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Probing the NADH- and Methyl Red-binding site of a FMN-dependent azoreductase (AzoA) from *Enterococcus faecalis*

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Abstract

AzoA from *Enterococcus faecalis* is a member of the polymeric flavin-dependent NADH-preferred azoreductase group. Little is known about the binding and interaction of NADH and azo dye in the azoreductase group. A synergetic strategy based on computational prediction, reverse genetics validation coupled with site-directed mutagenesis, and reconstruction of mutation network was used to investigate the binding and interaction of NADH and a model azo dye, Methyl Red, with AzoA. Methyl Red and NADH interacted in a unique binding mode in which the benzoic acid moiety of Methyl Red and the nicotinamide ring of NADH were not parallel to the flavin isoalloxazine ring, but lay against it at angles of $\sim 45^\circ$ and $\sim 35^\circ$, respectively. The adenine ribose moiety of NADH was surrounded by loop D on chain B and $\alpha 3$ on chain A in a typical Rossmann fold. There were 12 and 19 amino acid residues that could participate in the binding of Methyl Red and NADH, respectively, especially the residues Tyr-129 and Asp-184. The functional perturbation effects of 13 residues, including Tyr-129 and Asp-184, were mapped to reconstruct the mutation network, which confirmed the proposed binding modes and also provided insights into the interaction among NADH, FMN and Methyl Red.

Keywords

Enterococcus faecalis; Azoreductase; Azo dye; NADH; Site-directed mutagenesis

Introduction

Thousands of azo dyes, characterized by one or more $R_1-N=N-R_2$ bonds, are used in various consumer products including food, cosmetics, pharmaceuticals, textiles, and paper [1–4]. The human intestinal microbiota is composed of several thousand species of bacteria [5–7]. They play a crucial role in the metabolism of ingested azo dyes, with reduction of the azo dyes being the most important reaction related to the toxicity and mutagenicity of these

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dyes [8–11]. Azoreductase, which is widely distributed in the microbiota, is involved in the reductive cleavage of azo dyes [1,2,12].

The azoreductase family is made up of enzymes with diverse structure, function, and evolution that catalyze an NAD(P)H-dependent reductive cleavage of the azo bond of azo dyes [13]. Based on structure, flavin dependency, and dinucleotide preference, three major groups of azoreductases have been classified; (i) the polymeric flavin-dependent NADH-preferred azoreductases (Group I); (ii) the polymeric flavin-dependent NADPH-preferred azoreductases (Group II); and (iii) the monomeric flavin-free NADPH-preferred azoreductases (Group III). Azoreductases of Group I and II share structural and functional features in both NAD(P)H and substrate (azo dye) binding for the sequential transfer of four electrons via flavin (FMN or FAD) [14–17]. On the other hand, flavin-free azoreductases of Group III have an active site, enabling them to simultaneously accept both NAD(P)H and substrate in the catalytic cycle [13,18].

AzoA from *Enterococcus faecalis*, a typical Group I azoreductase, is one of the most active azoreductases of the human intestinal bacteria [14,15,19]. Since its ability to cleave azo dyes will depend on the chemical nature and size of the dye, the DNA sequence and 3-D structure (PDB 2hvp) of AzoA have been extensively studied [14,15,19]. The homodimeric AzoA has two separate active sites located at the interface between the two monomers, and FMN lies inside each active site. More than twenty amino acid residues contribute to the FMN binding, especially Trp-105, which participates in both stabilization and destabilization [19]. The *si* face of the isoalloxazine ring probably provides room for binding NADH and azo dye substrates such as Methyl Red, Orange II, Ponceau BS, and Ponceau S [14]. Despite the structure-based biochemistry of AzoA, which is consistent with its broad substrate specificity, FMN-dependency, and dimerization; co-crystallization of the enzyme with NADH and Methyl Red has not been successful and little information on the binding of NADH and the substrate is available. Because Group I azoreductases, including AzoA, lack a conserved characteristic motif and because very limited information on the binding of the nucleotide of the enzymes is available, they pose a challenge for finding binding site(s) and binding mode(s), which are essential for full understanding the enzyme kinetics and mechanism [14,15].

In this study, we aimed to better understand the binding and interaction of Methyl Red and NADH to AzoA by systemically integrating and interpreting *in silico* prediction and experimental validation studies using site-directed mutagenesis (Fig. 1). Secondly, we reconstructed the mutation network of AzoA, based mainly on the perturbation effects of the selected individual residues in the active site. Thirdly, the resulting mutation network enabled us to integrate disparate observations to draw conclusions for a global understanding of the perturbation effects in the AzoA system.

Materials and methods

Materials

Methyl Red, NADH, FMN, dimethylsulfoxide² (DMSO), and iso-propyl β -D-1-thiogalactopyranoside (IPTG) were obtained from Sigma-Aldrich. *Escherichia coli* BL21-

Gold (DE3) pLysS and pET-11a (Stratagene) were used as the host and expression vector, respectively. Luria-Bertani medium was used for the cultivation of recombinant *E. coli* with chloramphenicol and ampicillin (50 µg/ml of each antibiotic), and IPTG at 1 mM. All other reagents were reagent grade and high purity.

