Expansion of Substrate Specificity and Catalytic Mechanism of Azoreductase by X-ray Crystallography and Site-directed Mutagenesis*

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AzoR is an FMN-dependent NADH-azoreductase isolated from *Escherichia coli* as a protein responsible for the degradation of azo compounds. We previously reported the crystal structure of the enzyme in the oxidized form. In the present study, different structures of AzoR were determined under several conditions to obtain clues to the reaction mechanism of the enzyme. AzoR in its reduced form revealed a twisted butterfly bend of the isoalloxazine ring of the FMN cofactor and a rearrangement of solvent molecules. The crystal structure of oxidized AzoR in a different space group and the structure of the enzyme in complex with the inhibitor dicoumarol were also determined. These structures indicate that the formation of a hydrophobic part around the isoalloxazine ring is important for substrate binding and an electrostatic interaction between Arg-59 and the carboxyl group of the azo compound causes a substrate preference for methyl red over *p*-methyl red. The substitution of Arg-59 with Ala enhanced the *V*_max value for *p*-methyl red 27-fold with a 3.8-fold increase of the *K*_m value. This result indicates that Arg-59 decides the substrate specificity of AzoR. The *V*_max value for the *p*-methyl red reduction of the R59A mutant is comparable with that for the methyl red reduction of the wild-type enzyme, whereas the activity toward methyl red was retained. These findings indicate the expansion of AzoR substrate specificity by a single amino acid substitution. Furthermore, we built an authentic model of the AzoR-methyl red complex based on the results of the study.

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The atomic coordinates and structure factors (codes 2Z98, 2Z9B, 2Z9C, and 2Z9D) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡ AzoR is an FMN-dependent NADH-azoreductase isolated from *Escherichia coli* (1) as a protein responsible for the reduction of azo compounds. AzoR exists as a homodimer composed of 23-kDa subunits. The reaction follows a ping-pong mechanism requiring 2 mol of NADH to reduce 1 mol of methyl red (4′-dimethylaminoazobenzene-2-carboxylic acid), a typical azo dye, into 2-amino benzoic acid and N,N′-dimethyl-p-phenylenediamine. AzoR also can reduce ethyl red (4′-diethylaminoazobenzene-2-carboxylic acid) and menadione (vitamin K₃, 2-methyl-1,4-naphthoquinone) and is inhibited by dicoumarol (3,3′-methylene-bis(4-hydroxycoumarin)). Biochemical studies also revealed that, compared with other azoreductases so far reported, AzoR is different in several regards: in its requirements for cofactors, electron donors, substrate specificity, and amino acid sequence. On the other hand, although the physiological function of AzoR remains unknown, genome projects have inferred its importance from the wide distribution of highly homologous genes in many microorganisms (2–7).

We have previously reported the crystal structure of oxidized AzoR at 1.8 Å resolution (8). That was the first report on the structure of AzoR orthologues and of FMN-dependent NADH-azoreductase. This structure has provided a wealth of information, including the overall fold, the nature of the dimer, and the interactions of the FMN cofactor. A structural similarity search revealed that the overall structure of AzoR resembles that of mammalian NQO1 (FAD-dependent NAD(P)H:quinone oxidoreductase 1, originally called DT-diaphorase) (9), ROO from *Desulfovibrio gigas* (FAD-dependent rubredoxin:oxygen oxidoreductase) (10), and yeast YLR011wp (FMN-dependent NAD(P)H:ferric iron oxidoreductase) (11) without the explicit overall amino acid sequence similarity. They are homodimeric flavodoxin-like proteins.

Many types of azoreductases have been isolated and extensively studied in the field of environmental biotechnology (12–20). However, those studies have focused mainly on the screening of microorganisms exhibiting azoreductase activity. Because their detailed molecular reaction mechanisms remain largely unknown, AzoR is an interesting case for azoreductase research. Structural and enzymatic insights into AzoR provide...
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knowledge of the molecular mechanism underlying the reduction of azo compounds and would also provide important clues for substrate-specificity expansion techniques that will be essential for industrial use of the enzyme in the biodegradation process of azo compounds.

