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Mutation network-based understanding of pleiotropic and epistatic mutational behavior of *Enterococcus faecalis* FMN-dependent azoreductase

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ABSTRACT

We previously identified a highly active homodimeric FMN-dependent NADH-preferred azoreductase (AzoA) from *Enterococcus faecalis*, which cleaves the azo bonds (R-N=N-R) of diverse azo dyes, and determined its crystal structure. The preliminary network-based mutational analysis suggested that the two residues, Arg-21 and Asn-121, have an apparent mutational potential for fine-tuning of AzoA, based on their beneficial pleiotropic feedbacks. However, epistasis between the two promising mutational spots in AzoA has not been obtained in terms of substrate binding and azoreductase activity. In this study, we further quantified, visualized, and described the pleiotropic and/or epistatic behavior of six single or double mutations at the positions, Arg-21 and Asn-121, as a further research endeavor for beneficial fine-tuning of AzoA. Based on this network-based mutational analysis, we showed that pleiotropy and epistasis are common, sensitive, and complex mutational behaviors, depending mainly on the structural and functional responsibility and the physicochemical properties of the residue(s) in AzoA.

1. Introduction

Azo dyes, characterized by the presence of one or more azo groups, are the largest and most versatile dye class [1–4]. Since an azo group in a natural product is rare [1] and industrially produced azo dyes usually resist biodegradation in conventional aerobic sewage treatment plants, they are considered persistent pollutants [2,5]. Azoreductase, widely distributed in the bacterial world, is a key enzyme responsible for biodegradation of azo dyes by breaking of the nitrogen double bonds (-N = N-) to generate aromatic amines via hydrazo intermediates [6–15]. Studying the molecular mechanism of azoreductase might lead to better understand toxicity, mutagenicity and carcinogenicity of the azo dyes and the development of effective bioremediation method to degrade azo dyes in environment.

AzoA from *Enterococcus faecalis* is a typical group I azoreductase, the polymeric flavin-dependent NADH-preferred azoreductases [16]. In our previous synergetic studies based on three-dimensional structure, computational analysis, and reverse genetics coupled with site-directed mutagenesis deciphered the binding site and binding mode of the substrates, FMN, NADH and a model azo dye, methyl red, and the catalytic mechanism of AzoA [6,7,17]. As shown in Fig. 1, AzoA is homodimeric with two separate active sites at the interface between the two monomers, and the FMN lies inside each active site [17]. FMN, stabilized by 22 amino acid residues, transfers four electrons from NADH to the azo dye substrate, resulting in the degradation of the azo dye substrate [6]. In AzoA, the substrates NADH and methyl red are stabilized by about 12 and 19 amino acid residues, respectively, and lie against the flavin isoalloxazine ring of FMN at angles of 45° and 35°, respectively. Conclusively, the sequential functional interaction of three substrates, FMN, NADH, and methyl red is essential for successful biodegradation of azo dyes via azo reduction (Fig. 1).

Pleiotropy (mutations at loci that affect more than one trait) and epistasis (interaction between mutations) have been recognized to play a prominent role in enzyme engineering in the laboratory (i.e., directed evolution, semi-rational or rational design) and in natural enzyme evolution [18–20]. In our previous study, pleiotropy and epistasis were used to analyze the interaction between AzoA and its substrates. The phenotypic feedbacks of 13 single mutations (e.g., substrate binding and azoreductase activity) were mapped to a mutation network [7]. The network-based mutational analysis suggested that the two residues,

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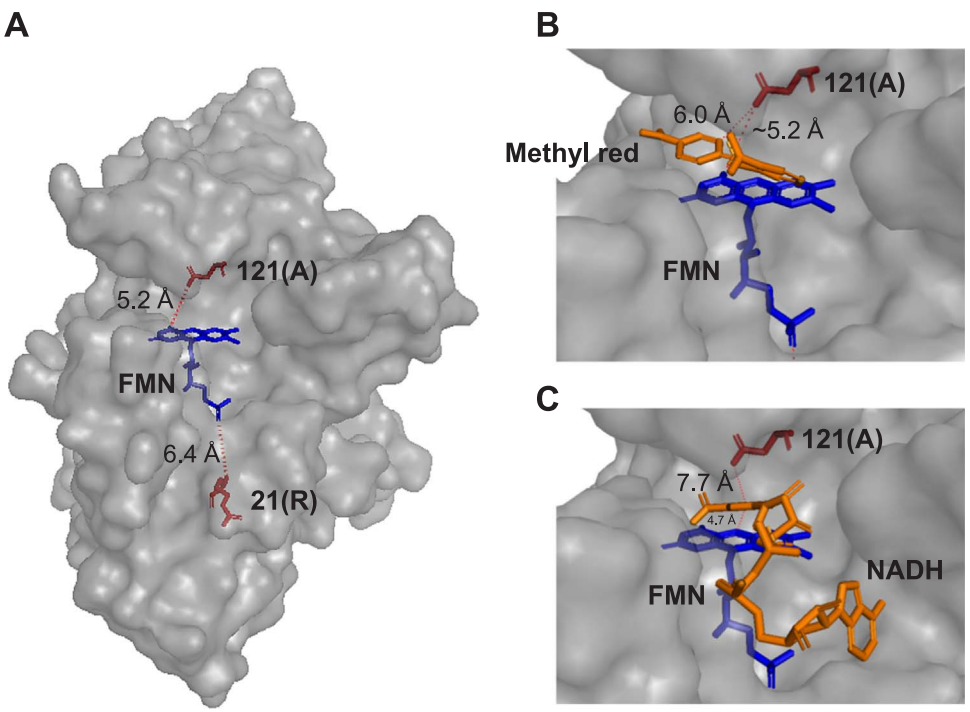