Site-directed mutagenesis of azoreductase

The original construct pAZOA [14] was used as the template in reactions of site-directed mutagenesis by using the QuikChange II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. In brief, the primers containing the corresponding mutations (Table 1) were used in the PCR reactions. PCR was performed in a Mastercycler gradient (Eppendorf). The amplification conditions were one cycle of 95 °C for 2 min, 20 cycles with each cycle including 50 s of melting at 95 °C, 50 s of annealing at 60 °C, and 7 min of extension at 68 °C, and one final extension cycle at 68 °C for 10 min. DpnI was used at 37 °C for 1 h to digest the parental plasmids and then the DNA was transformed to XL10-Gold competent cells. The plasmids containing the corresponding mutations were extracted by the QIAprep Spin Miniprep Kit (Qiagen) and confirmed by sequencing on a model 3100 Genetic Analyzer (Applied Biosystems) using T7 promoter and terminator primers. All of the mutant plasmids were used to transform competent *E. coli* BL21-Gold (DE3) pLysS cells.

Recombinant protein expression and purification

The mutant azoreductases were expressed in *E. coli* as described previously [19]. Cells were harvested by centrifugation at 4000g for 15 min, washed twice with 20 ml Tris-HCl buffer (25 mM, pH7.5) and resuspended in 20 ml of the same buffer. 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 12,000g for 20 min and the supernatants were carefully collected and ready for the protein purification steps. Ammonium sulfate was added to the crude enzyme to a final concentration of 0.5 M. The mixture was stirred for 1 h on ice before being centrifuged and filtered. The filtered enzyme sample was applied to a HiPrep 16/10 phenyl FF column (GE Healthcare). The column had been equilibrated with Tris-HCl buffer (25 mM, pH7.5) containing 0.5 M ammonium sulfate and then the column was washed by 3 column volumes of 0.5 M (NH₄)₂SO₄ to remove the unbound proteins, the azoreductase was eluted by three column volumes of 0.05 M (NH₄)₂SO₄. All of the active fractions were collected, concentrated and diluted by 25 mM Tris-HCl buffer (pH 7.5). Next, the concentrated sample was applied to a Mono Q HR 5/5 column (GE Healthcare) equilibrated with 25 mM Tris-HCl buffer (pH 7.5). The column was washed with 2 ml of the starting buffer and was eluted with 10 ml of a NaCl gradient from 0 to 1 M in the same buffer. Final purification of the enzymes was by gel filtration on a HiLoad 16/60 Superdex 75 column (GE Healthcare) run with 25 mM potassium phosphate buffer (pH 7) containing 0.1 M NaCl and 1 mM FMN. All of the active fractions were collected. The access FMN in the liquid of the purified proteins was removed by quickly passing the samples through a

²Abbreviations used: DMSO, dimethylsulfoxide; IPTG, isopropyl β-D-1-thiogalacto-pyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LPD, Ligand-Protein Contacts; RMSD, Root-Mean-Square-Deviation; AMP, adenine monophosphate; NMN, nicotinamide mononucleotide; NAD, nicotinamide adenine dinucleotide.

PD-10 desalting column. The purification steps were performed at 4 °C and all of the chromatography steps were performed with an AKTA purifier 10 system with UNICORN 4.10 software (GE Healthcare). The FMN content of each purified mutant protein was measured using a Beckman Coulter DV 800 spectrophotometer and by comparing with that of the wild type as described in our previous work. The oligomerization state of the protein was determined by gel filtration chromatography on a HiLoad 16/60 Superdex 75 column. BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) (Amersham Biosciences) were used as the standards [14].

Azoreductase assays

Azoreductase activity assays and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were carried out as described before [14,19]. In brief, the activities of AzoA and of the mutants were assayed by measuring the reduction of Methyl Red at 430 nm at room temperature ($\epsilon_{430} = 23.36 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture was in 2 ml potassium phosphate buffer (pH 7), containing 25 μM Methyl Red, 0.1 mM NADH, 0.5 μM FMN, and the enzyme. One unit of activity was defined as the amount of enzyme needed for the reduction of 1 μmol Methyl Red per minute. Bicinchoninic acid assay (Pierce) was used to quantify the proteins with bovine serum albumin (BSA) as the standard. SDS-PAGE was carried out with 12.5% polyacrylamide in Laemmli's buffer [20]. Prestained protein markers (Bio-Rad) were used, and the gel was stained by Coomassie brilliant blue R-250 (Bio-Rad).

Kinetic parameters of the mutant azoreductase

Initial velocities of the enzymatic reaction were performed by varying the concentrations of one substrate, Methyl Red (from 1.1 to 45 μM) or NADH (from 0.07 to 2.2 mM), while the concentration of the other substrate was kept constant (NADH: 1 mM or Methyl Red: 30 μM). Apparent K_m and $V_{\max}$ values were obtained from Lineweaver-Burk plots.