In this study, we have determined the structure of reduced AzoR. This work reveals the structural changes upon reduction of the enzyme and is the first report on the crystal structure of homodimeric flavodoxin-like proteins in the reduced state. In addition, we have determined the crystal structure of oxidized AzoR in a different space group as well as the crystal structure of the enzyme in complex with the inhibitor dicoumarol. Substrate-specificity analysis and site-directed mutagenesis were also performed. According to these analyses, we succeeded in expanding AzoR substrate specificity, and we have built an authentic model of an AzoR-methyl red complex. Finally, the mechanisms underlying the reductive cleavage of azo compounds are discussed.

EXPERIMENTAL PROCEDURES

Protein Preparation for Crystallization—The recombinant AzoR used for crystallization was expressed and purified as described previously (1, 21).

Improvement of the Quality of the Tetragonal Crystals of Oxidized AzoR—The improved oxidized tetragonal crystals (P4₃2₁2) were obtained in the same crystallization method as reported previously (21), except that 0.1 mM warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin) was added to the crystallization solution as an additive reagent for crystal growth (the electron density of warfarin was not observed). The crystals grew to full size (0.1 × 0.1 × 0.6 mm) within 1 week.

Chemical Reduction of the Tetragonal Crystals of Oxidized AzoR—Crystals of the enzyme in the reduced state were prepared by transferring the oxidized tetragonal crystals to a degassed reducing solution containing 200 mM MgCl₂, 100 mM HEPES, pH 7.5, 30% (v/v) 2-propanol, 30% (v/v) ethanol glycol, and a saturated concentration of sodium dithionite at 4 °C. After 10 min, the crystals were transferred to fresh reducing solution. This procedure was repeated two more times. The crystals lost their bright yellow color and became transparent over the course of the treatment. They were then immediately flash-cooled in a stream of nitrogen and stored in liquid nitrogen prior to data collection. It was verified that the crystal remained transparent after data collection.

Co-crystallization with Dicoumarol—The purified protein was dialyzed against a solution containing 10 mM Tris-HCl, pH 8.0, 0.1 mM FMN, 4% (v/v) pyridine, and 2 mM dicoumarol. Crystals of AzoR in complex with dicoumarol were obtained from a drop made by mixing 16 mg/ml of the protein solution mentioned above and an equal volume of reservoir solution containing 200 mM NaCl, 100 mM HEPES, pH 7.5, and 20% (w/v) polyethylene glycol 3000. The drop was equilibrated over the reservoir solution by the hanging-drop vapor diffusion method at 25 °C. The crystals grew to full size (0.03 × 0.05 × 0.5 mm) within 2 weeks.

Data Collection and Processing—All diffraction data were collected under cryogenic conditions at 100 K. Prior to data collection, the crystals were soaked in a reservoir solution containing 30% (v/v) ethylene glycol or 25% (v/v) glycerol as a cryoprotectant. All diffraction data were collected at KEK (Tsukuba, Japan). The beamlines used are shown in Table 1. Data were reduced with MOSFLM, SCALA, and TRUNCATE from the CCP4 program suite (22).

Structure Determination—The initial structures of all the crystals were obtained by molecular replacement with MOLREP (23) using the 1.8 Å resolution structure of oxidized AzoR as a search model (8). The solutions were then improved by ARP/wARP (24) followed by iterative manual model building with XtalView (25). All the stages of maximum likelihood refinement were carried out with REFMAC5 (26). For the refinement of the oxidized and reduced tetragonal crystal structures, the restraint of the planarity of the isalloxazine ring was removed from the standard REFMAC5 library to allow the model to adopt the omit density map more precisely.

Model Analysis—The quality of the model was checked with PROCHECK (27). LSQKAB was used to superpose the molecules and to calculate the root mean square deviation between pairs of equivalent Ca atoms and all atoms of the proteins (28). Structure figures were prepared with PyMOL (29).

Mutant Preparations—For enzymatic analyses, mutations and C-terminal His tag were introduced into the AzoR gene by two rounds of PCR with pETacPD as a template (1). The NADH-methyl reductase activity of the His-tagged wild-type AzoR was very similar to that of the non-tagged wild-type AzoR.