Fig. 1. Structure and active site stereochemistry of AzoA with the substrates. (A) Surface structure of AzoA with FMN, in which the target residues (Arg-21 and Asn-121) for mutation are colored (red). (B and C) Surface diagram of the active site with FMN and the stick representation of the bound substrates methyl red (B) and NADH (C).

Arg-21 and Asn-121, have an apparent mutational potential for fine-tuning of AzoA, based on their beneficial pleiotropic feedbacks, i.e., enhancing ligand binding affinity and azoreductase activity. However, epistasis between the two promising mutational spots in AzoA has not been obtained in terms of substrate binding and azoreductase activity. In this study, we further quantified, visualized, and described the pleiotropic and/or epistatic behavior of six single or double mutations at the positions, Arg-21 and Asn-121, as a further research endeavor for beneficial fine-tuning of AzoA.

2. Materials and methods

2.1. Materials

Methyl red, NADH, FMN, dimethyl sulfoxide (DMSO) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich. *Escherichia coli* BL21-Gold (DE3) pLysS and pET11a (Stratagene) were used as the host and expression vectors, respectively. Luria-Bertani liquid medium with chloramphenicol (50 μg/ml) and ampicillin (50 μg/ml) was used for cultivation of recombinant *E. coli*.

2.2. Site-directed mutagenesis of azoreductase

The original construct, wild type pAzoA [16], was used as the template for site-directed mutagenesis using the QuikChange II XL site-directed mutagenesis kit (Stratagene). The primers with the corresponding mutations were used in the PCR reactions (Table 1). PCR reactions were performed in a Mastercycler gradient (Eppendorf). The amplification conditions were one cycle of 95 °C for 2 min, 20 cycles with 50 s of melting at 95 °C, 50 s of annealing at 60 °C, and 7 min of extension at 68 °C for each cycle, and one final extension cycle at 68 °C for 10 min. The parental plasmids were digested using *Dpn*I at 37 °C for 1 h. Then the DNA was transformed to XL-10Gold competent cells. The plasmids with the desired mutations were extracted using QIAprep Spin Miniprep Kit (Qiagen) and confirmed by sequencing. All the mutant plasmids were used to transform to competent *E. coli* BL21-Gold (DE3) pLysS cells.

Table 1
Primers used for site-directed mutagenesis of *E. faecalis* AzoA.

Mutant	Primer Sequences (5′→3′)
R21G	TCACGCTCAGTTGGTGCGTTAGAAACAT
	ATGTTTCTAACGCACCAACTGAGCGTGA
N121A	GTAGATACAATCGCAGTTGCTGGAAAAAC
	GTTTTTCCAGCAACTGCGATTGTATCTAC
N121Q	GTAGATACAATCCAAGTTGCTGGAAAAAC
	GTTTTTCCAGCAACTTGGATTGTATCTAC
R21K	TCACGCTCAGTTAAAGCGTTAGAAACAT
	ATGTTTCTAACGCTTTAACTGAGCGTGA

Underlined nucleotides indicate mutations incorporated into primers.

2.3. Recombinant protein expression and purification

The mutant azoreductases were expressed in *E. coli* as described previously [6]. Cells were harvested by centrifugation at 4000 × *g* for 15 min and resuspended in 20 ml potassium phosphate buffer (25 mM, pH 7.1). The cells were disrupted by sonication for 5 min at 0 °C with a Vibra-Cell VCX 400 sonifier (Sonics and Materials). The cell debris was removed by centrifugation at 12,000 × *g* for 20 min and ammonium sulfate was added to the collected supernatants to a final concentration of 0.5 M. Then the mixture was centrifuged and filtered for purification. The azoreductase samples were purified as described previously [7].

2.4. Azoreductase activity assays

Azoreductase activity assays were performed as described previously [6]. The activities of AzoA wild type and mutants were analyzed by measuring the reduction of methyl red at 430 nm at room temperature. The reaction mixture was 1956 μl potassium phosphate buffer (pH 7.1), 2 μl methyl red (25 μM), 20 μl NADH (0.1 mM), 2 μl FMN (0.5 μM), and 20 μl azoreductase. The amount of azoreductase needed for the reduction of 1 μmol methyl red per minute was defined as one unit of activity. Specific azoreductase activity is defined as units of activity per mg protein. The protein was quantified using the Bicinchoninic acid assay (Pierce) with bovine serum albumin (BSA) as the standard. SDS-PAGE was carried out using precast gels (12%, Bio-Rad).