In silico analysis

SuperPose (version 1.0) was used for structural superposition and RMSD (Root-Mean-Square-Deviation) calculation [21]. PyMOL (0.99RC6) (<http://www.pymol.org/>) was used to visualize all structure figures in this paper. The protein-FMN-NADH interactions were verified and plotted using the Ligand Protein Contacts (LPC) online server (<http://bip.weizmann.ac.il/oca-bin/lpcsu>) and Lig-Plot⁺ [22]. To align the wild-type and mutant AzoA with the related proteins of known 3D structure in the PDB (Protein Data Bank), SAS (<http://www.ebi.ac.uk/thornton-srv/databases/sas/>) was used. Automated docking of Methyl Red and NADH to AzoA was performed using AutoDock vina [23]. The grid box (Center_x = 50.28, Center_y = 44.073, Center_z = 32.404) was centered in the catalytic active region (size_x = 28, size_y = 28, and size_z = 28). The maximum number of binding modes to generate was 1000 for all docking analysis.

Results

Experimental strategy

Fig. 1 outlines the experimental strategy to better understand the ligand binding and interaction of FMN, NADH and Methyl Red in AzoA; Step 1: prediction of the feasible

binding site(s) and mode(s) of Methyl Red and NADH (Fig. 2 and Fig. 3); Step 2: reverse genetic validation of the prediction by site-directed mutagenesis (Fig. 4); and Step 3: reconstruction of mutation network based on the kinetic parameters of mutants (Fig. 5).

Step 1: Prediction of NADH and Methyl Red binding in AzoA

A top-down docking using NADH and a bottom-up docking with the moieties of adenine monophosphate (AMP) and nicotinamide mononucleotide (NMN) of NADH were conducted for a more reasonable prediction of NADH in AzoA, since the flexibility of NADH with 13 rotatable bonds allows more docking reliability of rigid molecules than of flexible ones (Fig. 1). Using Methyl Red as a model azo dye compound, a top-down docking approach was conducted (Fig. 1). Initial attempts to choose the functional binding modes (in a productive manner) were guided by the reaction distance ($<5 \text{ \AA}$) from N5 of FMN, forming an electron transfer-competent complex. For AzoA and Methyl Red docking experiment, we prepared the input file for the enzyme AzoA with oxidized FMN by adding only polar hydrogens. In this study, the experimentally determined K_m values were used as an estimate of substrate binding affinity in docking studies [24–27]. Note that, as mentioned below, the calculated binding energies of the most stable productive structures (Methyl Red and NADH) bound to AzoA were consistent with the experimental K_m values for the respective substrates.

Methyl Red binding

The AutoDock docking simulation of Methyl Red to AzoA proposed 575 possible binding solutions. Among them, three conformations satisfy the distance to accept electrons from FMN, in which the reaction center ($-\text{N}=\text{N}-$) of Methyl Red lies within less than $\sim 5 \text{ \AA}$ from N5 of FMN, an appropriate position for direct hydride transfer. Among the three functional binding modes, the two binding modes with the lowest binding energy (-6.1 kcal/mol) in the dimeric active site of AzoA show very similar conformations (Fig. 2A and B), while the third shows a conformation in the opposite direction with a binding energy of 6.0 kcal/mol (data not shown). However, interestingly, all of the aromatic benzoic acid moieties in the three functional binding modes lie between the side chains of Tyr-129 and Asp-184 on the *si* face of isoalloxazine ring of FMN at angles of similar degree ($\sim 30^\circ$ to $\sim 45^\circ$).

Fig. 2B shows a functional binding mode of Methyl Red with the lowest binding energy of -6.1 kcal/mol . In the binding site of Methyl Red, which is generally cationic (Fig. 2B), the anthranilic acid ring sits in a parallel mode on the *si* face of the isoalloxazine ring of FMN. Whereas, interestingly, the aromatic benzoic acid moiety lies between the side chains of Tyr-129 and Asp-184 on the *si* face of isoalloxazine ring of FMN at an angle of $\sim 45^\circ$, placing the substrate in an ideal position for electron acceptance. The binding of the two aromatic moieties of Methyl Red led to conditions for an efficient direct hydride transfer, in which the azo group is placed just $\sim 4.69 \text{ \AA}$ from the N5 of the isoalloxazine ring (Fig. 2C). Methyl Red-AzoA interaction analysis revealed that the bound Methyl Red is stabilized by about 12 residues (6 in chain A and 6 in chain B), which form an interaction network, including hydrogen bonds and hydrophobic interactions (Fig. 3A and Table 2).

NADH docking

In the top-down docking approach using NADH, 1000 possible binding conformations of NADH to AzoA (2HPV) were generated. Among them, twenty one solutions satisfy the distance requirement to transfer the hydride, in which the nicotinamide ring is positioned within less than ~ 5 Å from N5 of FMN, with a binding energy of ~ -5.7 kcal/mol. Fig. 2D shows a functional arrangement of NADH in the active site, keeping a distance of < 5 Å between the NADH C4 and N5 of FMN. In the bottom-up docking with the NMN moiety, several binding modes satisfy the functional distance for electron transfer and can also be superimposed on the productive binding solution of top-down binding. In the AMP moiety docking analysis, although dispersed binding positions are shown, several solutions are superimposed on the productive binding solution of top-down docking (data not shown).

