTG) in *Escherichia coli* BL21(DE3) cells and purified using Ni-NTA affinity chromatography.

2. Functional characterization

To estimate potential opine dehydrogenase activity, AaOdhB3 was incubated with nopaline or octopine in the presence of *p*-iodonitrotetrazolium violet (INT) and phenazine methyl

sulfate (PMS) as an artificial electron acceptor and mediator, respectively. The yield of L-arginine was measured using an amino acid analyzer.

3. Preliminary crystallization

All crystallization trials were performed at 20 °C using the sitting-drop vapor-diffusion method. Drops (0.5 μL) of ~16 mg/ml AaOdhB3 protein were mixed with equal amounts of reservoir solution and equilibrated against 70 μL of the same reservoir solution by vapor diffusion. The initial trial was performed using Index HT and Crystal Screen (Hampton Research). The best crystal of AaOdhB3 was obtained with Index HT A4, C3, G2, and Crystal Screen HT B3, F10, G2, and H2 conditions.

4. Optimization of crystallization conditions

Among several crystallization conditions, Index HT G2 (0.2 M lithium sulfate monohydrate, 0.1 M BIS-TRIS (pH 5.5), 25% (w/v) PEG 3350) was further optimized.

5. Data collection of native crystals

Diffraction data of native crystals of AaOdhB3 were collected using the PILATUS 6M detector of BL45XU at SPring-8 (Hyogo, Japan). Although the resolution was significantly high (2Å>), it was unsuccessful in determining the structure, possibly due to the low sequence identity with the model structure for the molecular replication method.

6. Determination of the structure using Se-Met derivative protein

Alternatively, the crystal structure was elucidated by the single-wavelength anomalous dispersion (SAD) method using selenomethionine (SeMet)-substituted crystal and subsequently refined to a resolution of 1.5 Å against the data set from the native crystal.

7. Site-directed mutagenic analysis

Based on this structure, several alanine mutants of amino acids close to the putative active pocket were designed and their activity elucidated.

Results

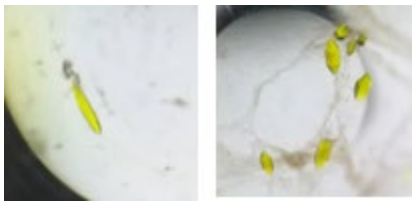

Figure 1. Crystals for native (left) and SeMet-substituted forms (right)

All crystals of AaOdhB3 were yellow, suggesting the binding of flavin nucleotides. The crystal structure was determined at a 1.5 Å resolution by the SAD method using a selenomethionine (SeMet)-substituted crystal. AaOdhB3 is a homodimer and each monomer contains a bidomain architecture composed of a FAD-binding domain and a

substrate-binding domain. The overall structural folding of AaOdhB3 was similar to those of flavin-dependent dehydrogenases (oxidases), including glycine oxidase, D-arginine dehydrogenase, and L-glutamate oxidase. On the other hand, there was low sequence similarity (~30%) between AaOdhB3 and them. FAD binds the enzyme tightly by interacting with Ile11, Trhr38, Ser39, Trp45, Val178, Val340, and Thr341 within the FAD-binding domain (Fig. 2, right panel). Among the two chains A and B, only chain A contained polyethylene glycol in the putative active pocket, derived from the crystallization conditions (Fig. 3). Superimposition of the two chains revealed that the side chains of Tyr57, His337, and Arg313 in chain A appeared to undergo significant appears conformational changes compared to chain B, and the loop between β3 and α6 was partially disordered.

