Structure of Dihydromethanopterin Reductase, a Cubic Protein Cage for Redox Transfer*†‡

Dihydromethanopterin reductase (Dmr) is a redox enzyme that plays a key role in generating tetrahydromethanopterin (H₄MPT) for use in one-carbon metabolism by archaea and some bacteria. Dmr is a bacterial enzyme understood to reduce dihydromethanopterin (H₂MPT) to H₄MPT using flavins as the source of reducing equivalents, but the mechanistic details have not been elucidated previously. Here we report the crystal structure of DmrB from Burkholderia xenovorans at a resolution of 1.9 Å. Unexpectedly, the biological unit is a 24-mer composed of eight homotrimers located at the corners of a cubic cage-like structure. Within a homotrimer, each monomer-monomer interface exhibits an active site with two adjacent flavin mononucleotide (FMN) ligands, one deeply buried and tightly bound and one more peripheral, for a total of 48 ligands in the biological unit. Computational docking suggested that the peripheral site could bind either the observed FMN (the electron donor for the overall reaction) or the pterin, H₂MPT (the electron acceptor for the overall reaction), in configurations ideal for electron transfer to and from the tightly bound FMN. On this basis, we propose that DmrB uses a ping-pong mechanism to transfer reducing equivalents from FMN to the pterin substrate. Sequence comparisons suggested that the catalytic mechanism is conserved among the bacterial homologs of DmrB and partially conserved in archaeal homologs, where an alternate electron donor is likely used. In addition to the mechanistic revelations, the structure of DmrB could help guide the development of anti-obesity drugs based on modification of the ecology of the human gut.

Tetrahydromethanopterin (H₄MPT)² is a folate analog that functions as a C1 carrier in diverse organisms (1–3). H₄MPT is essential to the energy-yielding metabolism of many archaea and is required by virtually all methanogens (2, 4). Within the domain Bacteria, the use of H₄MPT (dephospho form) is somewhat restricted. The only established role at this time is in formaldehyde oxidation by methylotrophic bacteria (5–9). Because of its wide use in prokaryotic C1 metabolism, H₄MPT is critical to the cycling of greenhouse gasses and environmental biodegradation. Also of interest is the possibility that inhibition of microbial H₄MPT biosynthesis might be used to treat obesity in humans (10–12). The methanogenic archaea have a major effect on the breakdown of organic matter in anaerobic environments by acting as H₂ sinks (13), and recent studies indicate that the most prominent methanogen in the human gut (Methanobrevibacter smithii) enhances the fermentation of indigestible fiber to short-chain fatty acids, thereby supplying added calories to the host and increasing adiposity (11). Consequently, it is suggested that control of M. smithii in the gut might provide a novel approach to treat obesity (12). Enzymes involved in the synthesis of H₄MPT are inviting targets for selective inhibition of methanogenic bacteria, because H₄MPT is essential to methanogens but is absent from other prominent gut bacteria. Moreover, most of the archaean enzymes used for H₄MPT synthesis are unrelated in sequence to those used for folate production (14).

The final step of H₄MPT biosynthesis is the conversion of dihydromethanopterin (H₂MPT) to H₄MPT by H₂MPT reductase (Dmr) (Fig. 1). Prior studies have indicated that the Bxe_B2440 protein from Burkholderia xenovorans and the DmrX protein from the archaeon Methanosarcina mazei are Dmr enzymes.

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The atomic coordinates and structure factors (codes 3WIS and 4MWG) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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Two abbreviations used are: H₄MPT, tetrahydromethanopterin; DmrB, dihydromethanopterin reductase; H₂MPT, dihydromethanopterin; HPDC, homo-oligomer flavin-containing decarboxylase; SEC-MALS, size exclusion chromatography coupled with multi-angle light scattering; ITC, isothermal titration calorimetry; +FMN, crystals grown in the presence of added FMN; −FMN, crystals grown in the absence of added FMN; MR, molecular replacement; PDB, Protein Data Bank.
(15, 16). DmrX and Bxe_B2440 (the latter often annotated as AfpA (archaeal flavoprotein) but which we shall refer to as DmrB (dihydromethanopterin reductase)) are 239 and 192 amino acids in length, respectively. They are 35% identical over 144 N-terminal amino acids, although DmrX has a C-terminal insertion that includes two 4Fe-4S binding motifs not found in DmrB (17). Within the domain Bacteria, DmrB-type enzymes are found only within a relatively small group of methylotrophs (15). In contrast, DmrX-type enzymes are absent from bacteria but present in most methanogenic archaea (17). Many archaea also contain a paralog of DmrX known as AfpA. This enzyme is proposed to function in electron transport and has no known role in H4MPT biosynthesis (17).

Sequence analyses indicate that DmrB is a member of the homo-oligomeric flavin-containing cysteine decarboxylase (HFCD) superfamily (18–20). Members of this superfamily are widespread in nature and functionally diverse. Superfamily representatives MrsD and EpiD catalyzes the decarboxylation of peptidyl-cysteine residues during the synthesis of antibiotics (19, 21). Dfp catalyzes the decarboxylation of 4’-phosphopantothenoylcysteine for coenzyme A biosynthesis (20). Other HFCD members catalyze the decarboxylation of aromatic amino acids, an oxidative step in dipicolinic acid synthesis, and electron transfer reactions in the methanogens or are involved in signal transduction in Arabidopsis (17, 18, 22–24). In some cases, HFCD-related sequences are found as domains in multidomain proteins (20). In addition, many HFCD family members have unknown functions.

A characteristic feature of the HFCD superfamily is their unique mode of flavin binding (18, 20). FMN is bound at a subunit interface with the re side of the isoalloxazine ring facing the active site (18, 19). Two semi-conserved motifs (TGSG and PATANT) play key roles in flavin binding and can be used to identify HFCD family members (17). The tertiary structure of HFCD monomers is based on the well known Rossmann fold. The structure is also generally similar to flavodoxin, but the mode of flavin binding and the order of secondary structural elements are distinctly different (18). The quaternary structures of HFCD members are diverse and include dimers (17), trimers (18, 25), and in several cases dodecameric cages (20–23).

Here we present the crystal structure of DmrB from the bacterium B. xenovorans at a resolution of 1.9 Å using phases determined by anomalous scattering methods. This is the first reported structure of a Dmr enzyme. Comparing it with other HFCD family members, we show that DmrB has two distinctive features. It binds two molecules of FMN per active site, and the biological unit is a large cage-like 24-mer composed of eight homotrimers positioned at the corners of a cube. Based on the DmrB structure and on biochemical and biophysical characterization of substrate binding, as well as modeling studies, we propose a catalytic mechanism and predict the key catalytic residues involved in redox transfer.

