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The Flavoenzyme Azobenzene Reductase AzoR from *Escherichia coli* Binds Roseoflavin Mononucleotide (RoFMN) with High Affinity and Is Less Active in Its RoFMN Form

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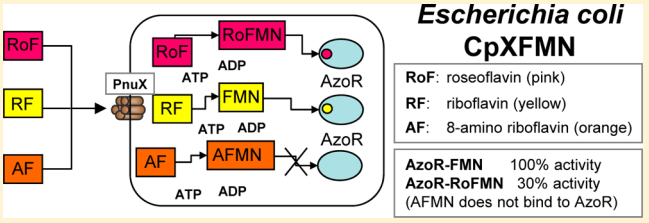
Supporting Information

ABSTRACT: The Gram-positive bacterium *Streptomyces davawensis* is the only organism known to produce the antibiotic roseoflavin. Roseoflavin is a structural riboflavin analogue and is converted to the flavin mononucleotide (FMN) analogue roseoflavin mononucleotide (RoFMN) by flavokinase. FMN-dependent homodimeric azobenzene reductase (AzoR) (EC 1.7.1.6) from *Escherichia coli* was analyzed as a model enzyme. *In vivo* and *in vitro* experiments revealed that RoFMN binds to the AzoR apoenzyme with an even higher affinity compared to that of the "natural" cofactor FMN. Structural analysis (at a resolution of 1.07 Å) revealed that RoFMN binding did not affect the overall topology of the enzyme and also did not interfere with dimerization of AzoR. The AzoR−RoFMN holoenzyme complex was found to be less active (30% of AzoR−FMN activity) in a standard assay. We provide evidence that the different physicochemical properties of RoFMN are responsible for its reduced cofactor activity.



In all organisms, riboflavin (vitamin B_2) serves as the precursor for the flavoprotein cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).[1] FMN is synthesized from riboflavin and ATP by flavokinase (EC 2.7.1.26), whereas FAD is synthesized from FMN and ATP by FAD synthetase (EC 2.7.7.2).[2] The antibiotic roseoflavin (8-dimethylamino-8-demethyl-riboflavin) is produced by the Gram-positive bacterium *Streptomyces davawensis*.[3,4] Roseoflavin is toxic to Gram-positive but also to Gram-negative bacteria if roseoflavin is able to enter the cell.[4−7] Roseoflavin was reported to have a profound inhibiting effect on the growth and infectivity of the human bacterial pathogen *Listeria monocytogenes* at very low concentrations.[8] Many organisms contain riboflavin uptake systems that also account for the import of roseoflavin.[6,7,9,10] Following import, flavokinases/FAD synthetases are responsible for the subsequent activation of roseoflavin to roseoflavin mononucleotide (RoFMN) and roseoflavin adenine dinucleotide (RoFAD) within the cytoplasm of target cells.[11]

In this work, we set out to study exemplarily the FMN-containing azobenzene reductase (EC 1.7.1.6) AzoR from *Escherichia coli* to understand the molecular mechanism of action of roseoflavin and its direct precursor 8-amino-8-demethyl-riboflavin, which is produced by a *rosA* deficient *S. davawensis* strain.[12] AzoR from *E. coli* is an oxidoreductase that is able to degrade azo compounds.[13−15] Moreover, AzoR was reported to be involved in resistance to thiol-specific stress caused by the accumulation of electrophilic quinones.[16] AzoR exists as a homodimer composed of 23 kDa subunits and routinely is tested using the azo compound methyl red as a substrate. The reduction of methyl red by AzoR follows a ping-pong mechanism requiring 2 mol of NADH to reductively cleave 1 mol of methyl red into 2-aminobenzoic acid and *N*,*N*′-dimethyl-*p*-phenylenediamine[13−15] (Scheme 1).

We found that, in contrast to 8-amino-8-demethyl-riboflavin mononucleotide (AFMN), RoFMN bound with high affinity to AzoR. Furthermore, our results suggest that the different physicochemical properties of RoFMN were responsible for rendering AzoR less active.

MATERIALS AND METHODS

Chemicals. Roseoflavin was obtained from MP Biomedicals. 8-Amino-8-demethyl-riboflavin was a gift of S. Ghisla

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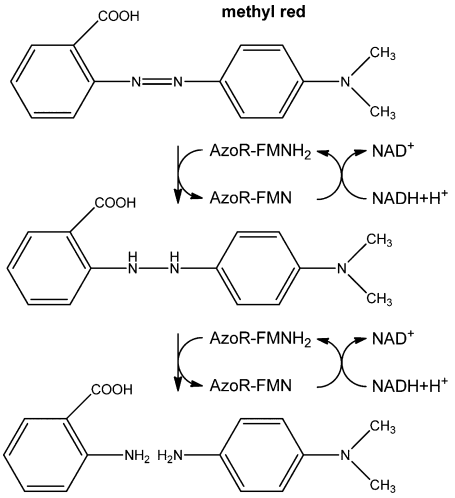
Scheme 1



(University of Konstanz, Konstanz, Germany). Roseoflavin mononucleotide (RoFMN) and 8-amino-8-demethyl-riboflavin mononucleotide (AFMN) were enzymatically prepared using recombinant human flavokinase as described previously.[17] All other chemicals were from Sigma-Aldrich. Restriction endonucleases and other cloning reagents were purchased from Fermentas.