To mutate Arg-59 to Ala, the following pairs of oligonucleotide primers were used for the first PCR: 5'′-terminal sense primer for AzoR (5′-GGGAATTCATATGAGCAAAGTAT-TAGTTCTTAATCCAGC-3′ containing an Ndel site) and R59A mutation antisense primer (5′-CGGCGCATGCGTGGGAGG-GACGCGGCACCACACAGGGTC-3′) were used to amplify the 5′-terminal part of the AzoR gene. R59A mutation sense primer (5′-GAACHTTGTTGCGCCTGTGGTGGCGGCA- TGCCCG-G-3′) and 3′-terminal antisense primer for AzoR (5′-AAAACCGTCAAGTGTGGTTGATGGTGGTGATGGTG- GAGAAAATGCTGTGGACGC-3′ containing an Xhol site and His₆ tag sequence) were used to amplify the 3′-terminal part of the AzoR gene. PCR products were used as templates for the second PCR, which used the 5′-terminal sense primer and 3′-terminal antisense primer for AzoR. To

Crystallization of the Orthorhombic Crystals of Oxidized AzoR—The purified protein was dialyzed against a solution containing 10 mM Tris-HCl, pH 8.0, and 0.1 mM FMN. The orthorhombic (P₂₁₂₁) crystals of oxidized AzoR were obtained from a drop made by equal volumes of three solutions: 8 mg/ml of the protein solution mentioned above, 100 mM NAD⁺ solution (the electron density of NAD⁺ was not observed), and a reservoir solution containing 200 mM NaOAc, 200 mM sodium cacodylate, pH 6.7, 15% (w/v) polyethylene glycol 8000, and 3% (v/v) dimethyl sulfoxide. The drop was equilibrated over the reservoir solution by the hanging-drop vapor diffusion method at 25 °C. The crystals grew to full size (0.03 × 0.05 × 0.5 mm) within 2 weeks.
generate other mutants, the following primers were used in place of R59A mutation sense and antisense primers: Y120A mutation sense primer (5'-GCAGGGCTTACTTTCCGGCTACCGAGAAGCCGTCG-3') and Y120A mutation antisense primer (5'‐CGGACCGTTCTCGGTAGCGCGGAAAGTAACCGCTGC-3') for Tyr-120 to Ala mutant; F162A mutation sense primer (5'-CCACGTTCCTCGGCGTATC-CCACGTTCCTCGGCGCAGGT-3') and F162A mutation antisense primer (5'-CATCGGTAATGCCGATAGCGCCGAGGAAGTGG-3') for Phe-162 to Ala mutant. To generate the His-tagged wild-type AzoR gene as a control that is catalytically very similar to AzoR, the 5'-terminal sense primer and the 3'-terminal antisense primer for AzoR described above were used for PCR with pETacpD as a template. The PCR product of each mutated AzoR was inserted between the NdeI and the XhoI sites of the expression vector pET-22b (Novagen). The entire DNA sequence was confirmed by DNA sequencing.

His-tagged wild-type and mutant AzoR proteins were expressed in E. coli BL21(DE3) at 37 °C in Luria Bertani medium containing 100 µg/ml ampicillin. Protein expression was induced by adding 1 mm isopropyl-β-D-thiogalactopyranoside to early exponential phase cultures (A600 ~ 0.5) for 3 h. Bacteria were lysed in a solution containing 50 mm Tris-HCl, pH 7.5, 2 mm 2-mercaptoethanol, 500 mm NaCl, 20 mm imidazole, and 10 mg/ml lysozyme by sonication. The lysate was centrifuged, and the His-tagged proteins were purified by gravity-flow chromatography using nickel-nitrilotriacetic acid-agarose (Qiagen) according to the manufacturer’s instructions. Eluted proteins were stored at ~80 °C in a solution containing 20 mm Tris-HCl, pH 7.5, 2 mm 2-mercaptoethanol, 200 mm NaCl, and 0.1 mm FMN. Protein samples were analyzed on SDS-PAGE and were more than 95% pure.