**EXPERIMENTAL PROCEDURES**

**Media and Chemicals**—The rich medium used was lysogeny broth (LB), also known as Luria-Bertani medium (EMD Millipore, Gibbstown, NJ) (26). Antibiotics, DNase I, FMN, FAD, and lysozyme were from Sigma. IPTG and DTT were from Gold Biotechnology, Inc. (St. Louis, MO).
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Protein Methods—SDS-PAGE and native-PAGE were performed using Bio-Rad Ready gels and Bio-Rad Mini-Protean Tetra electrophoresis cells according to the manufacturer’s instructions (Bio-Rad). Following gel electrophoresis, Coomasie Brilliant Blue R-250 was used to stain proteins. The protein concentration of solutions was determined using Bio-Rad protein assay reagent (Bio-Rad), which is based on the method of Bradford, with bovine serum albumin as the standard (27).

His$_{6}$-DmrB Production and Purification—DmrB from *B. xenovorans* was codon-optimized for expression in *Escherichia coli* and fused to an N-terminal His$_{6}$ tag by gene synthesis (GenScript, Piscataway, NJ). His$_{6}$-DmrB was subcloned into T7 expression vector pET41a (Novagen, Darmstadt, Germany) via Ndel and HindIII sites using standard protocols (28). The sequence of the clone was verified by the dyeoxy chain termination method, and it was transformed into BL21DE3 RIL (Strategene, La Jolla, CA) to produce the protein production strain used in these studies (*E. coli* BL21 (DE3)-RIL/pET41a-His$_{6}$-DrmB).

The His$_{6}$-DmrB production strain (MER3) was grown at 37 °C with shaking at 240 rpm in 2-liter baffled flasks containing 1 liter of LB with 25 μg ml$^{-1}$ kanamycin and 10 μg ml$^{-1}$ chloramphenicol. When the *A$_{600}$* of the culture reached 0.6–0.8, the growth temperature was reduced to 30 °C, and protein production was induced by the addition of IPTG to 0.5 mM. Cells were grown an additional 6 h and then harvested by centrifugation using a Sorvall RC-5C Plus centrifuge and Sorvall SLC-4000 rotor at 5,000 rpm and 4 °C. Cells were washed with 50 mM Tris HCl, pH 8.0, and 200 mM NaCl, 5% glycerol, 20 mM imidazole, 1 mg ml$^{-1}$ lysozyme, 0.01 mg ml$^{-1}$ DNase, 4 mM DTT, and Roche Complete protease inhibitor. Resuspended cells were broken using an Emulsiflex (Avestin, Inc., Ottawa, Ontario, Canada) as described (29). The cell lysate was clarified by centrifugation at 32,000 g for 30 min. The supernatant (~40 ml) was passed through a 0.45-μm syringe filter (Millipore), and His$_{6}$-DmrB was purified from the filtered cell lysate as follows. Buffer A contained 50 mM Tris HCl, pH 8.0, 200 mM NaCl, 5% glycerol, and 4 mM DTT (added just before use). Buffer B was similar to buffer A but also included 500 mM imidazole. A 5-ml HisTrap HP column (GE Healthcare) was equilibrated with 4% buffer B. About 40 ml of clarified cell lysate (~1 g of protein) was loaded using a 50-ml Superloop (GE Healthcare). The column was developed with 15 column volumes of 4% buffer B followed by 10 column volumes of 15% buffer B and a 15-column volume linear gradient from 4 to 100% buffer B. The elution of proteins from the column was followed by monitoring *A$_{280}$*. DmrB eluted at 125–300 mm imidazole. The best fractions obtained from the nickel affinity chromatography were pooled and concentrated to 20 mg ml$^{-1}$ using a 30-kDa cutoff centrifugal concentrator (Millipore). Concentrated His$_{6}$-DmrB was further purified using an AKTA purifier and S200 16/300 column (GE Healthcare) loaded with 250 μl of sample and developed at 0.4 ml min$^{-1}$ with 20 mM Tris HCl, pH 8, 100 mM NaCl, 5% glycerol, and 4 mM DTT. A minor void peak was observed, but the major His$_{6}$-DmrB peak was centered at 10.3 ml with a small upstream shoulder. The best fractions were concentrated using a 30-kDa centrifugal filter to give a protein concentration of 13 mg ml$^{-1}$ as determined with a Bio-Rad protein assay reagent. Concentrated DmrB was frozen in liquid N$_{2}$ and stored at −80 °C before being used to set crystal trays.

Size Exclusion Chromatography Coupled to Multi-angle Light Scattering (SEC-MALS)—Purified His$_{6}$-DmrB was analyzed with SEC-MALS. Thawed protein was diluted to a 4 mg ml$^{-1}$ concentration, and 100 μl of protein was loaded onto a WTC-030S5 analytical size exclusion column (Wyatt Technology Co., Santa Barbara, CA) using an AKTA purifier (GE Healthcare). The Rayleigh ratio and differential refractive index measurements were performed with a miniDAWN TREOS and an Optilab T-rEX system, respectively (Wyatt Technology Co.). The weight average molecular mass was calculated in ASTRA 6 software (Wyatt Technology Co.) with a differential index of refraction of 0.185 ml g$^{-1}$ for protein components.

Extraction of the Flavin Cofactor from DmrB—The flavin content of DmrB was determined as described using ε$_{448}$ nm = 12,200 M$^{-1}$ cm$^{-1}$ (30). Briefly, a microcentrifuge tube containing purified DmrB (13 mg ml$^{-1}$) was placed in a boiling water bath in the dark for 10 min and then cooled on ice. Precipitated proteins were pelleted by centrifugation at 4 °C and 16,200 g in an Eppendorf 5418-R microcentrifuge. The supernatant was filtered through a 0.2-μm syringe filter (Millipore), and UV-Vis spectra were determined using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA).