As shown in Fig. 2A, the binding site is fully formed only in the dimer, and about 19 amino acid residues could contribute to stabilization of the NADH. As shown in Fig. 2D, the nicotinamide ring is packed with a 35° angle against the isoalloxazine ring of FMN with a distance of 4.68 Å between the C4 of NADH and the N5 of FMN. In the LPC analysis (Fig. 3B), the 7 residues Arg-66(A), Ala-123(A), Phe-127(A), Tyr-129(A), Arg-18(B), Asp-184(B), and His-185(B), together with FMN were indicated to contact the moiety. Interestingly, as revealed in binding of Methyl Red, the stacking system of Tyr-129 and Asp-184 on the isoalloxazine ring of FMN seems to be responsible for keeping the nicotinamide ring within a direct reactive distance from the N5 of FMN. The pyrophosphate moiety could interact with the side chain atoms of the four residues Arg-66(A), Arg-18(B), Ile-183(B), and Pro-187(B). Although, in the arrangement, the negatively charged pyrophosphate moiety of the bound NADH is positioned near the phosphate moiety of FMN, because of the interaction of the positively charged side chain of Arg-18 with the phosphates, there seems to be no interaction with the negative charges on the phosphate of FMN. The adenine ribose moiety is surrounded by loop 2 on chain B (residues 10–16 between $\beta 1$ and $\alpha 1$) and $\alpha 3$ on chain A. The residues Leu-59(A), Ser-60(A), Gly-63(A), and Arg-66(A) of $\alpha 3$ and Leu-12(B), Glu-15(B), and Glu-16(B) of loop 2 appear to contribute to the adenine ring binding. As shown in Fig. 2, the active site pocket containing the bound FMN is Y-shaped. The hydrophobic pocket on the isoalloxazine is open toward the solvent regions, allowing access to the substrate binding pocket via three openings.

Step 2: Reverse genetic validation of the proposed complexes

For validation of the proposed functional binding of the NADH and Methyl Red, a reverse genetic approach based on site-directed mutagenesis has been adopted to look for the effect of such alterations in the AzoA enzyme system. We selected 13 functionally important amino acids based on the proposed ligand-protein complex structures (Fig. 2B and C), individually replaced them by different amino acids as described below, and analyzed their biochemical effects with the hierarchical viewpoint from primary to quaternary structure (Table 2). Acidic and polar amino acids Glu-16 (B), Asp-184 (B) were replaced with neutral and non-polar amino acid Gly; basic and polar amino acids Arg-18 (B), Arg-21 (B), Arg-66 (A) were replaced by non-polar amino acids Gly or Ala; long aliphatic amino acid Leu-59 (A) was replaced by short aliphatic amino acid Gly; polar amino acids Asn-106 (B), Asn-121 (A) were replaced by non-polar amino acid Ala; aromatic amino acids Trp-62 (A),

Phe-127 (A), and Tyr-129 (A) were replaced by aliphatic amino acids Ala or Gly; aliphatic amino acids Val-122 (A) and Ala-123 (A) were replaced by aromatic amino acids Tyr or Phe (Table 3). Especially, the four mutations (Arg-21, Trp-62, Asn-106 and Val-122) that are not in contact with Methyl Red and NADH were included to obtain some degree of indirect mutation effects which is critical for evaluation and understanding of the mutation effects in terms of the ligand binding, enzyme activity and their connection.

Overexpression and purification of the mutant proteins

Thirteen residues, which cover both ligand binding motifs, were substituted by site-directed mutagenesis and all of the mutants were overexpressed in *E. coli*. The levels of the expressed protein produced for the wild type and each mutant were similar (about 13% of the protein present in the crude cell extract). No inclusion body of the expressed protein was found in any sample. As shown in Fig. 4, a total of 14 enzymes, including the wild-type, were purified to homogeneity for more detailed biochemistry study. The yields at the end of the purification of the wild-type and mutants were about 11%. Overall, most of the purified mutant enzymes showed azo dye reduction activities. The activity of 3 mutants, F127G, Y129G and D184G, was lost completely (Table 3).