Molecular Cloning. The gene for AzoR was amplified by polymerase chain reaction (PCR) using pCA24N-azoR[18] as a template and the modifying primers 5′-CAG AGC ATA TGA GCA AGG TAT TAG TTC TTA-3′ and 5′-ATA TCT CGA GTG CAG AAA CAA TGC TG-3′. The restriction endonuclease sites *Nde*I and *Xho*I are underlined. The *Nde*I- and *Xho*I-treated PCR product was ligated to *Nde*I- and *Xho*I-digested expression vector pET24a(+) (Invitrogen) to give pET24a(+)C-azoR. The latter plasmid produced a C-terminally His_6-tagged version of AzoR (AzoR-CHis6). The N-terminally His_6-tagged version of AzoR [produced by pET24a(+)N-azoR] was found to have only 50% of AzoR-CHis6 activity and thus was not employed in further experiments.

Synthesis and Purification of Recombinant AzoR. *E. coli* CpXFMN harboring either pET24a(+)C-azoR or pET24a-(+)N-azoR was grown in Luria-Bertani (LB) broth (containing 4 μM riboflavin) to an OD_{600} of 0.5. Synthesis of recombinant AzoR-CHis6 was stimulated by adding 0.1 mM isopropyl thiogalactopyranoside (IPTG) to the cultures. At the same time, riboflavin, roseoflavin, or 8-amino-8-demethyl-riboflavin (50 μM each) was added (to stimulate holoenzyme formation) and the cultures were grown for an additional 12 h to the stationary phase. Cells were harvested by centrifugation (3500*g*) and stored at −20 °C. Frozen cell paste (6 g) was resuspended in 30 mL of HisTrap (GE Healthcare) binding buffer [50 mM Na_2HPO_4 (pH 7.4), 500 mM NaCl, and 20 mM imidazole]. Cells were passed twice through a French press at 2000 bar. Centrifugation (10000*g* and 4 °C) for 45 min removed cell debris and unbroken cells. The lysate was cleared by ultracentrifugation (106000*g* for 30 min at 4 °C) and applied to a 5 mL HisTrap column after equilibration with loading buffer. Chromatographic steps were performed using the ÄKTA purifier system (GE Healthcare). When the UV signal returned to baseline, elution of the His_6-tagged protein was induced by increasing in a stepwise fashion the concentration of the elution buffer [50 mM Na_2HPO_4 (pH 7.4), 500 mM NaCl, and 500 mM imidazole]. Aliquots of the fractions were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and staining with Coomassie Brilliant Blue G-250. The protein concentration was determined by the method of Bradford using bovine serum albumin (BSA) as a standard. Expression and purification of the AzoR apoenzyme essentially were performed as described above; however, at the time point of induction with IPTG, no additional flavin was added to the *E. coli* CpXFMN culture overproducing AzoR. Holoenzyme formation of AzoR was achieved *in vitro* by incubating the AzoR apoenzyme for 1 h at 4 °C in a 100-fold molar excess of FMN, RoFMN, or AFMN. Subsequently, FMN, RoFMN, or AFMN was separated from the AzoR holoenzyme by affinity chromatography as described above. Imidazole was removed from AzoR preparations by gel filtration, and the enzyme was stable in 50 mM potassium phosphate (pH 7.5) for several weeks.

Enzyme Assay. AzoR activity was followed spectrophotometrically in a final volume of 1 mL containing 25 mM Tris-HCl (pH 7.4), 0.1 mM methyl red, 0.4 mM NADH, and 0.08−0.8 μM AzoR.[15] One unit of methyl red reductase activity was defined as the amount of AzoR catalyzing the reduction of 1 μmol of methyl red/min at 30 °C using a molar absorption coefficient of 23360 M^{-1} cm^{-1}. Steady-state kinetic parameters were determined as follows. Rates of individual reactions were obtained by incubating varying amounts of substrates or cofactors with AzoR. The initial rates were plotted against the substrate or cofactor concentrations. To determine the kinetic constants K_m/K_D and V_{max}, the data were fit to the Michaelis−Menten equation using SigmaPlot (Erkrath, Germany).

Preparation of Cell-Free *E. coli* Extracts. Cell-free extracts of *E. coli* CpXFMN were prepared by passing the cells twice through a French press at 2000 bar. Centrifugation (10000*g* and 4 °C) for 45 min removed cell debris and unbroken cells. The lysates were cleared by ultracentrifugation (106000*g* for 30 min at 4 °C) and treated with trichloroacetic acid to fully denature all proteins. The samples were centrifuged (10000*g* and 4 °C), and the flavins in the supernatant were analyzed by high-performance liquid chromatography coupled to mass spectrometry (HPLC−MS).

HPLC Analysis of Flavins. Flavins were analyzed by HPLC−MS essentially as described previously.[12] A Poroshell 120 EC-C18 column [2.7 μm particle size, 50 mm × 3 mm (Agilent, Santa Clara, CA)] was employed. The following solvent system was used at a flow rate of 5 mL/min: 18% (v/v) methanol, 20 mM formic acid, and 20 mM ammonium formate (pH 3.7). Riboflavin, FMN, and FAD were detected photometrically at 445 nm, and roseoflavin, RoFMN, and RoFAD were detected photometrically at 503 nm.

HPLC Analysis of Flavin Cofactors Released from AzoR. Samples of the AzoR holoenzyme were denatured by being treated with 20% trichloroacetic acid and centrifuged at 14000*g* and 4 °C for 10 min. The supernatants of the corresponding samples were analyzed for their flavin content by HPLC−MS (see above).