High Pressure Liquid Chromatography (HPLC)—To distinguish between FMN and FAD, the cofactors extracted from DmrB were analyzed by reverse phase HPLC. Buffer A consisted of double deionized water containing 1 g liter$^{-1}$ trifluoroacetic acid (Thermo Fisher Scientific). Buffer B consisted of 95% acetonitrile (Thermo Fisher Scientific) in aqueous 0.1% trifluoroacetic acid. The HPLC system included a Waters model 620 pump and model 2487 UV detector. Separation was effected with a 250 × 4.6-mm C18 column (Western Analytical, Lake Elsinore, CA) equilibrated with buffer A and developed with a linear gradient for 0–40% B over 30 min at a flow rate of 1 ml min$^{-1}$. Flavins were detected by monitoring absorbance at 260 nm.

Crystallography—DmrB was crystallized using the hanging drop vapor diffusion method. Initial crystallization trials were performed at the UCLA Crystallization Facility using 96-well plates and commercially available sparse matrix screens. Trays were set with 210-nl drops using a Mosquito liquid handling device (TTP LabTech), and optimizations were done manually using 24-well Linbro plates. The crystals used for structure determination were obtained by optimization of the Qiagen AmSO$_{4}$ suite, well G2. Reservoirs contained 500 μl of (NH$_{4}$)$_{2}$SO$_{4}$ from 0.8 to 1.2 M. Hanging drops (3 μl) contained His$_{6}$-DmrB (in purification buffer at 13 mg ml$^{-1}$) and reservoir in the following ratios (v/v): 1:2, 1.5:1.5, and 2:1. Crystals took about 7–14 days to develop at room temperature and were yellow in color. Crystals of DmrB were also grown in the presence of added FMN. In this case, FMN and DmrB (0.5 mM each) were incubated on ice.
for 1 h, and then crystallization was carried out as described for crystal optimization above.

Data Collection—Diffraction data were collected at 100 K at APS-NECAT beamline 24-ID-C on a DECTRIS-PILATUS 6M detector. Single crystals were mounted with CrystalCap HT Cryoloops (Hampton Research, Aliso Viejo, CA). DmrB crystals were cryoprotected in mother liquor containing 33% glycerol and flash-frozen in liquid nitrogen prior to data collection.

Data Processing, Phasing, and Refinement—Data sets were collected from three types of crystals: 1) DmrB; 2) DmrB soaked for 10 s in a 1 μl KI solution (31); and 3) DmrB co-crystallized in the presence of equimolar FMN (DmrB-FMN) (Table 1). The KI dataset was collected at a wavelength of 1.46 Å. Data were indexed, integrated, and scaled using XDS/XSCALE with conservative resolution limits to balance the calculated I/σ(I), RSym, and CC1/2 in the highest resolution shell (32). The structure of DmrB was solved by SIRAS (single isomorphous replacement with anomalous scattering) using the graphic interface for the SHELXC/D/E programs. Two iodide sites were found by SHELXD (34). SHELXE (35) was used to assign the hand, produce the first set of phases, and perform solvent flattening. The final figure of merit was 0.667. A map based on the new set of phases was processed with Buccaneer (36), which traced 95% of the protein residues. The DmrB model was refined to 2.2 Å. This model was further refined against the high resolution 1.9 Å DmrB-FMN data set. More than 92% of the residues in the model were found in the most favored regions of the Ramachandran plot by PROCHECK (37). Final iterative rounds of model building and refinement were carried out using Coot (38) and PHENIX (39) with TLS refinement (40) and BUSTER (41). Data collection, phasing, and refinement statistics are presented in Table 1. There are one molecule of DmrB and 2 molecules of FMN in the asymmetric unit. The coordinates of the final models and the merged structure factors have been deposited in the Protein Data Bank with PDB codes 3WIS and 4MWG. All images of protein structures were made using PyMOL (42).

Quantification of Free and Bound FMN in Crystallization Drops with Spectrophotometry and X-ray Crystallography—To estimate bound FMN, additional crystals of DmrB harvested from hanging drops without and with added FMN (383 μM) were characterized structurally. Diffraction data were collected from four crystals (two with and two without added FMN), and the data were processed as described above, except that phasing was done by molecular replacement (MR) using the program Phaser (43) and the deposited DmrB structure (PDB code 3WIS) with the FMN molecules removed as the search model. Successful MR solutions were subjected automatically to rigid body refinement and immediately visualized in Coot to determine the mFo – DFc difference density in the FMN binding region (38). The FMN binding region was defined by aligning the FMN-bound DmrB structure with the MR solutions. The structure factors and models from this process were not subjected to further refinement and deposition.

To determine the FMN concentration in drops (from which crystals were harvested) 6 μl of well solution was added to each 3 μl of hanging drop. Following centrifugation at 14,900 × g for 5 min, 5 μl of the supernatant was removed and added to 5 μl of well solution. The final diluted hanging drop (15% of original concentration) was filtered of remaining crystals by centrifugation for 2 min at 12,000 × g with 0.1 μm microcentrifugal filters (Millipore). The concentration of FMN in the filtered solution was determined measuring absorbance at 448 nm using a Cary 60 UV-Vis spectrophotometer equipped with a small volume tray cell adapter (Agilent Technologies) by comparison with a standard curve.

Amino Acid Analysis—Briefly, purified DmrB was dialyzed repeatedly into double deionized water overnight using a 3500 molecular weight cutoff dialysis cassette (Thermo Fisher Scientific). TriPLICATE samples underwent vapor-phase hydrolysis in 6 N HCl for 22 h at 110 °C. Free amino acids were fractionated and quantitated by reversed phase HPLC following precolumn fluorescent derivatization. The extinction coefficient was determined experimentally by comparison of the average molar protein concentration and by measuring the absorbance at 280 nm of the same sample used for hydrolysis.

Isothermal Titration Calorimetry (ITC)—Purified DmrB (15 μM) was titrated with FMN (323 μM, >98% purity) at 37 °C using a MicroCal iTC200 (GE Healthcare). A series of 26 injections was performed including one 0.5-μl injection and 25 1.4-μl injections with 3 min between each injection. A titration of 323 μM FMN into buffer was used to subtract the background heat of dilution from the raw data, which were subsequently integrated with the program NITPIC (44). The corrected, integrated data with the initial 0.5-μl injection removed was fit to a single-site binding thermodynamic model using nonlinear regression in the program SEDPHAT (45). The estimated errors for nonlinear regression with a single-site binding model are based on the critical value for chi-squared at a 68.3% confidence level.