Kinetic characterization the mutant proteins

Table 3 shows the comparison of the kinetic parameters of the wild-type and mutated enzymes. The K_m values for NADH of the E16G, R21G and N121A mutants were 250, 380, and 292 μM , respectively, and were similar to that of the wild-type (143 μM). The azoreductase specific activities for these mutants were 46%, 382%, and 170% compared with that of the wild-type. The K_m values of R18G, L59G, R66A, N106A, V122Y, A123F and D184G mutants were higher than that of the wild-type, ranging from 405 to 2298 μM . Among these 7 mutants (Fig. 3B), the Arg18, Leu59, Asn106 and Asp184 residues were predicted by computational analysis (Table 2) to contact both FMN and nicotinamide adenine dinucleotide (NAD). This contact was confirmed by the lack of strong binding of FMN to the purified mutated enzymes. Thus, their activities were no more than 10% that of the wild-type. For the rest of mutants, the change in residue Val-122 decreased the reduction activity. No K_m values are available for, the F127G and Y129G mutants, since no detected activities were found. As we have mentioned before, the F127G mutant was expressed without bound FMN, so the residue may also affect FMN binding. Both Phe-127 and Tyr-129 contributed in binding the NMN moiety of NADH and the anthranilic acid moiety of Methyl Red as shown in Fig. 3, therefore, it is reasonable that both mutants lost their azo reductive activities. The L59G mutant had no effect upon the binding of the Methyl Red, but caused a significant decrease in NADH binding; the K_m values for Methyl Red and NADH were 28.8 and 2298 μM . Since the isoalloxazine ring of FMN is responsible for the binding of both NADH and Methyl Red, this observation indicates that although the FMN binding affinity of the mutant was decreased, there was no significant conformational change of FMN in the mutant, allowing the isoalloxazine ring to keep the functional contribution for NADH and Methyl Red binding.

Step 3: Reconstruction of mutation network

For a global view of mutation effect on binding and interaction with substrates and on enzyme activity, we explored the relationships among the mutants to reconstruct the mutation network based on their protein–ligand interaction and kinetics information. Fig. 5 shows a reconstructed mutation network of the 13 mutants showing relationships among the four functional categories—NADH binding, Methyl Red binding, FMN binding, and enzyme activity. Generally, the correlation between the stability of the AzoA system and the functional perturbation of the network validates the reverse genetic approach. In the network, the R21G, N121A, E16G, and R66A mutants are located in the inner region, indicating that the AzoA system has relatively high tolerance toward the perturbation. On the other hand, the F127G, D184G, and Y129G mutants, which completely lost their enzyme activity, are positioned in the outer region. The residues appear to be responsible for the binding of both NADH and Methyl Red in AzoA system. The lack of robustness of the AzoA system to the perturbation of these residues is in agreement with their critical functional role in the system. The other mutants showing significant fluctuation in the kinetic parameter lie in the middle layers.

In the network, the overall connection reveals NADH, FMN and Methyl Red- and location-dependent contributions, consistent with the contact information of the residues (Table 2). With respect to NADH binding, all mutations affected the AzoA system in a deleterious way, causing increased K_m values, with the exception of W62A, which decreased the K_m to 3 μ M. The correlation between the mutation and decreased binding affinity further supports the proposed NADH binding mode in AzoA. On the other hand, the mutations show apparent position-dependent effects on the Methyl Red and FMN binding. In Methyl Red binding, locations of the R66A, N121A, N106A, R21G, and R18G mutants are closer to the center in the mutation network than the wild-type (Fig. 5), which indicates increased binding affinity for the ligand. In FMN binding, AzoA showed relatively high tolerance toward several mutations including E16G, R21G, N121A, and A123F.

Discussion

In this study, we aimed to develop a global overview of AzoA interaction among FMN, NADH, and Methyl Red. As shown in Fig. 1, a synergetic strategy based on reverse genetics coupled with site-directed mutagenesis was essential to improve accuracy and reduce errors in computational prediction (Step 1) and empirical validation (Step 2) to gain new and deepened insights into the mutation effects in AzoA with a holistic perspective (Step 3).

Substrate Methyl Red-binding site

Methyl Red is held firmly in the active site of AzoA, as evidenced by its higher binding affinity than that of NADH [14]. In the proposed AzoA-Methyl Red complex, the *N,N'*-dimethylaniline moiety is sandwiched in the hydrophobic pocket formed by the isoalloxazine ring and the side chains of the aromatic residues (Phe-127, Tyr-129, Pro-134, and Phe-169). Indeed, such a sandwiching system of aromatic electron acceptors by means of the isoalloxazine ring and some aromatic side chains is known to be employed by several flavoproteins, including AzoR (FMN-dependent NADH-azo-reductase) and NQO1 (FAD-

dependent NAD(P)H:quinone oxidoreductase 1) [28,29]. Although AzoA has features in common with flavoproteins, with respect to the aromatic electron acceptor binding, the difference occurs in the binding of the benzoic acid moiety on the isoalloxazine ring. As revealed in NADH binding, the same sandwiching system of Tyr-129 and Asp-184 on the isoalloxazine ring of FMN should be involved in the binding of the nicotinamide moiety of NADH.