Enzyme Assays—The NADH-methyl red reductase activity for each mutant was determined spectrophotometrically by a method described previously (1). The initial reaction rates were fitted to the Equation 1

$$\frac{1}{V} = \frac{1}{V_1} + \frac{K_A}{V_1} + \frac{K_B}{V_2}$$  
(Eq. 1)

where $v$ is the initial reaction rate, $V$ is the maximum reaction rate at infinite substrate concentrations, $A$ and $B$ are the concentrations of methyl red and NADH, respectively, and $K_A$ and $K_B$ are their corresponding Michaelis constants. The initial reaction rate of p-methyl red reduction was determined in the same manner as methyl red, except that a 460-nm wavelength was used to monitor the decrease in absorbance of p-methyl red and a molar absorption coefficient of 17310 M⁻¹ cm⁻¹ was used. $K_B$ was applied to obtain Michaelis constants for p-methyl red because $K_B$ does not depend on the kind of azo compound. All of these assays were performed in triplicate.

RESULTS AND DISCUSSION

Structures of Oxidized and Reduced AzoR in Tetragonal Crystals—The structure of oxidized AzoR in tetragonal crystal was previously determined at 1.8 Å resolution (8). We have now improved the crystallization conditions and increased the resolution to 1.4 Å. In addition, we have determined the structure of reduced AzoR at 1.7 Å resolution using chemically reduced tetragonal crystals. The results allow the stringent comparison of the two states, especially the conformation of the isoalloxazine ring of FMN. The structure of this ring was unambiguously determined without the restraint of planarity for both the oxidized and reduced enzymes (Fig. 1). The reduced crystals turned colorless, indicating that two-electron reduction of the flavin occurred. The data collection and the final refinement statistics are summarized in Table 1.

The AzoR structures are nearly identical between the oxidized and reduced states. The root mean square deviations

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**FIGURE 1. Comparison of the active site of oxidized and reduced AzoR.** A–D, the omit $F_o - F_e$ electron density maps surrounding the FMN contoured at 3.5σ. FMN molecules in oxidized (A and C) and reduced (B and D) AzoR are shown in a ball-and-stick model, with carbon atoms in yellow, oxygen atoms in red, nitrogen atoms in light blue, and phosphorus atoms in orange. C and D show the molecules rotated ~90° around the vertical axis from A and B, respectively. E, superposition of the active site of oxidized and reduced AzoR. FMN, water molecules on top of the isoalloxazine ring, and the amino acid residues participating in hydrogen bonds with the isoalloxazine ring are shown in stereo view. The color coding of atoms in oxidized AzoRs is the same as above. All atoms in the reduced enzyme are shown in blue. Hydrogen bonds common to both oxidized and reduced AzoR are shown as gray dashed lines. Hydrogen bonds formed only in oxidized and reduced AzoR are shown as red and blue dashed lines, respectively. F, chemical structure and atom numbering of the isoalloxazine moiety of FMN, viewed from the si-face.
between equivalent 193 Ca atoms and all atoms of the amino acid residues, except hydrogens, in the two states are 0.282 and 0.582 Å, respectively. However, prominent structural differences are found in the active site. In the oxidized enzyme, the isoalloxazine ring of the flavin is nearly planar but shows a slight twist conformation (Fig. 1, A and C). Upon reduction of the enzyme, the isoalloxazine ring adopts a butterfly bend conformation along the N5-N10 axis caused by a shift of the dimethyl pyrimidine rings toward the re-face by an angle of ~15° (Fig. 1, B, D, and F). The slight twist conformation found in the oxidized state is retained in the reduced state, and N5 and N10 of the isoalloxazine ring move up toward the si-side. Therefore, the central ring of the isoalloxazine is distorted to a twist-boat conformation upon reduction. The movement of N10 is small relative to that of N5. The other structural changes associated with reduction are a significant movement of a water molecule and a loss of two spherical electron density peaks on FMN. The water molecule, which is hydrogen-bonded to Oδ-1 of Asn-97 in the oxidized state, still forms a hydrogen bond in the reduced state (Fig. 1E). This water molecule, however, moves by 2.4 Å and makes an additional hydrogen bond with N5 of the isoalloxazine ring upon reduction. The protonation of the N5 of the reduced isoalloxazine ring has been proved biochemically (30). This hydrogen bond may help stabilize the upward movement of the N5 atom, which adopts sp³ hybridization in the reduced state (31). The two spherical electron density peaks on FMN, which were also found in the oxidized 1.8 Å resolution structure as reported previously, are currently modeled by water molecules, although they are slightly larger than the electron density peaks of water molecules. The loss of two peaks upon reduction may be attributable to electronic restructuring of the reduced flavin.