Bioinformatics Methods—Ligand and receptor PDBQT files for the docking simulations were prepared with AutoDockTools (46), where the partial atomic Gasteiger charges were added to both the receptors and ligands. Rotatable bonds were set for the ligands, except for the atoms forming the rings, which were made non-rotatable. The grid box was set as a 64 × 64 × 64 Å cube centered on a homotrimer. AutoDock Vina was run on eight CPUs with an exhaustiveness parameter of 24 for docking simulations. Proposed docking models were collected from the cluster with the best binding affinity reported by Vina. Contacts between DmrB and the ligands were measured with a Python script implemented in PyMOL (42).

To construct multiple sequence alignments, homologs of DmrB and DmrX were identified by BLASTp searches of the complete microbial genomes available at the National Center for Biotechnology Information (47). Alignments were prepared with MUSCLE (48) and analyzed with Jalview (49).

RESULTS

Purification and Flavin Content—N-terminally His-tagged DmrB from B. xenorovans was produced at high levels in E. coli and purified by nickel-nitritolriacetic acid and gel filtration chromatography. SDS-PAGE indicated the purity of DmrB was >95%, and the yield obtained was about 2 mg g−1 of wet cell paste. DmrB was bright yellow in color, and its UV-visible spectrum had absorbance maxima at 454 and 383 nm, indicative of
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a bound flavin cofactor (FMN or FAD) (data not shown) (30). The cofactor of DmrB was released by boiling and analyzed by reversed phase HPLC. Its retention time was nearly identical to that of an FMN standard (25.52 versus 25.53 min) and different from that of FAD (24.06 min). For the released cofactor, the absorbance maxima shifted from 454 and 383 nm to 449 and 377 nm, which were very close to the values obtained for authentic FMN under the same conditions (448 and 377 nm). Thus, HPLC and UV-visible spectroscopy indicated that FMN is the cofactor of DmrB.

To determine the FMN content of DmrB under conditions of heterologous overexpression in E. coli, we quantitated the extracted flavin using the absorbance at 448 nm and determined the protein concentration by the Bradford method (30). Based on these values, the flavin occupancy was calculated to be 10%. Some flavin might have been lost during purification, or perhaps the E. coli expression host could not synthesize enough flavin to saturate DmrB, which was produced at very high levels via a T7 expression system. On the basis on that finding, the addition of FMN was included as an experimental variable during crystallization experiments.

Crystal Structure Determination—Crystals of DmrB were grown without and with added equimolar FMN (0.5 mM). Without added FMN, crystals were yellow in color, rectangular in shape, and ranged in size from about 50 to 250 μm. With added FMN, crystals were similar in shape but darker yellow and smaller (25–150 μm). Crystals grown in the presence of FMN were better ordered and gave good diffraction to 1.9 Å resolution. Phasing by MR failed using the top 10 homology-based models obtained via Phyre2 (50); the best model was 25% identical in amino acid sequence with 82% coverage. Subsequently, phases were obtained by SIRAS using diffraction data sets collected from native and KI-soaked crystals. Using data from crystals grown in the presence of added FMN, the structure of DmrB was built and refined to 1.9 Å resolution with residual errors of Rwork = 0.16 and Rfree = 0.18 (Table 1).

Subunit and Quaternary Structure—As expected, the tertiary structure of DmrB is similar to that of other members of the HFCD family, being based on a Rossmann fold, which typically consists of a six-stranded parallel β-sheet flanked by α-helices (20, 30). DmrB has a central five-stranded parallel β-sheet flanked by eight helices and a two-stranded anti-parallel β-sheet (Fig. 2A).

The quaternary structure of DmrB is remarkable. The biological unit is a 24-mer with a cage-like structure formed from eight trimeric units (Fig. 2D). The trimeric building block for DmrB is very similar to the trimeric units reported for other members of the HFCD superfamily (Fig. 2B). Some of those have appeared as 12-subunit structures composed of four trimers (20–23), but the 24-subunit cage formed by DmrB has not been observed previously. In DmrB, eight trimers are situated at the corners of a cube. The overall assembly obeys octahedral symmetry, as it sits at the origin of the cubically symmetric unit cell. The cage has a large central cavity 55 Å in diameter and wide openings (about 30 Å in diameter at the narrowest point) on the six faces of the cube. Within each trimer, each subunit buries 1664 Å² of its surface according to the program Pisa (51). Along the 12 edges of the cube, two monomers from different trimers form interfaces that hold the eight trimers together in the complete 24-mer. Each of the 12 dimeric interfaces that stabilize the 24-mer buries surfaces of 1388 Å². In total, 15.2% of the total DmrB surface is buried by assembly of the 24-mer.

Solution studies substantiate that the biological unit is the 24-mer. SEC-MALS indicated a weight average molecular mass of 507 ± 19 kDa for the complete assembly (Fig. 2C). Given that the mass of His6-DmrB is 22.1 kDa, the estimated oligomeric state (n ≈ 23 ± 1) is in excellent agreement with the conclusion from crystal structure analysis that the quaternary structure comprises 24 subunits. The symmetric portion of the retained peak was used to determine the highly monodisperse calculated mass by SEC-MALS. In addition, native-PAGE of DmrB (Fig. 2C, inset) indicated one major species in solution.

DmrB Binds Two Molecules of FMN—At each of the monomer-monomer interfaces within a trimer, we observed a deep active site cleft for FMN binding. The presence of this interfacial binding cleft is conserved across the functionally diverse HFCD superfamily. However, the structure of DmrB (crystallized in the presence of FMN) revealed a novel and unexpected feature. In contrast to the numerous structures of other proteins from the HFCD family, which bind a single flavin cofactor,

### TABLE 1 X-ray data collection and refinement statistics

| Data collection | DmrB-FMN | DmrB | DmrB + iodide |
|-----------------|---------|------|--------------|
| PDB accession code | 3W15 | 4MWG | F343 |
| Space group | F432 | F432 | F432 |
| Unit cell dimensions | | | |
| a = b = c (Å) | 183.84 | 184.18 | 183.01 |
| α = β = γ (°) | 90.0 | 90.0 | 90.0 |
| Reflections observed | 536,788 | 353,276 | 134,492 |
| Unique reflections | 21,516 | 14,076 | 9,943 |
| Wavelength (Å) | 0.9797 | 0.9797 | 0.9797 |
| Resolution (Å) | 91.9–1.9 | 65.1–2.2 | 91.5–3.0 |
| Highest resolution shell (Å) | 1.95–1.90 | 2.26–2.20 | 3.08–3.00 |
| Rwork (%) | 7.1 (95.4) | 4.2 (59.8) | 12.0 (67.8) |
| CC(1/2) | 100.0 (93.1) | 100.0 (96.6) | 99.7 (95.5) |
| Δρmax (e Å⁻³) | 36.23 (5.04) | 57.17 (7.74) | 18.0 (5.21) |
| Completeness (%) | 99.9 (99.6) | 99.5 (96.1) | 99.8 (100.0) |
| Wilson B-value (Å²) | 28.75 | 42.68 | 63.25 |