UV−Vis Absorbance Spectroscopy. UV−vis absorbance spectra were recorded with a specord 210 spectrophotometer (Analytic Jena, Jena, Germany). Difference titrations were conducted at 25 °C in tandem cuvettes by the addition of flavin to the enzyme in the measurement cell (60 μM AzoR for FMN and 35 μM AzoR for RoFMN) and to buffer [50 mM Tris-HCl (pH 8.0)] in the reference cell.

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pH Dependence of the Activity of AzoR. Methyl red was not soluble at pH <5. Thus, anthraquinone-2-sulfonic acid was used to test the pH dependence of AzoR.[16] AzoR activity as a function of pH was determined in 0.1 M McIllvaine buffer over the pH range of 3.5−8.0. AzoR activity was followed spectrophotometrically in a final volume of 1 mL containing 0.4 mM anthraquinone-2-sulfonic acid, 0.4 mM NADH, and 0.08−0.8 μM AzoR. One unit of reductase activity was defined as the amount of AzoR catalyzing the reduction of 1 μmol of anthraquinone-2-sulfonic acid/min at 30 °C using a molar absorption coefficient of 6220 M^{-1} cm^{-1}.

Crystallization. To prepare the protein solution used for crystallization, gel-filtration chromatography was performed with a Superdex 200 column (GE Healthcare) and a solution containing 20 mM Tris-HCl (pH 8.0), 20 mM NaCl, and 1 mM DTT. Fractions containing AzoR were pooled and concentrated using VIVASPIN columns (3000 molecular weight cutoff, Sartorius). Before crystallization, the concentrated protein solution was mixed with an equal volume of a RoFMN or FMN solution (1 mM). Crystals suitable for X-ray analysis were obtained from a drop made by mixing a solution containing 20 mg/mL protein in 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.5 mM DTT, 1.8 mM RoFMN or FMN, and an equal volume of reservoir solution containing 150 mM $MgCl_2$, 38% (v/v) 2-propanol, and 100 mM HEPES-NaOH (pH 7.5). The drop was equilibrated over a 500 μL reservoir solution by the sitting-drop vapor-diffusion method for 1 week at 20 °C.

X-ray Data Collection and Processing. A crystal of the AzoR-CHis6−RoFMN complex was transferred to a cryoprotectant solution [38% (v/v) 2-propanol, 100 mM HEPES-Na (pH 7.5), 150 mM $MgCl_2$, and 30% (v/v) glycerol] and flash-cooled in a nitrogen stream (100 K). An X-ray diffraction data set was collected on the NW-12A beamline at the Photon Factory (PF, Tsukuba, Japan) using an ADSC Quantum 210 detector and an X-ray wavelength of 1.00000 Å. The data set consisted of 600 images with an oscillation angle of 0.3°. The data were indexed, integrated, and scaled using XDS.[19] The crystal belonged to primitive tetragonal space group $P2_1$, with the following unit cell parameters: a = 44.2 Å, b = 105.0 Å, c = 50.9 Å, and β = 111.5°. The crystal contained two molecules per asymmetric unit according to the Matthews coefficient (V_M = 2.44 $Å^3$/Da), which corresponds to a solvent content of 49.6%. The structure determination by molecular replacement in the CCP4 suite was conducted using the coordinates of the AzoR−FMN complex (Protein Data Bank entry 2Z9B) as a search model. Several cycles of manual model rebuilding and refinement were performed using the REFMAC5 module[20] of the CCP4 suite[21] and the graphics suite COOT.[22] The figures showing the three-dimensional protein structure for AzoR were generated using PyMol.

Redox Potential Determination. Redox potentials were determined using the dye equilibration method with the xanthine/xanthine oxidase electron delivering system as described by Massey.[23] All experiments were performed in 50 mM potassium phosphate buffer (pH 7.0) at 25 °C containing benzyl viologen (5 μM) as a mediator, 300 μM xanthine, and xanthine oxidase in catalytic amounts (approximately 1 nM). To maintain anoxic conditions, all experiments were conducted with a stopped-flow device (SF-61DX2, Hi-Tech) positioned in a glovebox from Belle Technology. Spectra were recorded with a KinetaScanT diode array detector (MG-6560). FAD (E_0' = −219 mV) and indigodisulfonic acid potassium salt (E_0' = −125 mV) were used as dyes for redox potential determination. The potentials were calculated from plots of log([ox]/[red]) of the respective variant protein versus log([ox]/[red]) of the dye according to the method of Minnaert.[24]