**The Conformational Change of the Isoalloxazine Ring and the Solvent Rearrangement upon Reduction**—The butterfly bending direction in reduced AzoR is the opposite of the conformation predicted for reduced free flavin (32–34). This considerable disagreement suggests that the peptide moiety may greatly influence the equilibrium conformation of the isoalloxazine system in AzoR. On the other hand, although it is not clear why the central ring of the isoalloxazine adopts the twist-boat form in the reduced enzyme, which is energetically less favorable than a chair form, this conformation would affect the redox potential of AzoR. In addition, because free oxidized isoalloxazine is planar (35), the twisted form of the oxidized isoalloxazine of AzoR may favor reduction.

In the reduced state of the enzyme, the up-moved protonated N5 of the isoalloxazine ring is stabilized by the hydrogen bond with Oδ-1 of Asn-97 through a bridging water molecule (Fig. 1E). The protonation of the N5 of the reduced isoalloxazine ring has been proved biochemically (30). If methyl red binds to reduced AzoR on top of the isoalloxazine ring as described below, this water molecule must be replaced, resulting in the disruption of the hydrogen bond network. This may cause destabilization of the protonated state of the N5 atom, and a hydride anion would then be released efficiently from the N5 position to an electron acceptor azo compound.

**Structure of AzoR in Complex with Dicoumarol**—Dicoumarol inhibits the azo reduction activity of AzoR (1). To obtain clues to the substrate binding mode, we co-crystallized AzoR with dicoumarol and have determined the structure at 2.3 Å resolution. Although the average completeness of the data of the AzoR-dicoumarol complex is 81.1% (Table 1), there was not any uninterpretable electron density region in model building. The crystal structure shows that dicoumarol is bound to the space above the isoalloxazine ring mainly by hydrophobic interactions (Fig. 2A). In their interactions, the two coumarin rings of dicoumarol are sandwiched as a result of ring-stacking interactions. The one ring is sandwiched between the phenyl group of Phe-162° and the isoalloxazine ring of FMN,³ and the other

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³ AzoR is a homodimeric enzyme (1). The redox center FMN is found in the dimer interface, and both monomers contribute to form the two identical catalytic sites (8). When we refer to one catalytic site, residues will be primed (one monomer) or non-primed (the other monomer), but they are
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The activity is expressed as the mean ± S.E. (n = 3). The relative values to wild type (WT) are indicated in parentheses. MR, methyl red; PMR, p-methyl red.

| Enzyme | $K_m$ (μM) | $K_m$ (μM) | $V_{max}$ (units/mg) | $K_m$ (μM) | $V_{max}$ (units/mg) |
|--------|------------|------------|----------------------|------------|----------------------|
| WT     | 70.6 ± 8.1 | 41.9 ± 3.4 | 393 ± 35             | 266 ± 2.8  | 9.11 ± 0.14          |
| Y120A  | 637 ± 180 (×0.9) | 10.2 ± 3.2 (×0.24) | 53.0 ± 16.2 (×0.13) | 230 ± 5.3 (×0.86) | 6.02 ± 0.16 (×0.66) |
| F162A  | 739 ± 214 (×0.5) | 101 ± 30 (×2.4) | 124 ± 32 (×0.32)    | 867 ± 124 (×3.3)  | 6.18 ± 0.67 (×0.68)  |
| R59A   | 403 ± 32 (×5.7) | 84.9 ± 7.1 (×2) | 1336 ± 129 (×3.4)   | 1006 ± 188 (×3.8) | 248 ± 41 (×27)       |