| Refinement | | | |
| Resolution (Å) | 91.9–1.90 | 65.1–2.20 |
| Resolution (Å) | 1.98–1.90 | 2.37–2.20 |
| Reflections used | 25,514 | 14,073 |
| Rwork (%) | 16.1 (18.8) | 18.4 (21.8) |
| Protein molecules in asymmetric unit | 1 | 1 |
| No. of non-H atoms | | |
| Protein | 1,413 | 1,361 |
| Non-protein | 141 | 44 |
| r.m.s. deviations | | |
| Bond lengths (Å) | 0.008 | 0.009 |
| Bond angles (°) | 1.11 | 1.02 |
| Average B-factor (Å²) | 34.3 | 55.0 |
| Protein atoms | 41.7 | 55.6 |
| Ramachandran plot regions | | |
| Most favored | 92.4 | 90.0 |
| Allowed | 7.6 | 10.0 |
| Generously allowed | 0.0 | 0.0 |
| Disallowed | 0.0 | 0.0 |

* Highest resolution shells shown in parentheses.

† Rwork = Σ||Fo|−|Fc||/Σ|Fo|.

‡ Rfree was calculated using 5% of the data.

§ Root mean square.

Percentage of residues in Ramachandran plot regions was determined using PROCHECK (37).
in each active site, DmrB was found to bind two molecules of FMN, in chemically distinct environments, face-to-face within each active site. This is clearly demonstrated by an $F_o - F_c$ simulated annealing map (Fig. 3B). We will refer to the two distinctly bound flavins as FMN-1 and FMN-2. FMN-1 is buried deeply in the DmrB binding cleft in a manner characteristic of other HFCD family members (Fig. 3A) (20, 30). Two semi-conserved motifs are critical to FMN-1 binding: TGSG (residues 23–26) and PATSNT (residues 96–101) (17). These motifs form numerous H-bonds with the ribitol phosphate side chain and the isoalloxazine ring. Both motifs are located on the same DmrB monomer, and this subunit plays the major role in FMN-1 binding. In addition, however, the neighboring monomer interacts with FMN-1 via five residues, four of which form H-bonds to its ribitol moiety (Ser-76, Ser-77, Ser-110, and Asn-116) and one of which interacts with O2 of the isoalloxazine ring (Lys-123) (Fig. 1). Overall, 14 residues of DmrB interact directly with FMN-1.

FMN-2 makes far fewer contacts with DmrB than does FMN-1 (Fig. 3, C and D). Its isoalloxazine ring sits in the active site cleft, but its ribitol phosphate tail extends outward into solvent. The tail of FMN-2 makes four protein contacts, and its isoalloxazine ring contacts three residues, which likely affects catalysis as described below. In total, seven DmrB residues contact FMN-2, in contrast to FMN-1, which contacts 14 residues.

FMN-1 and FMN-2 also interact with one another (Fig. 3). The two isoalloxazine rings are nearly planar and are stacked face-to-face with each other but in a slightly tilted orientation. The re-face of FMN-1 contacts the re-face of FMN-2. The proximal parts of the ring systems are only about 3.2–3.5 Å apart. Hence, there are numerous atomic interactions, including π stacking between the dimethylbenzene rings. Most notably, the N5 position of FMN-1 and the N5 position of FMN-2 are properly situated to allow hydride transfer between the two flavins.

Characterization of Flavin Binding Sites in DmrB—For characterization of the FMN binding site of DmrB, we first experi-
mentally determined the extinction coefficient of DmrB at 280 nm to be 87,700 M⁻¹ cm⁻¹ using a protein concentration determined from amino acid analysis. Subsequently, this extinction coefficient was used to determine the molar concentration ratios for ITC experiments (Fig. 4A). Under the conditions examined, the results indicated that DmrB possesses one high-affinity site (n = 0.96 ± 0.02 sites) for FMN with a Kd of 40 ± 20 nM. The titration performed at 37 °C revealed a large exothermic change in enthalpy of 11.5 ± 0.47 kcal mol⁻¹. The unfavorable change in entropy upon binding at this temperature was −3.08 ± 0.0029 cal mol⁻¹ deg⁻¹. A second transition for binding the second FMN was not evident by ITC.

To further examine FMN binding by DmrB, we measured the concentration of free FMN in equilibrium with bound FMN in crystallization drops. Using UV-visible spectroscopy (see “Experimental Procedures”), we determined that drops containing 383 μM added FMN contained 40 μM free FMN after the formation of DmrB crystals. To estimate the amount of FMN bound by DmrB crystals, structures were determined for two crystals from drops with and without added FMN. The four crystals examined were nearly isomorphous with the crystals used for determination of the deposited structures (data not shown). The best quality diffraction data of +FMN and −FMN crystals were processed to 2.2 and 2.1 Å resolution, respectively, with overall Rsym equal to 14.9 and 12.1%, I/σ equal to 2.8 and 2.2, and CC1/2 equal to 80.9 and 69.4 in the highest resolution shells. The difference electron density was visualized following molecular replacement using the coordinates of the refined DmrB model (without FMN), and the resulting coordinates were essentially identical, as expected. In difference maps for +FMN crystals, unambiguous electron density was present for both molecules of FMN (Fig. 4B). A small amount of positive density was present at the position of the FMN-1 terminal phosphate (Fig. 4C). As expected, −FMN crystals showed only limited electron density in the FMN binding sites. Taken together, these studies indicated that both FMN sites of DmrB had high occupancy when the concentration of free FMN was 40 μM.