Azo dye substrate diversity

The AzoA-Methyl Red complex also accounts for the substrate preference and diversity of AzoA. Despite its catabolic ability for azo dyes, AzoA shows apparent substrate preference for Methyl Red and relatively low azo reduction activity for dyes of relatively large structure, such as Orange II, Amaranth, and Ponceau S [14,15,19,30]. Considering the hydrophobic environment of the active site and functional alignment of the azo group and N5 of FMN, a charged functional group in proximity to the azo group or the presence of a second polar group seems likely to be problematic from the standpoint of productive binding, consistent with the previous speculation [14]. In addition, based on the productive binding mode of Methyl Red, two benzene ring-containing azo dyes such as Orange G and Amaranth should be not suitable substrates for AzoA, due mainly to its relatively limited space for the binding [14]. The side chain of Arg-59 of an FMN-dependent NADH-azoreductase (AzoR) from *E. coli*, has been considered to determine the substrate specificity [28]. However, although the amino acid corresponding to Arg-59 from AzoR, Arg-66, is spatially conserved in AzoA, due to its distance of $>8 \text{ \AA}$ from the carboxyl group of Methyl Red, its functional significance for the substrate specificity could be ruled out. Actually, the carboxyl group in AzoA interacts with the residues of Tyr-129, Asp-184, and His-185 by contacts including hydrogen bond and hydrophobic interaction. As revealed in the contact analysis and the mutation network, rather, the positively charged side chain of Arg-66 could provide contacts that stabilize and orient the NMN moiety of the NADH toward the flavin for favorable hydride transfer. The overview in terms of the sequential binding of NADH and azo dye substrates can be extended to further support why dimeric azoreductases, including AzoA, may accept azo dyes that are relatively diverse in size and structure more readily than monomeric azoreductases [13].

NADH binding site

With respect to NADH binding, since the solvent-accessible room on the *si* face has a height of $\sim 6 \text{ \AA}$, it may not be enough to accommodate the folded conformation of NADH, as shown in the flavin reductase PheA2 from *Bacillus thermoglucosidasius* A7 (PDB IRZ1) with the NADH compactness of $3.8 \text{ \AA}$ [31]. This speculation suggests that the active site should accommodate the extended conformation, and hence the adenine base should be located in another binding region. This opinion is further supported by the secondary structure of AzoA, as each monomer of AzoA has a typical NAD(P)-binding Rossmann fold with parallel five-stranded β -sheets (strand order $\beta 2$, $\beta 1$, $\beta 3$, $\beta 6$, and $\beta 7$) and α -helices flanked on both sides of the sheets ($\alpha 1$ and $\alpha 8$ on one side and $\alpha 6$ and $\alpha 7$ on the other side) [15].

Interestingly, the binding of NADH to the active site in AzoA is analogous to that observed in the NADH:FMN oxidoreductase (EmoB, PDB 2vzj) from *Mesorhizobium* sp. BNC1[32],

whose RMSD (Ca) superimposed on the AzoA is 1.69 Å. The bound FMN cofactors of the two enzymes also have very similar conformations. However, the difference between the bound nucleotides is the orientation of the NMN moiety. Unlike EmoB, in AzoA, the nicotinamide ring is packed with about a 35° angle against the isoalloxazine ring of FMN. As revealed in Methyl Red binding, the sandwiching system of Tyr-129 and Asp-184 seems to have a critical role, not only in Methyl Red binding but also in orienting the NMN moiety of the bound nucleotide to favor hydride transfer.

As revealed by the bottom-up docking analysis, in which several solutions for the moieties of NMN and AMP were superimposed on that of the productive binding mode of NADH, each moiety of NADH seems to be able to interact with the enzyme in a partially independent fashion. Such a binding disposition further supports the proposed NADH-binding mode, consistent with its structural interpretation. In addition, based on the observations, we can hypothesize that the NADH could interact by a bipartite binding mode with AzoA, in which both moieties, adenosine-ribose and nicotinamide, bind separately, similar to what has been seen for other NAD(P)H-dependent enzymes [33–35]. Considering that Leu-59 is involved only in the binding of the adenosine portion of NADH, the decreased enzyme activity of the mutant can be thought that the local perturbation of the adenine moiety binding might be enough to disturb the proper binding of NADH and hence the interactions between NADH and FMN. The specific binding of the AMP moiety seems to help place the remainder of the dinucleotide in a perfect position to interact with the flavin and form an electron transfer-competent complex. This speculation could be extended to explain that AzoA shows apparent preference for NADH compared to NADPH. As a whole, the coordinated contribution of each part seems to be essential for the productive binding mode. More experimental evidence could clarify a leading role of the AMP moiety in AzoA-NADH interaction as an anchor, which enables the nicotinamide moiety to search locally for reactive configuration—conformational sampling, as observed in biological electron transfer between redox partner proteins formed with electron transferring flavoproteins [36–38].

Mutation network

Since functional perturbation could affect the whole system of the enzyme, the effect of induced mutation should extend to FMN-, NADH-, and Methyl Red-binding and hence interaction, ultimately resulting in changing the enzyme activity. In this context, the mutation network reduces the unnecessary complexity of the net mutation effects, which enables researchers to rationalize current mutational results with structural and kinetic studies [14,15,19].