TABLE 2

Kinetic parameters of wild-type and mutant AzoR

The $K_m$ values for NADH of Y120A and F162A mutants are ≈10-fold larger than those of the wild-type enzyme, whereas the difference between the $K_m$ values for both the azo compounds of Y120A and F162A mutants are not significant compared with the case of NADH (Table 2). Although the $V_{max}$ value for methyl red of the Y120A mutant is moderately changed, the other $V_{max}$ values are comparable with those of the wild type. These results indicate that Tyr-120 and Phe-162 are mainly responsible for NADH binding rather than methyl red binding.

Substrate Specificity—To investigate the substrate recognition mechanism of AzoR, substrate specificity was analyzed by the steady-state kinetic procedures for methyl red and its analogue using the wild-type enzyme.

The $K_m$ values for NADH of Y120A and F162A mutants are ≈10-fold larger than those of the wild-type enzyme, whereas the difference between the $K_m$ values for both the azo compounds of Y120A and F162A mutants are not significant compared with the case of NADH (Table 2). Although the $V_{max}$ value for methyl red of the Y120A mutant is moderately changed, the other $V_{max}$ values are comparable with those of the wild type. These results indicate that Tyr-120 and Phe-162 are mainly responsible for NADH binding rather than methyl red binding.

Structures of Dimeric AzoR from the Orthorhombic Crystal—We have also determined the structure of oxidized AzoR from the orthorhombic crystal at 2.1 Å resolution. In this structure, Asp-62 of the crystallographically related neighboring molecule penetrates the active site and forms a salt bridge with Arg-59’ on top of the isoalloxazine ring of FMN (Fig. 2D). This salt bridge fixes a loop that includes Arg-59’ and permits the full ring to sandwich between the phenolic groups of Tyr-120’ and Tyr-178. The distances between these planes are within 4.0 Å and are stabilized by π-stacking systems. The phenolic groups of Phe-98 and Phe-118’ are also within 4.0 Å from and interact with dicoumarol, forming a hydrophobic pocket with the phenolic group of Phe-162’ in the deep part of the active site cavity. In addition, the Cα of the carboxyl group of Gly-141, the Cα of Gly-142, and the CB of Ala-177 of AzoR directly interact with dicoumarol in the range of 3.4 to 3.8 Å and contain no hydrogen bond. These observations suggest that the hydrophobic part around the isoalloxazine ring participates in the binding of hydrophobic substrates.

**Mutational Analysis of AzoR—**To elucidate the role of residues in the active site of AzoR, a site-directed mutagenesis study was performed. Tyr-120 and Phe-162 were chosen because they are predicted to participate in the substrate binding on top of the isoalloxazine ring, as observed in the AzoR-dicoumarol structure. In addition, a structural comparison of the active site with that of NQO1 revealed the conservation of these amino acid residues, and they are thought to play a similar role in AzoR (8). That is, in NQO1 the side chain of Tyr-128 interacts with electron acceptor duroquinone (2,3,5,6-tetramethyl-quinone) by hydrogen bond and hydrophobic interactions (Fig. 2C) (9, 36). Phe-178’ of NQO1 stacks onto duroquinone, resulting in the sandwiching of its aromatic ring between the isoalloxazine ring and Phe-178’.

The $K_m$ values for NADH of Y120A and F162A mutants are ≈10-fold larger than those of the wild-type enzyme, whereas the difference between the $K_m$ values for both the azo compounds of Y120A and F162A mutants are not significant compared with the case of NADH (Table 2). Although the $V_{max}$ value for methyl red of the Y120A mutant is moderately changed, the other $V_{max}$ values are comparable with those of the wild type. These results indicate that Tyr-120 and Phe-162 are mainly responsible for NADH binding rather than methyl red binding.
tracing of this loop, which is not observed in other crystals because of poor electron density.