Structural Features Indicative of Hydride Ion Transfer between the Two Flavins—Analysis of DmrB revealed a number of conserved structural features typical of flavin dehydroge-
The N5 atom of FMN acts as a hydride ion donor/acceptor in electron transfer reactions (Fig. 1). In many cases, the substrate site (S) undergoing oxidation is ~3.5 Å from N5 of the flavin (52). In addition, the N10-N5-S angle is typically within the narrow range of 96 to 117° (52). The distance between N5 of FMN-1 and N5 of FMN-2 is 3.5 Å (Fig. 5). Hence, these sites are an ideal distance for hydride transfer between them. Assigning the N5 nitrogen of FMN-1 as the substrate site undergoing oxidation, the N10-N5-S angle is 76°. Regarding the N5 nitrogen of FMN-1 as the substrate site, the N10-N5-S angle is 74°. These angles are outside the typical range, but exceptions are known (52). In addition, in the prior cases that were examined the substrate site was a carbon atom (52). In DmrB, the substrate sites evaluated were the N5 atoms of FMN-1 and FMN-2. This difference might account for the atypical N10-N5-S angle in DmrB. Hence, we propose that the hydride is passed between the N5 positions of FMN-1 and FMN-2. Importantly, this idea is supported by multiple structural features of DmrB as described below.

A common feature of FMN dehydrogenases is that an H-bond donor (usually a backbone nitrogen) is located ~3.5 Å from N5 (on the side of the flavin away from the substrate) such that the angle between N5, N10, and the H-bond donor ranges from 116 to 170° (52). For FMN-1 of DmrB, the backbone amino group of Gly-26 is 3.1 Å from N5, and the aforementioned angle is 135° (Fig. 5). For FMN-2, the H-bond donor to N5 is the side chain nitrogen of Gln-120, which is 3.2 Å from N5 and the relevant angle is 160°. The presence of an H-bond donor to N5 of both FMN-1 and FMN-2 positioned at an appropriate angle is consistent with both FMNs having a role in hydride ion transfer.

A further characteristic of FMN-dependent dehydrogenases is that the N1-C2-O2 region of FMN is less than 3.5 Å from a positive charge (Fig. 1) (52, 53). This positive charge is significant for two reasons: it stabilizes the anionic form of the reduced cofactor, which increases the midpoint potential, and it strengthens flavin binding. For FMN-1, the side chain nitrogen of Lys-123 is 3.3 Å from O2, providing a positive charge that interacts with its N1-C2-O2 region (Fig. 5). This is consistent with FMN-1 acting as a hydride ion acceptor. In contrast, there is no interaction of a positively charged residue with FMN-2 at <3.5 Å. The lack of a charge here suggests that FMN-2 may have a lower redox potential than FMN-1. Moreover, π-π stacking between Tyr-85 and the dimethylbenzene moiety of FMN-2 is expected to drop its midpoint potential further (54). These features suggest that FMN-2 donates a hydride to FMN-1. In addition, the absence of a positive charge in proximity to its N1-C2-O2 region emphasizes the weaker binding of FMN-2 to DmrB compared with FMN-1. This supports the idea that FMN-2 acts as an electron donor and a co-substrate for DmrB, whereas FMN-1 acts as a tightly bound cofactor.

In addition to the interactions described above, the DmrB structure shows that the backbone oxygen of Gly-24 is 3.6 Å from the N5 of FMN-1 and that the Tyr-85 side chain hydroxyl is 3.0 and 3.4 Å from the N5 and N10 of FMN-2, respectively. Given the key role of N5 in catalysis, these interactions likely
modulate the reactivity of FMN-1 and FMN-2. These interactions as well as the structural features described above indicate that FMN-1 and FMN-2 participate directly in catalysis. They also support the idea that FMN-2 functions as a hydride donor that reduces FMN-1 during the first stage of the DmrB catalytic mechanism.

Identification of the H$_2$MPT Binding Site by Computational Docking—Prior studies indicate that the DmrB flavoprotein is a H$_2$MPT reductase (15). Because H$_2$MPT (and related compounds) is not commercially available, we used computational docking to identify potential H$_2$MPT binding sites in DmrB. First, as a control calculation, we tested whether the AutoDock Vina suite of programs (46) could correctly identify the second binding site, which was occupied by FMN in the crystal structure. FMN-2 was removed from the atomic coordinate set and was then used as the search ligand against DmrB containing FMN-1. The atoms of the ligand were set as rotatable with the exception of those in the rings. The grid space was a 64 Å cube encompassing a complete homotrimer. The program was able to automatically place the FMN remarkably close to the crystallographically observed position of FMN-2; the root mean square deviation was only 0.04 Å. The computationally predicted standard state binding free energy was 10.1 kcal mol$^{-1}$.

Supported by the positive computational docking result given above, we then investigated the presumptive binding of the H$_2$MPT substrate to DmrB. For this study, the flexible tail of H$_2$MPT (the ribitol-ribitol phosphate-hydroxyglutarate moiety) was truncated to decrease the search space to be explored by Vina (Fig. 1). First we investigated whether H$_2$MPT could plausibly be docked into the structure of DmrB when both FMN molecules were present, i.e. at a possible third binding site. As the best solution, the Vina program placed H$_2$MPT remote from the known binding cleft and with a notably weaker predicted binding affinity of −6.4 kcal mol$^{-1}$. We interpreted this to reflect a biologically insignificant result, inferring that there were no additional binding sites for H$_2$MPT. We then reasoned that H$_2$MPT might instead occupy the second flavin site, in place of FMN-2, during the second stage of the DmrB reaction cycle. To perform a docking calculation to test this possibility, the FMN-2 was removed from the coordinate set, and the Vina program was used again to search for a H$_2$MPT bind site. In this case, the program automatically placed H$_2$MPT in the site otherwise occupied by FMN-2. The atomic contacts were plausible, and the calculated binding affinity was −8.8 kcal mol$^{-1}$. Hence, the second site provides nearly as good a fit for the pterin as for the flavin. Most compelling however was the observation that the computationally predicted binding mode for H$_2$MPT placed this substrate in a manner fully consistent with hydride transfer from the tightly bound FMN-1.

Physiological Relevance of the Predicted H$_2$MPT Binding Site—A remarkable outcome of the computational docking is that the predicted H$_2$MPT binding site is ideally shaped to effect catalysis, and the specific contacts between H$_2$MPT and DmrB suggest a likely catalytic mechanism (Fig. 6). The reduction of H$_2$MPT to H$_2$MPT is expected to involve two key steps: protonation of H$_2$MPT at the N5 position and hydride ion transfer to H$_2$MPT (Fig. 1) (1). In addition, the known stereochemistry of C6a requires that the hydride approach from the front of H$_2$MPT, as shown in Fig. 1. In our computationally docked model, the N5 position of FMN-1 (hydride donor) is 3.2 Å from...
C6a of H2MPT and positioned to donate the hydride to yield the correct stereochemistry (1). The angle formed by FMN-2, N10-N5-C6a, is 121°, which is very close to the geometry of 97°–116° that is conserved in many flavin dehydrogenases (52). The second key step in the reduction of H2MPT to H4MPT is protonation at N5. For DmrB, the side chain hydroxyl group of Tyr-85 is suitably positioned to act as the catalytic acid that protonates N5; its hydroxyl group is 3.0 Å from N5. This arrangement is very similar to that found in the well studied flavin reductase called Old Yellow Enzyme (55). In that enzyme, the catalytic acid (the Tyr-196 hydroxyl) is 3.5 Å from the substrate site (55). Thus, structural evidence indicates that computational docking properly placed H2MPT within the DmrB active site and implicates Tyr-85 as a catalytic acid.