RESULTS

***E. coli* CpXFMN Is Used for the *in Vivo* Generation of AzoR Loaded with RoFMN and AFMN.** *E. coli* does not contain an uptake system for flavins and thus naturally is resistant to the antibiotic roseoflavin (minimal inhibitory concentration of >50 μg/mL).[4] The functional introduction of (heterologous) riboflavin transporters, however, generates *E. coli* strains that are able to import flavins and consequently are roseoflavin sensitive.[5−7,25] *E. coli* strain CpXFMN constructed and employed in this work to generate *in vivo* AzoR loaded with RoFMN and AFMN (instead of FMN) is a derivative of CmpX131.[25] The latter strain overproduces the flavin transporter PnuX from *Corynebacterium glutamicum*[6] and is riboflavin auxotrophic (*rib*$^-$) because of the chromosomal deletion of the *ribC* gene encoding riboflavin synthase (EC 2.5.1.9). Riboflavin auxotrophy was crucial for our present application to reduce the disturbing effect of endogenously synthesized FMN, (possibly) competing with RoFMN or AFMN for AzoR apoenzyme binding. *E. coli* CpXFMN is different from CmpX131 in that it carries the gene for the monofunctional flavokinase (*FMN1*) from *Schizosaccharomyces pombe*.[26] *FMN1* was introduced to enhance intracellular synthesis of FMN and FMN analogues. The details of the construction of this strain will be published elsewhere. Growth of CpXFMN was tested using LB broth in the presence of 50 μM roseoflavin or 50 μM 8-amino-8-demethyl-riboflavin. The strains showed limited growth in the presence of roseoflavin (final cell density OD_{600} of 0.6 ± 0.1) or 8-amino-8-demethyl-riboflavin (final cell density OD_{600} of 0.8 ± 0.1). Notably, LB broth contained 4 μM riboflavin, which was sufficient to support growth of riboflavin auxotrophic *E. coli* CpXFMN (final cell density OD_{600} of 3.0 ± 0.3).

The Cofactor Analogues RoFMN and AFMN Are Present in the Cytoplasm of *E. coli* CpXFMN. *E. coli* CpXFMN was grown to an OD_{600} of 0.5 and pulsed with riboflavin, roseoflavin, or 8-amino-8-demethyl-riboflavin (50 μM each). The cells were grown for an additional 2 or 20 h, thoroughly washed, and disrupted, and the cell-free extracts subsequently were analyzed with regard to their cofactor content. The data revealed that cells pulsed with riboflavin contained 0.22 ± 0.04 μmol of FMN/g of total cellular protein when harvested after being grown for an additional 2 h and 0.30 ± 0.05 μmol of FMN/g of total cellular protein when harvested after being grown for an additional 20 h. The roseoflavin-pulsed cells contained 0.24 ± 0.03 μmol of RoFMN/g of total cellular protein when harvested after being grown for an additional 2 h and 0.24 ± 0.01 μmol of RoFMN/g of total cellular protein when harvested after being grown for an additional 20 h. The 8-amino-8-demethyl-riboflavin-pulsed cells contained 0.49 ± 0.06 μmol of AFMN/g of total cellular protein when harvested after being grown for an additional 2 h and 0.62 ± 0.03 μmol of AFMN/g of total cellular protein when harvested after being grown for an additional 20 h.

FMN and RoFMN but Not AFMN Bind to the AzoR Apoenzyme *in Vivo*. AzoR-CHis6 was overproduced in a recombinant *E. coli* CpXFMN strain pulsed with riboflavin (50 μM), roseoflavin (50 μM), or 8-amino-8-demethyl-riboflavin (50 μM). Subsequently, AzoR-CHis6 was purified and tested for activity (Figure 1). The cells that had been pulsed with

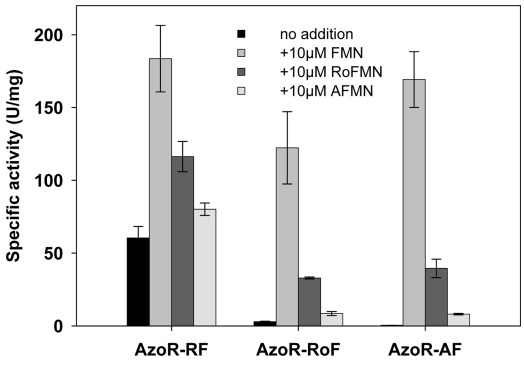

Figure 1. AzoR-CHis6 is less active when isolated from cells grown in the presence of roseoflavin (RoF) or 8-amino-8-demethyl-riboflavin (AF) than from cells grown with riboflavin (RF). AzoR-CHis6 was purified from different *E. coli* CpXFMN cultures that had been pulsed with either RF (AzoR−RF), RoF (AzoR−RoF), or AF (AzoR−AF). The different enzyme preparations (10 μg) were incubated for 30 min in the absence (black bars) or presence of a 100-fold molar excess of flavin mononucleotide (FMN), roseoflavin mononucleotide (RoFMN), or 8-amino-8-demethyl-riboflavin mononucleotide (AFMN). Subsequently, the samples were tested for AzoR activity using methyl red and NADH as substrates. The specific activity is expressed as means ± the standard error ($n \geq 3$).

riboflavin (Figure 1, AzoR−RF) produced active AzoR-CHis6. AzoR-CHis6 activity was enhanced by a factor of 3 (to a maximal level of 184 ± 13 units/mg of protein) upon addition of a 100-fold molar excess of FMN. This finding suggested that FMN was limiting in the cytoplasm of *E. coli* CpXFMN with regard to holoenzyme formation. The addition of RoFMN or AFMN to the latter AzoR preparation did not lead to a fully active enzyme. The cells that had been pulsed with roseoflavin produced essentially inactive AzoR-CHis6 (Figure 1, AzoR−RoF), which could be activated by adding FMN to a level of 122 ± 14 units/mg of protein. The maximal level [184 units/mg of protein (see above)] of activity, however, was not reached. The latter finding suggested that AzoR-CHis6 purified from cells grown with roseoflavin was loaded *in vivo* with inactive RoFMN and that FMN could not displace RoFMN *in vitro* and fully activate the enzyme. AzoR-CHis6 isolated from 8-amino-8-demethyl-riboflavin-pulsed cells was activated upon addition of FMN to almost the maximal level (169 ± 11 units/mg of protein), suggesting that AFMN did not bind to AzoR *in vivo*.