The lack of tolerance of the AzoA system toward the functional perturbation of the residues of Phe-127, Tyr-129, and Asp-184 validates the proposed binding modes of the substrates and at the same time, indicates that the stacking interaction between Tyr-129 (and Phe-127) and Asp-184 on the isoalloxazine could be a key contact in the active site of AzoA. In addition, the network suggests that the mutants appear to be connected to FMN binding. However, due to no direct contribution of Phe-127 and Tyr-129 on FMN binding, the decreased binding affinity of FMN may be related to structural perturbations induced by the loss of the bulky side-chains. As revealed in a previous study, at least in part, local

adjustments within the FMN binding site are likely to be responsible for the changed enzyme activities observed in the mutants in this study. Changes in the position of the bound FMN, especially in the isoalloxazine ring should affect the interactions with NADH and/or substrate, which causes its enzyme activity to fluctuate [19]. In this context, the mutants R21G and N121A are of particular interest. As shown in Fig. 5, both R21G and N121A mutants show enhanced enzyme activity, about 3.8 and 1.7 fold higher than that of wild type, respectively, which can be explained in terms of the substrates binding in the mutation network at a slightly decreased NADH binding affinity (the K_m values were 380 and 292 μM , respectively), but increased Methyl Red binding affinity (the K_m values were 12.6 and 8.2 μM , respectively) with no significant change in FMN binding. Unlike other mutants, R21G and N121A demonstrated a moderately higher level of tolerance of the perturbation made by the side chain substitution. Rather, the mutations seem to improve the stability of the interaction without significantly affecting their geometries, hence resulting in enhancing enzyme activity.

Conclusion remark

In this study, the reverse genetic approach based on reliable computational prediction and experimental confirmation allowed us to connect the structure to its function in terms of the binding and interaction of FMN, NADH, and Methyl Red. We suggest the structures of NADH and Methyl Red as bound to AzoA. The localization and characterization of the ligand binding sites completes our general geometric knowledge of the active site, allowing for a deeper functional understanding of AzoA.

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Fig. 1.

Overview of the systematic approach based on the reverse genetics coupled with a site-directed mutagenesis. This process is composed of three steps: *in silico* prediction (Step 1), experimental validation by analysis of the biochemical data of 13 mutant proteins (Step 2) and reconstruction of the mutation network (Step 3). In this study, NADH was divided into two fragments (the moieties of NMN and AMP) for the bottom-up docking analysis.

Fig. 2. Active site stereochemistry of AzoA with substrates. (A and B) The dimeric active site of AzoA with FMN. (C and D) Electrostatic surface diagram of the substrate binding pocket, colored by charge, and the stick representation of the bound substrates Methyl Red (C) and NADH (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Close-up view of the interaction between the amino acid residues in the active site and the substrates—Methyl Red (A) and NADH (B). Hydrogen bonds are indicated with green broken lines (green residue numbers) and van der Waals' interactions by red hatching (black residue numbers). Each atom is colored according to atom type. The figures were generated by LigPlot⁺. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)[22].

Fig. 4. SDS-PAGE of protein profiles of cell extracts (A) and purified protein preparations (B) from wild-type and mutant AzoA expressed in *E. coli*. M, protein molecular mass standards; lane 1, wild-type; lane 2, E16G; lane 3, R18G; lane 4, R21G; lane 5, L59G; lane 6, W62A; lane 7, R66A; lane 8, N106A; lane 9, N121A; lane 10, V122Y; lane 11, A123F; lane 12, F127G; lane 13, Y129G; lane 14, D184G; lane 15, control sample from non-induced *E. coli*. Lanes 1–14 are the samples extract from the induced culture.

Fig. 5.

The mutation network reconstructed from 13 mutants and the wild-type. Connection for wild-type and the mutants with enhanced enzyme activity (R21G and N121A) is represented by black thick solid lines and red solid lines, respectively, while thin dashed lines show the connection of the other mutants. The color scale ranges from saturated green for lowest values to saturated red for highest values for the binding affinity or enzyme activity. In the network, the white color means complete loss of ligand binding affinity or enzyme activity. The black color indicates the wild-type enzyme for ligand binding and enzyme activity. Each mutant or wild-type enzyme is represented by a single row of colored boxes. The network was generated with the K_m values and relative specific activities of wild-type and the mutants.

Table 1

Primers used for site-directed mutagenesis of AzoA.