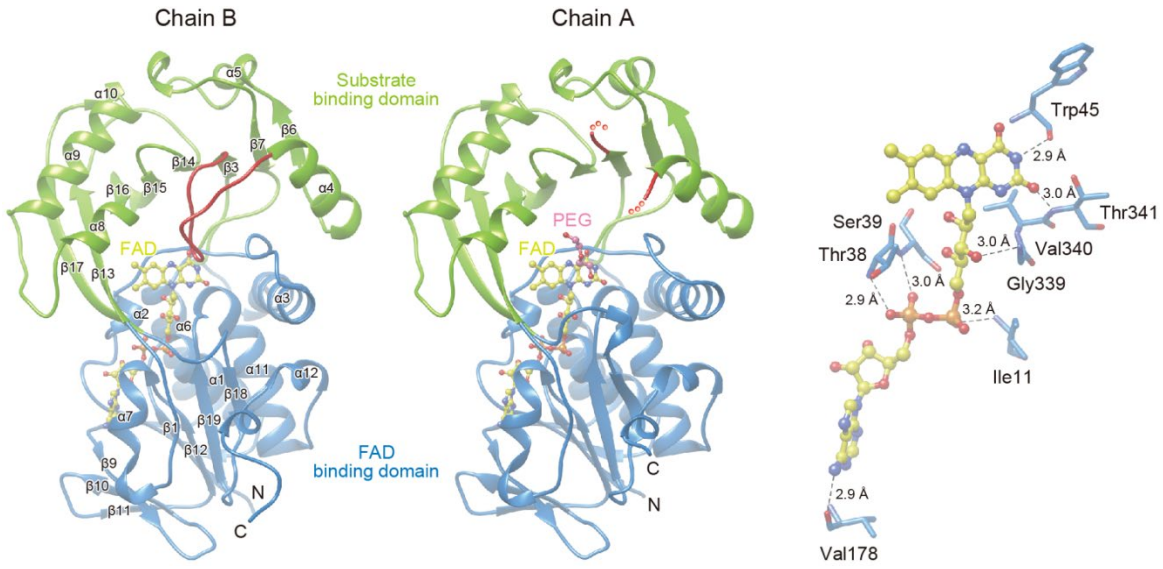

Figure 2. Overall structure (left) and FAD binding site (right)

Among the commercially available nopaline and octopine, AaOdhB3 only catalyzed the dehydrogenation reaction of nopaline to produce α-ketoglutarate and L-arginine. However, it was not possible to obtain crystals in complexes with these compounds. It is likely that the conformational difference between chains A and B partially mimics substrate binding. Therefore, we selected the 17 amino acid residues close to putative active site, which were substituted to alanine. Among them, W45A, R312A, H337A, S338A, and T341A were completely inactive, and E50A, Y57A, R247A, and P315A dramatically decreased the activity. Since a conformational change was observed in the above underlined residues, it is likely that some of them actually play a role as active sites. Furthermore, these residues are found only in AaOdhB3 but not in other enzymes belonging to the same protein superfamily, indicating the importance of these residues in recognizing opine compounds with complex structures.

Conclusion

In this study, the crystal structure of flavin-dependent opine dehydrogenase was determined for the first time. However, there are two possibilities for trace activity toward nopaline. First, nopaline is not the best substrate for AaOdhB3. Opine is synthesized by the reductive condensation of α-keto acid and amino acids. α-Ketoglutarate is necessary for AaOdhB3 as an α-keto acid, whereas L-arginine may be not usually best as an amino acid. Second, AaOdhB3 corresponds to a catalytic subunit of a heteromeric enzyme. I am currently working on elucidating these hypotheses.

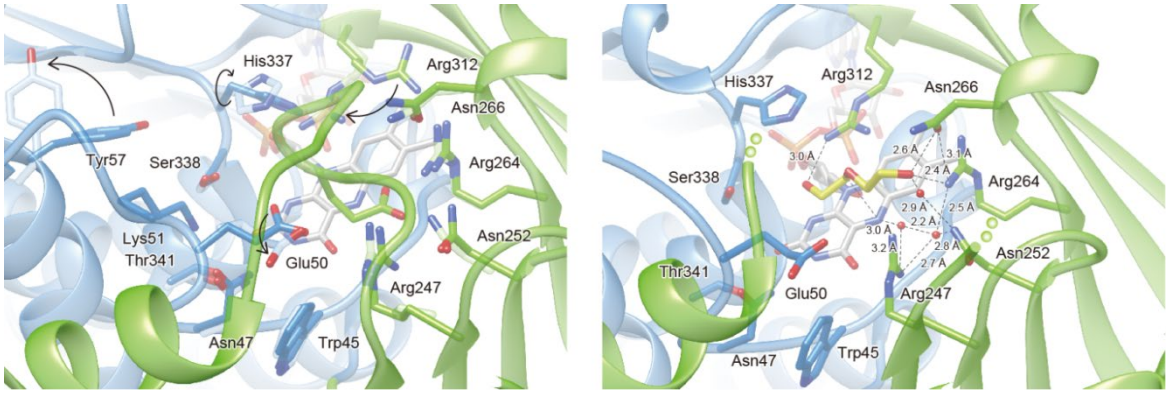

Figure 3. Conformational change between chains A and B (left) and PEG binding (right)

References

None.