FMN and RoFMN but Not AFMN Bind to the AzoR Apoenzyme *in Vitro*. The AzoR-CHis6 apoenyzme was purified from *E. coli* CpXFMN grown in LB broth (4 μM riboflavin) in the absence of additional flavins, and thus (because of the very limited availability of FMN), AzoR-CHis6 had only little activity (1.4 ± 0.1 units/mg of protein). The pure apoenzyme (10 μg) was incubated for 30 min in the presence of a 100-fold molar excess of FMN, RoFMN, or AFMN or a 1:1 mixture of FMN and RoFMN. Subsequently, unbound flavins were removed by affinity chromatography (Figure S1A of the Supporting Information). SDS−PAGE of the repurified enzyme preparations revealed that AzoR-CHis6 was still intact (and that no degradation of AzoR-CHis6 had occurred) (Figure S1B of the Supporting Information). The different holoenzymes were tested for activity. FMN-treated AzoR-CHis6 was fully active (165 ± 4 units/mg of protein). The RoFMN-treated enzyme had an activity of 47 ± 2 units/mg of protein. The AFMN-incubated enzyme had an activity (0.4 ± 0.1 unit/mg of protein) that corresponded to the residual activity present in the apoenzyme preparation (see above). When FMN (100-fold molar excess) was added to the latter enzyme preparation, AzoR activity increased to 97%. When RoFMN was added, only 30% of the maximal activity was reached. Adding a 1:1 FMN/RoFMN mixture to the AzoR apoenzyme resulted in an enzyme preparation that was active to a maximum of 50%. These results suggested that RoFMN was a good ligand for AzoR but was not fully active as a cofactor.

AzoR Is Less Active in Its RoFMN Form and Has a Higher Affinity for RoFMN Than for FMN. The AzoR-CHis6 apoenzyme (10 μg) was incubated with increasing amounts of FMN or RoFMN (AFMN was found to not bind to the enzyme and therefore was not tested). Subsequently, the activity of the reconstituted AzoR-CHis6 holoenzyme was measured (Figure 2). The data show that AzoR was less active

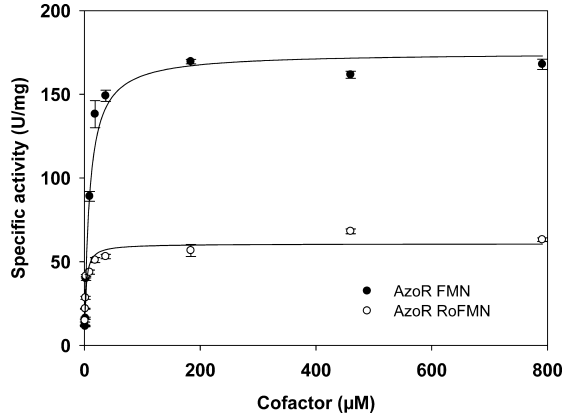

Figure 2. AzoR apoenzyme that binds roseoflavin mononucleotide (RoFMN) with high affinity but is less active in its RoFMN form. Time course of AzoR-catalyzed reduction of methyl red and determination of steady-state kinetic parameters. Rates of individual reactions were obtained by incubating varying amounts of flavin mononucleotide (FMN) or RoFMN with the AzoR apoenzyme. The initial rates were plotted vs the FMN and RoFMN concentrations. To determine the kinetic constants K_D and V_{max}, the data were fit to the Michaelis−Menten equation using SigmaPlot. The specific activity is expressed as means ± the standard error ($n \geq 3$).

in its RoFMN form [V_{max} = 61 ± 2 μmol min^{-1} (mg of protein)$^{-1}$] than in its natural FMN form [V_{max} = 175 ± 6 μmol min^{-1} (mg of protein)$^{-1}$]. The K_M values for the substrates methyl red and NADH+H$^+$ were determined for AzoR in complex with either FMN (AzoR−FMN; K_M methyl red = 27 μM; K_M NADH+H$^+$ = 260 μM) or RoFMN (AzoR−RoFMN; K_M methyl red = 2 μM; K_M NADH+H$^+$ = 269 μM). The data revealed that the AzoR−RoFMN complex has a higher apparent affinity for methyl red than does the AzoR−FMN complex.

Curve fitting (Figure 2) allowed the determination of apparent K_D values for RoFMN (2.2 ± 0.4 μM) and FMN (8.5 ± 1.4 μM). Moreover, difference titrations were conducted at 25 °C in tandem cuvettes by the addition of flavin to AzoR-CHis6 in the measurement cell and to buffer in the reference cell (Figure 3). These data revealed that RoFMN and FMN bind similarly with an upper limit for the dissociation constant of ∼20 μM. Thus, the reduced activity of AzoR in complex with RoFMN (AzoR−RoFMN) cannot be explained by the reduced level of binding of the cofactor analogue.