In addition to identifying residues likely to have key roles in catalysis, docking also implicated side chains likely to have secondary catalytic functions. Beyond its proposed role as a catalytic acid (see above), Tyr-85 is involved in π stacking interactions with the benzene ring of H2MPT, and its side chain hydroxyl is within H-bonding distance of N5 and N10 of H2MPT. The Lys-123 side chain amino group is within H-bonding distance to H2MPT, O4, and the Cys-133 backbone amide nitrogen and is positioned to form H-bonds with the amino group of H2MPT. Lastly, the side chains of Val-140 and Leu-150 are positioned to make van der Waals interactions with the benzene ring and C4a of H2MPT. These residues are likely important for precisely positioning H2MPT in the active site.

The H2MPT and FMN-2 Binding Sites Overlap—Several of the residues predicted by computer modeling to interact with H2MPT also interact with FMN-2 in our crystal structure. Tyr-85 interacts with N5 and N10 of FMN-2 or H2MPT. Gln-120 interacts with N5 and O4 of FMN or with O4 of H2MPT. Lys-123 is in close proximity to O4 of FMN-2 and O4 of H2MPT. Conversely, several residues of DmrB that interact with FMN-2 are unlikely to bind H2MPT. The backbone carbonyl of Cys-133 interacts with the amino group of H2MPT but not with FMN-2. Gln-142 interacts with the phosphate of FMN but not with H2MPT, at least in the truncated form used for modeling. According to the crystallographic data and the docking results, FMN-2 and H2MPT occupy overlapping, mutually exclusive binding sites.

Conservation of the H2MPT Binding Site of DmrB and Bacterial Homologs—A bioinformatic analysis was conducted to assess conservation of the key residues predicted to bind H2MPT in DmrB. Sequence homology searches showed that close homologs of DmrB are prevalent in the archaea but are relatively uncommon among bacteria. Among the bacteria, DmrB homologs are restricted almost exclusively to methylotrophic bacteria that synthesize H2MPT, and they often cluster with H2MPT biosynthetic genes (8, 15). BLASTp analysis of 1299 complete bacterial genomes identified 18 DmrB homologs that had 49–91% amino acid sequence identity. A multiple sequence alignment of these proteins showed that the three amino acid residues predicted from the structure to be most critical to H2MPT binding and catalysis in DmrB (Tyr-85, Gln-120, and Lys-123) were 100% conserved (supplemental Fig. S1A). In addition, two residues with side chains predicted to contribute to H2MPT binding were also conserved: Val-140 (80% Val or Ile) and Leu-150 (100% Leu or Ile). Thus, overall, the predicted H2MPT binding pocket of DmrB is conserved among its bacterial homologs.

Conservation of the H2MPT Binding Site of DmrB and Homologs in Methanogenic Archaea (DmrX)—Recent studies have shown that DmrX and its close homologs function as Dmr enzymes in methanogenic archaea (16). DmrX and DmrB enzymes have relatively high sequence identity (31–48%) over about 144 amino acids of their N termini. However, these enzymes diverge substantially in their C-terminal regions, where DmrX has a 50–60-residue insertion that includes two 4Fe-4S cluster motifs not found in DmrB. Thus, the degree to which DmrB and DmrX share a conserved catalytic mechanism is uncertain. Based on the analysis of a multiple sequence alignment, the flavin binding motif characteristic of the HFCD family is well conserved in DmrX homologs found among the methanogenic archaea (18, 19) (supplemental Fig. S1B). With regard to key catalytic residues, the following levels of conservation were observed: Tyr-85 (not conserved), Gln-120 (80% Gln), and Lys-123 (100% Lys). For residues having side chains proposed to have binding roles due to interaction with H2MPT, conservation levels were as follows: Val-140 (55% Val, or Ile) and Leu-150 (100% Ile, Val, or Met). Thus, important catalytic residues are partially conserved between DmrB and DmrX. However, there is clearly some divergence between these enzymes, and the absence of Tyr-85 (the proposed catalytic acid of DmrB) suggests mechanistic variations, which are addressed below under “Discussion.”

DISCUSSION

In this study, the crystal structure of DmrB was elucidated at 1.9 Å resolution. This is the first reported structure of a Dmr enzyme. An unusual feature of DmrB is that the biological unit is a 24-mer built from eight trimers positioned at the corners of a cube to form a cage-like structure (Fig. 2, C and D). Such a structure might be of interest in the nanomaterials field, because protein cages (which are uncommon in biology) have numerous potential uses as templates for the synthesis of nanomaterials (56), as enzymatic nanocontainers (57), as molecular delivery devices (58), and as scaffolds for synthetic vaccines (59). With respect to cell physiology, it is unclear why a large cage-like structure might be advantageous in transferring redox equivalents from FMN to the pterin substrate. It is possible that DmrB has unknown functions in addition to Dmr activity, such as serving as a metabolic scaffold, where its unusual quaternary structure might play an important role. Alternatively, there might be as yet undiscovered dynamic aspects of DmrB that require a large cage-like structure.

It is notable that several other HFCD proteins with diverse substrates and cellular functions also form cage-like structures. At least four distinct 12-subunit dodecameric cages have been reported (20–23), all assembled from four copies of the same conserved trimeric building block as seen here for DmrB. The four previous examples represent two very different assembly forms; opposite faces of the trimer face outward in the two types of dodecameric cages reported to date. In one type, seen in HFCD enzymes for lantibiotic synthesis (20, 21), the active site
clefts open toward the interior of the cage. In the other type, seen in HFCD enzymes that decarboxylate aromatic carboxylic acids (22, 23), the active sites are disposed to the exterior. The 24-mer cubic cage observed for DmrB has its trimers facing with their binding clefts toward the exterior, but the assembly is otherwise distinct from the two types of cages reported previously. In particular, the atomic contacts between trimers, which govern the shape of the overall assembly, are dissimilar in the three types of cages. Based on their geometric dissimilarity and the lack of conservation at the interfaces between trimers, it seems likely that these assembly forms arose independently of each other during evolution. The presumptive cellular advantage conferred by these structures remains obscure, and the multiple independent occurrences of (typically rare) cage-like structures within the same protein superfamily presents an especially intriguing puzzle.