As described under “Substrate Specificity,” the position of the carboxyl group of the azo compounds is the AzoR substrate-specificity determinant. On the other hand, Arg-59 is the only positively charged residue that can be positioned over the isoalloxazine ring, as has been demonstrated in the orthorhombic crystal structure. These facts imply an electrostatic interaction between Arg-59 and the carboxyl group of the azo compounds, which govern the substrate specificity.

Expansion of Substrate Specificity—As described above, the electrostatic interaction between Arg-59 and the carboxyl group of the azo compounds was predicted to determine the substrate specificity. In this case, the mutation of this residue was expected to contribute to a conversion of the substrate specificity. To investigate these observations, the kinetic parameters were determined (Table 2).

The R59A mutant resulted in a 27-fold increase in the $V_{\text{max}}$ value for $p$-methyl red reduction compared with that of the wild-type enzyme, which is comparable with the $V_{\text{max}}$ value for the methyl red reduction of the wild-type enzyme, with a 3.8-fold increase of $K_m$ value. This result indicates that the R59A mutant is capable of reducing $p$-methyl red much more effectively than the wild-type enzyme. On the other hand, the activity toward methyl red was retained. These results indicate the acquisition of AzoR’s $p$-methyl red reduction activity besides the methyl red reduction activity and demonstrate the interaction between Arg-59 and the carboxyl group of the azo compounds.

Azo Compound Binding—Although azoreductases have long been studied, there has been no report on the structure of the enzyme in complex with the azo compound nor has there been any spectroscopic study related to the azo compound binding mode. The very low solubility of azo compounds may make it difficult to prepare samples. However, the crystal structure of AzoR in complex with dicoumarol, the orthorhombic crystal structure, and the enzymatic analyses shown here provide the first view of this binding.

It has been demonstrated that the locations of some amino acid residues around the isoalloxazine ring are conserved between AzoR and NQO1 (8). In NQO1, one coumarin ring of dicoumarol is sandwiched between the isoalloxazine ring and Phe-178′ (Fig. 2B) (37), and this coumarin ring is in the same position as duroquinone (Fig. 2C) (9, 36), an electron acceptor of NQO1. This Phe-178′ of NQO1 exists in a similar position as Phe-162′ in AzoR (Fig. 2, A–C). In addition, NQO1 can exploit methyl red as an electron acceptor as well as AzoR (38). Therefore, the coumarin ring of dicoumarol sandwiched between the isoalloxazine ring and Phe-162 in AzoR can be expected to mimic the binding of the ring structure of the electron acceptor, such as the aromatic ring of methyl red. Indeed, a sandwiching system of aromatic electron acceptors by means of the isoalloxazine ring and an aromatic side chain over the isoalloxazine ring is seen in several flavoproteins (39–41). In addition to the sandwiching system, the second clue about the mechanism underlying azo binding is the interaction between Arg-59 and the carboxyl group of the azo compounds, as has been demonstrated by the substrate-specificity analysis and by the acquisition of the $p$-methyl red reduction activity of the R59A mutant.

![Figure 3. Chemical structures of azo compounds.](image)

![Figure 4. Modeling of methyl red in the active site of AzoR.](image)
protein from AzoR required 2 mol of NADH for the reductive cleavage of 1 mol of methyl red into 2-aminobenzoic acid and dimethyl-p-phenylenediamine (I). Furthermore, the flavin rings in the two identical catalytic sites are ~25 Å apart in AzoR, and there are not any possible pathways for intramolecular electron transfer between these sites. Thus, it seems that the reaction proceeds not by a simultaneous four-electron reduction but by two subsequent two-electron transfers via a hydrazo compound, namely, two cycles of a ping-pong mechanism or one cycle of a ping-pong mechanism followed by a non-enzymatic reduction in the presence of NADH. In the case involving two cycles, the aromatic ring of the hydrazo compound formed from methyl red may also be stacked on Phe-162 using a π-stacking interaction as well as in the case of methyl red, with its hydrazo group (-N,N'-diethylaniline moiety) almost on top of N5 of the isoaalloxazine ring. Attack by a hydride anion then breaks the -N,N'- bond and the respective two amines are produced. Further experiments are needed to elucidate the four-electron transfer mechanism.

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