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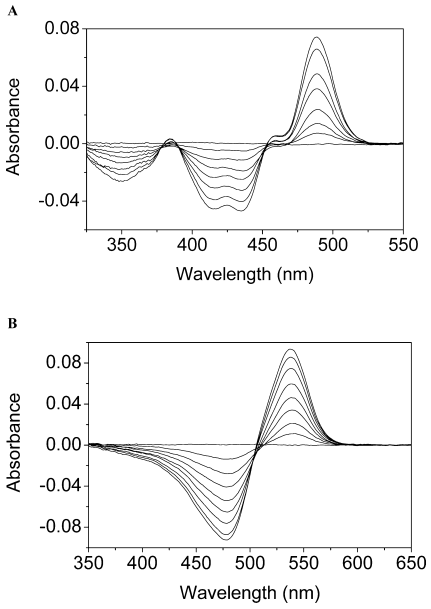

Figure 3. Cofactors flavin mononucleotide (FMN) and roseoflavin mononucleotide (RoFMN) bind with a similar affinities to the AzoR apoenzyme. UV−vis absorbance difference titrations were conducted at 25 °C in tandem cuvettes by the addition of flavin to the enzyme in the measurement cell and to buffer in the reference cell. Flavins were added to the following concentrations: (A) 0 (baseline), 6.9, 13.7, 23.9, 35.5, 40.4, 55.0, and 64.5 μM FMN and (B) 0 (baseline), 3.7, 7.4, 11.1, 14.8, 18.4, 22.0, 25.6, and 29.1 μM RoFMN.

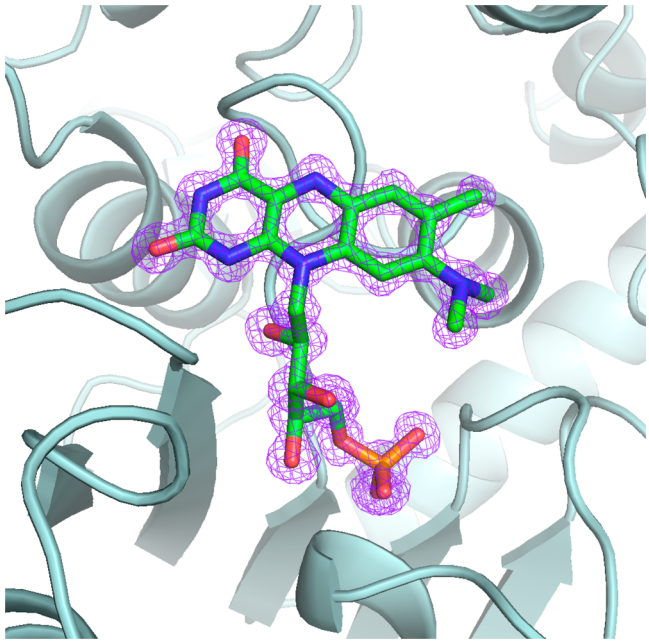

Figure 4. RoFMN binding in the active site of AzoR. Electron density maps surrounding RoFMN contoured at 1.5σ.

Structural Analysis of AzoR-CHis6 in the Presence of RoFMN. AzoR-CHis6 crystallized within 5 days in the presence of RoFMN (Figure S2 of the Supporting Information). The crystal structure of AzoR-CHis6 was determined in complex with RoFMN at a resolution of 1.07 Å and was compared to the structure in complex with FMN that had been determined previously.[13,14] The data collection statistics are summarized in Table S1 of the Supporting Information. The structure of the AzoR−RoFMN complex shows that RoFMN does not affect dimerization of the enzyme and that only minor structural changes occur upon RoFMN binding (Figure S3 of the Supporting Information). RoFMN fits perfectly well into the FMN binding site (Figure 4), whereby the dimethylamino group at C8 of RoFMN is able to make hydrophobic contacts to I10, L11, L50, and V55 (Figure S4 of the Supporting Information) and may also interact with R59. This is different in the case of the AzoR−FMN complex, where the methyl group at C8 was reported not to interact with the enzyme.[13] The dimethylamino group at C8 of RoFMN could also interact with the dimethylamino group of methyl red, which could explain the reduced K_M that was observed for the AzoR−RoFMN complex and the latter substrate. Moreover, in contrast to the AzoR−FMN complex, an asymmetric unit was detected for the AzoR−RoFMN dimer containing two different protomers, AzoR−RoFMN1 and AzoR−RoFMN2 (Figure S5 of the Supporting Information). Upon comparison of the two complexes in the asymmetric unit, a different orientation was found for R59. The electron density of R59, however, was weak, indicating that the side chain was disordered. We tried to match the electron density of R59 as well as possible to a structural model (Figure S4 of the Supporting Information). Interestingly, in RoFMN1, a water molecule was found to be present between R59 and the dimethylamino group of RoFMN (Figure S4A of the Supporting Information), which was not reported for the AzoR−FMN complex and not present in RoFMN2 (Figure S4B of the Supporting Information). The latter may be explained by the fact that in RoFMN2 amino acid residue R59 was found to be closer to the dimethylamino group of RoFMN than that of RoFMN1. Possibly, this water molecule leads to protonation of the cofactor analogue RoFMN, which in turn would lead to different redox properties.[27] No differences were found with regard to cofactor binding between the AzoR−RoFMN and AzoR−FMN complexes, except for the slightly bent dimethylamino group of RoFMN (Figure S6 of the Supporting Information). Figure S7 of the Supporting Information schematically displays interactions between RoFMN and amino acid residues in the active site of AzoR.

pH Dependence of the AzoR−RoFMN Complex. In contrast to FMN, RoFMN contains a dimethylamino group at C8 and thus was hypothesized to be affected differently by changes in pH when compared to FMN. The activities of AzoR−RoFMN and AzoR−FMN complexes were determined at different pH values (pH 3.5−8) using anthraquinone-2-sulfonic acid and NADH as substrates (Figure 5). Because on anthraquinone-2-sulfonic acid the AzoR−RoFMN complex was less active (7%) than the AzoR−FMN complex, the activities were normalized to 100%. The data revealed that the activity of the AzoR−RoFMN complex was slightly reduced at lower pH values compared to that of the AzoR−FMN complex. Moreover, the AzoR−RoFMN complex has an apparent pH optimum of 6.9, whereas the pH optimum of the AzoR−FMN complex is shifted to a more acidic value (pH 6.3). The pK_a of dimethylaniline is 5.8. If we presume that the dimethylamino group of RoFMN has a similar pK_a value, it is plausible that at pH <5.5 a substantial portion of the dimethylamino group at C8 of RoFMN is protonated. The redox potential of RoFMN probably changes significantly upon protonation,[27] which in turn could be an explanation for the reduced activity of the AzoR−RoFMN complex.

