

Structure of thermostable FMN-dependent NADH-indigo reductase

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1 Introduction

Japanese Aizome (indigo dyeing) is a traditional dyeing method in Japan. This dyeing method uses Sukumo made indigo leaves and performs fermentation reduction (Aidate fermentation). The indigo compound contained in Sukumo is insoluble in water and can not be used for dyeing. In indigo fermentation process, insoluble indigo (dark blue) is reduced by indigo reductase from thermophilic and alkalophilic *Bacillus* to change to water soluble leuco indigo (pale-yellow). Since, indigo is converted to soluble form by this enzymatic reaction, it can be used for dyeing. In our group, the microorganism was isolated from the polygonum alkaline liquor, and it was clarified that it was *Bacillus cohnii*, and the enzyme having indigo carmine reductase activity was partially purified. Since indigo is a insoluble compound that does not dissolve in water, the enzyme activity was measured using indigo carmine, which is a water-soluble indigo (model substrate). However, *B. cohnii* indigo reductase was very unstable, which halved active in 1 week and completely loses activity 10 min at temperatures up to 100°C (unpublished data). Due to instability, we could not analyze the function and structure of this enzyme. Recently, it has been reported that the identity of the indigo reductase from *Bacillus* sp. is a flavin mononucleotide (FMN)-dependent NADH-azoreductase. FMN-dependent NADH-azoreductase (EC 1.7.1.6) catalyzes the reductive cleavage of azo groups (-N=N-) in aromatic azo compounds, and also the enzyme can also use indigo compounds (not containing azo group) as substrates (Fig. 1). Requires NADH, but not NADPH, as an electron donor for its activity. To date, many azoreductases from several *Bacillus* species have been isolated and studied as enzymes involved in the azo dye biodegradation or decolorization. However, the enzymatic properties and physiological roles of azoreductases are barely understood. Therefore, we searched for highly stable indigo reductase (azoreductase) from thermophilic *Bacillus* using genomic information and found *B. smithii* type strain DSM 4216 (wide range of growth temperature 25-65°C) indigo reductase gene [1]. In this study, we performed cloning, expression and enzymological characterization of thermostable indigo reductase from thermophilic *Bacillus*. We also succeeded structural analysis of *B. smithii* indigo reductase and finding important amino acid residue related to the thermostability. The enzymological characterization and 3D structural information of thermostable

indigo reductase has significant potential application for the indigo dyeing (Aizome).

2 Experiment

Single-wavelength (1.0 Å) data for *B. smithii* indigo reductase was collected on the beamline AR-NE3A and AR-NW12A at the Photon Factory. The data were processed using HKL2000 and the CCP4 program suite.

3 Results and Discussion

Within the cofactor binding site of *B. smithii* indigo reductase, a clear electron density corresponding to the bound FMN was observed (Fig. 2) [1]. The phosphate group interact with the side chains of H10, S17 and S19 and with the main chain nitrogen of Y18. The OH groups of the ribityl chain form hydrogen bonds with the side chains of Q186 and N187 and the main chain nitrogen of G148. The O2 atom of the flavin ring interacts with the side chain of Y151 and the main chain nitrogen of G149, although the N3 and O4 atoms are hydrogen bonded to the side chain of Y151 and the main chain nitrogen of F105. The N1 and N5 atoms are hydrogen bonds with the main chain nitrogen atoms of G148 and N104, respectively. In addition, the isoalloxazine ring of FMN was covered with hydrophobic residues such as W103 and M102. The active site cavity of the *B. smithii* indigo reductase was formed by the residues N104, F105, Y127', Y172', F125', A119', I52', W60' and F57', and two water molecules (the prime indicates the neighboring subunit in the dimer) (Fig. 3). CHES, which is a buffer for crystallization, was bound in strongly hydrophobic pocket and was oriented to the *si*-face of the FMN, forming hydrophobic interactions. The sulfo group of the CHES molecule form hydrogen bonds with the side chains of N104 and the main chain oxygen of A119', and two water molecules (Fig. 3). The nitrogen atom of the CHES also forms a hydrogen bond to a N5 atom of the isoalloxazine ring moiety of the FMN. It has already been reported that Y151 residue of *Bacillus* sp. B29 azoreductase was not key residues in the electron-transfer mechanism and Y151 residue is to stabilize the binding of FMN by hydrogen bonding. In addition, substrate azo group was sandwiched and stabilized by π -stacking with Y127 of *Bacillus* sp. B29 azoreductase and FMN. The role of the amino residues of Y151 and Y127 in *B. smithii* indigo reductase seems to be the same as the *Bacillus* sp. B29 azoreductase.

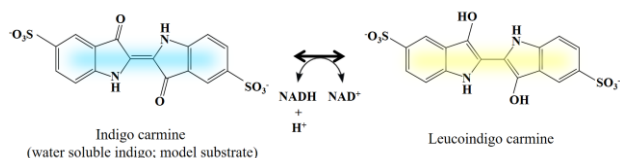


Fig. 1: Reaction scheme for indigo carmine reduction catalyzed by *B. smithii* indigo reductase.

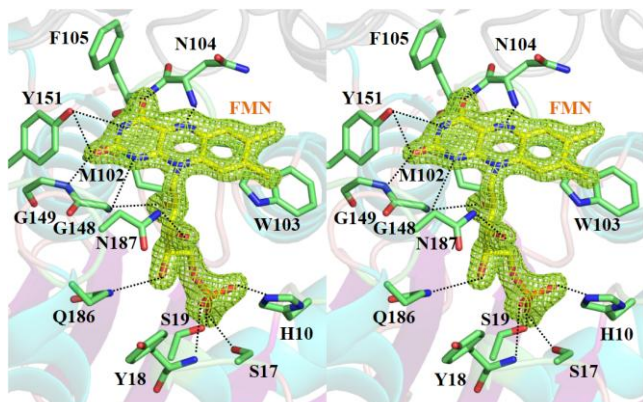


Fig. 2: Wall-eyed stereo view of the bound FMN molecule.

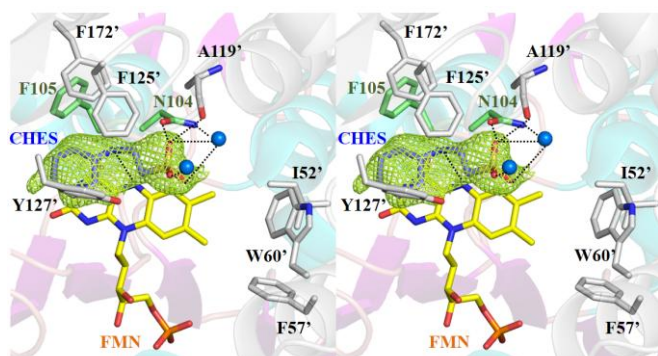


Fig. 3: Wall-eyed stereo view of the substrate binding site of *B. smithii* indigo reductase.

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Reference

[1] K. Yoneda *et al.*, International Journal of Biological Macromolecules, 164 (2020) 3259-3267. IF=5.16

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