Mutant	Primer Sequences (5' → 3')
E16G	CCATTAACAAAAGAAGGA-TCACGCTCAGTTCG CGAACTGAGCGTGATCCTTCTTTTGTAAATGG
R18G	CAAAAGAAGAATCAGGCTCAGTTCGTGCG CGCACGAACTGAGCCTGATTCTTCTTTTG
R21G	TCACGCTCAGTTGGTGCGTTAGAAACAT ATGTTTCTAACGCACCAACTGAGCGTGA
L59G	GAAATCGATGAAGAATTAGGATCTGCTTGGGGTGC GCACCCCAAGCAGATCCTAATTCTTCATCGATTTC
W62A	TTATTATCTGCTGCTGGTGCACTTCGAG CTCGAAGTGCACCAGCAGCAGATAATAA
R66A	TGGGGTGCACTTGCAAGCTGGCGCAGCAT ATGCTGCGCCAGCTGCAAGTGCACCCCA
N106A	GCTAATCCAATGTGGGCCTTAAACGTACCGAC GTCGGTACGTTTAAAGGCCACATTGGATTAGC
N121A	GTAGATACAATCGCAGTTGCTGGAAAAAC GTTTTTCCAGCAACTGCGATTGTATCTAC
V122Y	GATACAATCAACTACGCTGGAAAAACATTC GAATGTTTTTCCAGCGTAGTTGATTGTATC
A123F	GATACAATCAACGTTCCAGGAAAAACATTCC GGAATGTTTTTCCTGGAACGTTGATTGTATC
F127G	GCTGGAAAAACAGGCCAATATACTGC GCAGTATATTGGCCTGTTTTTCCAGC
Y129G	GAAAAACATTCCAAGGTACTGCAGAAGGAC GTCCTTCTGCAGTACCTTGGAATGTTTTTC
D184G	CATCGAAGGAATCGGTCATTTCCCTGATC GATCAGGGAAATGACCGATTCTTCGATG

* Underlined nucleotides indicate mutations incorporated into primers.

Table 2
Residues in contact with the ligands FMN, Methyl Red, and NADH in the wild-type AzoA (PDB 2HPV).

Residue	FMN		Methyl Red		NADH	
	D (Å)	S (Å ²)	D (Å)	S (Å ²)	D (Å)	S (Å ²)
54 A	ILE	3.5	32.8			
59 A	LEU	4.2	19.5		4.9	5.9
60 A	SER				6.2	0.6
62 A	TRP	4.6	11.4			
63 A	GLY				4.8	7.7
66 A	ARG			4.6	23.3	3.7
118 A	ASP	6	0.2			86.6
121 A	ASN	5.8	1.9	6	3.6	
123 A	ALA				6.2	2.5
127 A	PHE			4.6	8.7	5.7
129 A	TYR			3.4	59.7	3.4
134 A	PRO			4	28.5	42.7
169 A	PHE			4.9	23.3	
10 B	HIS	2.7	24.9			
12 B	LEU				4.8	10.1
15 B	GLU				4.8	2.2
16 B	GLU	4.9	4.2		3.8	49.3
17 B	SER	2.6	28.6			
18 B	ARG	2.8	58.8		3.6	36.9
19 B	SER	2.9	24.3			
103 B	PRO	3.6	13.7			
104 B	MSE	2.7	41.5			
105 B	TRP	3.6	43.3			
106 B	ASN	2.8	34.2			
107 B	LEU	2.7	36.2	5.2	5.8	
148 B	SER	2.6	46.1			
149 B	ASN	3.6	6.6			

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Residue	FMN		Methyl Red		NADH	
	D (Å)	S (Å ²)	D (Å)	S (Å ²)	D (Å)	S (Å ²)
150 B	GLY	3.2	20.1	4.4	5.6	3.5
151 B	GLY	3	25.6	4.5	1.3	11.7
153 B	TYR	3.6	28.9	3.6	21.7	
180 B	ILE	4.4	0.2			
183 B	ILE	5.8	0.2			
184 B	ASP	2.5	33.5	3.3	46	3.5
185 B	HIS			3.4	41.5	25
187 B	PRO					3.6
190 B	ALA					48.2
194 B	LEU					11.9
	FMN			3.4	97.8	3.8
						23.1
						5.3
						7.9
						6.3
						2.5
						81

Table 3

Kinetic parameters of wild-type and the mutants.

Enzyme	FMN*	Poly-merization	K_m (μ M, Methyl Red)	K_m (μ M, NADH)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat} / K_m^{MR} (μ M $^{-1}$ s^{-1})	k_{cat}/K_m^{NADH} (μ M $^{-1}$ s^{-1})	Relative specific activity (%)
Wild-type	1.8	Dimer	24	143	84	59.1	2.46	0.41	100
E16G	1.9	Dimer	ND**	250	29.6	20.8	ND	0.083	46
R18G	1.7	Dimer	16.7	624	4.5	3.17	0.19	0.005	9
R21G	1.9	Dimer	12.6	380	200	140	11.11	0.37	382
L59G	1.8	Dimer	28.8	2298	9.4	6.61	0.23	0.0029	10
W62A	1.7	Dimer	33.5	3	30	21.1	0.63	7.03	35
R66A	1.9	Dimer	5.3	581	110	77.3	14.58	0.13	47
N106A	1.6	Dimer	11.2	793	1.7	1.2	0.11	0.0015	3
N121A	1.9	Dimer	8.2	292	158	111	13.54	0.38	170
V122Y	1.7	Dimer	109.2	405	24.8	17.5	0.16	0.044	14
A123F	1.8	Dimer	48.5	411	350	245	5.05	0.6	36
F127G	-	Monomer	-	-	-	-	-	-	-
Y129G	-	Monomer	-	-	-	-	-	-	-
D184G	-	Monomer	-	-	-	-	-	-	-

* FMN per dimer of the purified protein.

** ND, not determined due to strong substrate Methyl Red inhibition.