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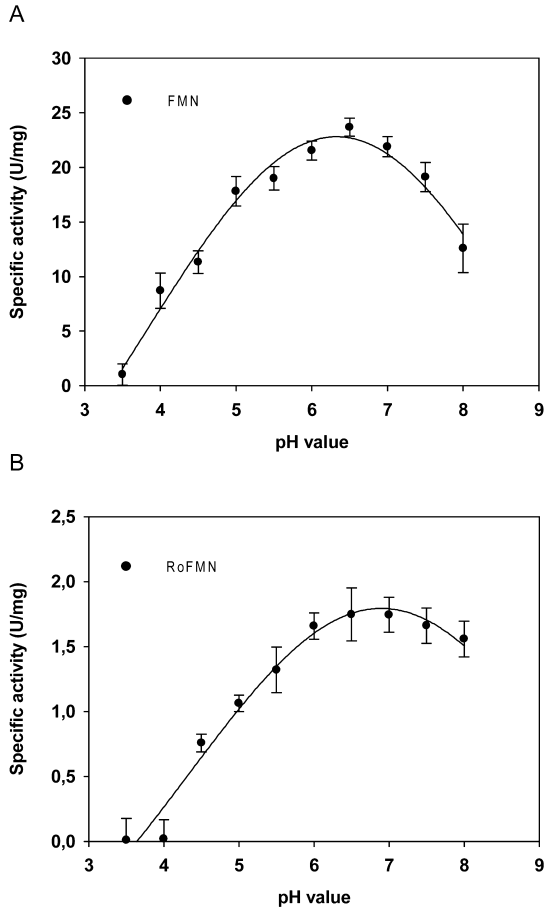

Figure 5. Specific activity of AzoR–FMN and AzoR–RoFMN complexes at different pH values. AzoR–FMN (A) and AzoR–RoFMN (B) complexes were tested at different pH values using anthraquinone-2-sulfonic acid and NADH as substrates. The AzoR–RoFMN complex was less active (7%) than the AzoR–FMN complex. The AzoR–FMN complex shows an apparent pH optimum of 6.3, whereas the AzoR–RoFMN complex has an apparent pH optimum of 6.9.

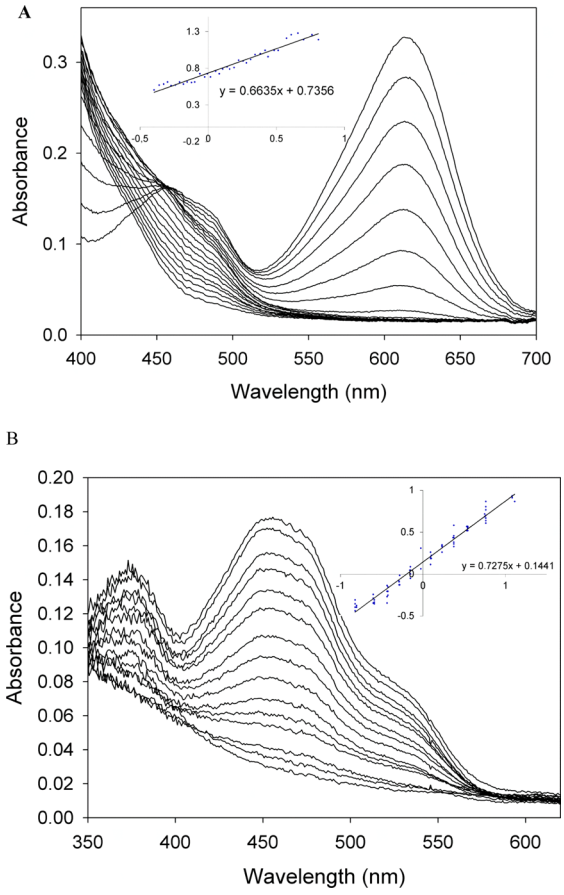

Figure 6. (A) Redox potentials of flavin mononucleotide (FMN) bound to AzoR with indigodisulfonic acid as the reporter dye (E_0 = −125 mV). The decreases in absorbance of the FMN cofactor and indigodisulfonic acid were followed at 480 and 615 nm, respectively. The inset shows the Nernst plot. The determined redox potential of FMN bound to AzoR is −145.4 ± 1.9 mV (three independent measurements). (B) Redox potentials of roseoflavin mononucleotide (RoFMN) bound to AzoR with FAD as the reporter dye (E_0 = −219 mV). The decreases in absorbance of the RoseoFMN cofactor and FAD were followed at 530 and 375 nm, respectively. The inset shows the Nernst plot. The determined redox potential of RoFMN bound to AzoR is −223.0 ± 0.4 mV (three independent measurements).