A second interesting feature of DmrB is the occurrence of two FMN molecules sitting face-to-face in the active site (Fig. 5). This is somewhat similar to FMN binding in the NADH:FMN oxidoreductase EmoB (60), but it has not been observed previously for the HFCD family. Based on the manner in which these FMN molecules interact with one another and with DmrB, we propose that FMN-1 functions as a tightly bound cofactor and FMN-2 is the hydride donor (Fig. 7). In this model, FMN-2-red enters the active site, reduces FMN-1, and then exits in the oxidized form (FMN-1-ox). Next, H₂MPT enters the active site and is reduced by FMN-1-red to H₄MPT which exits, supplying the cell with an essential cofactor. Finally, the reaction cycle is completed by regeneration of FMN-2-red from FMN-2-ox by flavin reductase (Fre) (61). Many features of the DmrB structure and our model for H₂MPT binding support this model. FMN-2 appears to bind DmrB relatively weakly and in a surface-accessible location, suggesting it functions as a co-substrate rather than a cofactor. FMN-1 and FMN-2 sit in the active site with their N5 atoms 3.5 Å apart, which is ideal for hydride transfer between them. A computational docking program automatically places H₄MPT in the second binding site, perfectly positioned to be reduced by FMN-1 once FMN-2 has exited. The site of H₄MPT that will accept a hydride ion (C6a) is an optimal distance from N5 of FMN-1 (3.2 Å) with a geometry near the ideal range (the N10-N5-S angle is 121°). Moreover, H₄MPT is positioned such that reduction of C6a will give the correct stereochemistry for H₄MPT (1).

This model is further supported by detailed studies of the FMN binding properties of DmrB. From ITC measurements, we determined that FMN-1 binds DmrB tightly with a $K_d$ in the mid-nanomolar range (Fig. 4A). The binding affinity of FMN-2 for DmrB could not be determined by ITC in our experiments, likely reflecting a minimal enthalpic contribution to binding. However, we did determine the equilibrium binding of FMN by DmrB crystals as a function of the concentration of free FMN left in the surrounding crystallization drop. Unbiased difference electron density maps showed both FMN sites as highly occupied when the free FMN concentration was 40 μM. Only spurious features were observed in the crystals without added FMN; DmrB was crystallized in the presence of ammonium sulfate, and the minor spherical difference density that was observed was likely a sulfate ion bound similarly as the phosphate group in FMN (Fig. 4C). Accordingly, we estimated the binding affinity for FMN-2 to be in the low to mid-micromolar range. Thus, DmrB binds both FMN-1 and FMN-2 with affinities that are physiologically relevant, supporting our proposed reaction cycle (Fig. 7).

The studies described here also allow us to propose a minimal catalytic mechanism (Fig. 6). In this mechanism, Tyr-85 acts as the catalytic acid, and a number of other amino acids serve to bind and activate H₄MPT, FMN-1, and FMN-2 (see above). To assess whether this mechanism is conserved across diverse organisms, bioinformatics analyses were conducted. A multiple sequence alignment showed that all key catalytic and H₂MPT binding residues of DmrB are highly conserved among bacterial homologs, suggesting a shared mechanism. For the more remote homologs of DmrB in the methanogen archaea (DmrX), the key catalytic and H₂MPT binding residues were 55–100% conserved except for Tyr-85, which was not conserved. The lack of conservation at Tyr-85 (the proposed catalytic acid of DmrB) might be explained by different electron donors. A major difference between DmrX and DmrB is that DmrX has two FeS clusters in its C-terminal region that could reasonably function as the electron donor to FMN-1. This is in contrast to DmrB, where results suggest that FMN-2-red is the electron donor to FMN-1. Hence, we suggest the following model to explain the lack of conservation of Tyr-85 in DmrX. We propose that in DmrB, Tyr-85 activates H₂MPT by donating a proton to N5 of H₂MPT. Subsequently, a hydride ion is transferred to C6a, completing the reaction. However, in the case of DmrX, the reaction mechanism would start with the donation of a hydride to C6a followed by protonation of N5 from solvent. Donation of the hydride ion to C6a would be made possible by a lower midpoint potential of FMN-1 in DmrX compared with DmrB. In turn, the reduction of FMN-1 in DmrX requires a lower potential electron donor such as a FeS center linked to ferredoxin. In other flavin dehydrogenases, both types of reaction sequences have been proposed (52, 53). Hence, we believe it is likely that some mechanistic features are conserved between DmrB and DmrX and that the main difference between these two enzymes is the electron donor. In this regard, it is also of note that H₂MPT biosynthetic genes found in the domain Bacteria are thought to have been acquired by horizontal gene transfer from the archaea (7). This suggests that the DmrB family may have arisen from a DmrX gene that was transferred from the Archaea to the Bacteria domain, where it adapted to its new environment with a change in the electron donor.
obtained from the structure of DmRB might be helpful in designing inhibitors of DmRX as treatments for obesity. *M. smithii* is the most prevalent methanogen in the gut (11). Recent studies in a mouse model showed that *M. smithii* stimulates the breakdown of indigestible fiber to short chain fatty acids, which serve as an added source of calories leading to increased adiposity (11, 12). Accordingly, it was proposed that inhibition of *M. smithii* might help prevent weight gain (12). Because H₄MPT is essential to the methanogenic archaea but absent from other major groups of intestinal bacteria (11), the inhibition of H₄MPT biosynthesis might allow selective control of *M. smithii*. Currently, the lack of an available structure for DmRX precludes the in silico design of inhibitors for this enzyme. However, because of their similarities, compounds that inhibit DmRB might also inhibit DmRX. Thus, the structure of DmRB determined here may allow the in silico design of inhibitors that also impair DmRX. In addition, DmRB might be helpful in screening libraries of potential inhibitors, as it is oxygen-stable, whereas DmRX is highly oxygen-sensitive due to its 4Fe-4S centers. In this regard, we also note that dihydrofolate reductase, the folate biosynthetic enzyme analogous to Dmr, has been studied extensively as an antimicrobial target (62).

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