The Redox Potential of AzoR-Bound FMN Is Different from That of AzoR-Bound RoFMN. The redox potentials (midpoint potentials) of AzoR-bound flavins and free flavins were determined at pH 7 using the dye equilibration method described by Massey.[23] The redox potential of enzyme-bound FMN was −145 mV (−207 mV for free FMN) (Figure 6A), and the redox potential of enzyme-bound RoFMN was −223 mV (−246 mV for free RoFMN) (Figure 6B). These different redox properties are very similar to what was reported for FMN/RoFMN-reconstituted L-lactate oxidase from *Aerococcus viridans*[28] and could explain the reduced activity of the AzoR–RoFMN complex compared to that of the AzoR–FMN complex.

DISCUSSION

Using AzoR[13−16] from *E. coli* as a model, we set out to study the molecular effect of the antibiotics roseoflavin and 8-amino-8-demethyl-riboflavin with regard to flavoenzymes *in vitro* and *in vivo*. Both flavin analogues were taken up by a recombinant *E. coli* strain and were converted to FMN analogues RoFMN and AFMN. Surprisingly, RoFMN was found to bind to AzoR with an apparent affinity even higher than that of the "natural" cofactor FMN. This excellent apparent binding of RoFMN can be explained by our structural data, which revealed additional contacts (when compared to FMN) between the dimethylamino group of RoFMN and amino acids of the cofactor binding site of AzoR. In contrast, AFMN was found to bind to the AzoR apoenzyme neither *in vivo* nor *in vitro*. AFMN contains a hydrophilic amino group at C8, which apparently is not compatible with the cofactor binding site "naturally" accommodating the hydrophobic dimethylbenzene portion of FMN.

It was hypothesized earlier that flavin cofactor analogues with electron-donating substituents at C8 (such as AFMN and RoFMN) may be good steric replacements for FMN and FAD but not catalytic substitutes and consequently may lead to inactivation of flavin-dependent proteins.[29] Our results with RoFMN merely revealed that AzoR is less active, and a complete inactivation of the enzyme was not observed. Possibly, the different redox properties of RoFMN are responsible for the reduced activity of our model flavoenzyme AzoR. At present, however, we cannot predict which of the AzoR partial reactions indeed are negatively affected by the different redox potential. The activity of the AzoR–RoFMN

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complex using the substrate methyl red was found to be reduced (30% of AzoR−FMN activity); when the substrate anthraquinone-2-sulfonic acid was tested, an even greater reduction in activity was found (7% of AzoR−FMN activity). The natural substrates for AzoR are not known,[16] and we therefore only can speculate about the effect(s) of roseoflavin on *E. coli* in a natural setting. Under laboratory conditions, cells grow constantly and relatively fast. In a natural setting, growth occurs only sporadically and many competing cells are present. If RoFMN or RoFAD would reduce the activity of other flavoproteins (~40 are predicted to be present in *E. coli*)[30] with a higher efficiency, this could indeed affect overall growth.

A few other enzymes were found to be less active in combination with RoFMN or RoFAD;[11,28,31] the molecular mechanism of the reduction of activity, however, was not studied. In principle, the toxic effect of roseoflavin could be complex and different for each flavoenzyme target. RoFMN and RoFAD may disturb the overall structure of flavoproteins, may affect multimerization, or may be inactive cofactors because of an altered reactivity. As shown for the AzoR−RoFMN complex and the substrate methyl red, cofactor analogues also have the potential to change the K_M for a certain substrate. In the case of AzoR a reduction in the K_M for methyl red was observed. However, cofactor analogue binding might also lead to an increased K_M for a certain substrate, which in turn might lead to large changes in enzyme activities at the substrate concentrations present in the cell.

Moreover, it is plausible that the activity and functionality of the roseoflavin-derived cofactors depend on the amino acid environment of the cofactor binding site. A very interesting work addressing this question was published by Shinkai and colleagues.[32] They argued that roseoflavin-derived cofactors should be inactive only in polar environments where the isoalloxazine ring loses its oxidizing ability because of a transfer of intramolecular charge from the dimethylamino group to the pteridine moiety. In a more hydrophobic cofactor binding site, however, they predicted that RoFMN could very well be active as a cofactor. In the case of the AzoR−RoFMN1 complex, a single water molecule may provide the polar environment leading to the inactive cofactor analogue (Figure S4 of the Supporting Information).

Flavoenzymes conduct a wide variety of different biochemical reactions.[33,34] The number of genes encoding flavin-dependent proteins varies greatly in the genomes analyzed and covers a range from approximately 0.1 to 3.5% of the predicted genes.[30] Accordingly, a bacterium with a genome of, e.g., 4 Mbp contains an average of 70 potential target proteins for roseoflavin. In addition, nonprotein cellular targets have been identified for roseoflavin and also 8-amino-8-demethyl-riboflavin. According to these works, RoFMN and AFMN block FMN riboswitches, rendering cells riboflavin auxotrophic.[8,35−37]

■ ASSOCIATED CONTENT

Supporting Information

Details of structural data collection and refinement statistics (Table S1), purification of AzoR−FMN and AzoR−RoFMN complexes (Figure S1), pictures of AzoR crystals (Figure S2), the overall structure of the AzoR−RoFMN complex (Figure S3), the two different protomers for the AzoR−RoFMN complex (Figure S4), interactions between RoFMN and the active site of AzoR (Figure S5), structures of flavin cofactors present in AzoR (Figure S6), and contacts of RoFMN with amino acid residues of AzoR (Figure S7). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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