Domain Engineering of the Reductase Component of Soluble Methane Monoxygenase from *Methylococcus capsulatus* (Bath)*

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Soluble methane monoxygenase (sMMO) from *Methylococcus capsulatus* (Bath) is a three-component enzyme system that catalyzes the conversion of methane to methanol. A reductase (MMOR), which contains [2Fe-2S] and FAD cofactors, facilitates electron transfer from NADH to the hydroxylase diiron active sites where dioxygen activation and substrate hydroxylation take place. By separately expressing the ferredoxin (MMOR-Fd, MMOR residues 1–98) and FAD/NADH (MMOR-FAD, MMOR residues 99–348) domains of the reductase, nearly all biochemical properties of full-length MMOR are retained, except for interdomain electron transfer rates. To investigate the extent to which rapid electron transfer between domains might be restored and further to explore the modularity of MMOR, MMOR-Fd and MMOR-FAD were connected in a non-native fashion. Four different linker sequences were employed to create MMOR reversed-domain (MMOR-RD) constructs, MMOR(99–342)-linker-MMOR(2–98), with a domain connectivity observed in other homologous oxidoreductases. The optical, redox, and electron transfer properties of the four MMOR-RD proteins were characterized and compared with those of wild-type MMOR. The linker sequence plays a key role in controlling solvent accessibility to the FAD cofactor, as evidenced by perturbed flavin optical spectra, decreased FADox/FADsq redox potentials, and increased steady-state oxidase activities in three of the constructs. Stopped-flow optical spectroscopy revealed slow interdomain electron transfer (k < 0.04 s\(^{-1}\)) at 4 °C, compared with 90 s\(^{-1}\) for wild-type MMOR-Fd protein with 7-residue linkers. A long (14-residue), flexible linker afforded much faster electron transfer between the FAD and [2Fe-2S] cofactors (k = 0.9 s\(^{-1}\) at 4 °C).

**The first step in the catabolic pathway of methanotrophs is the conversion of methane to methanol by soluble or membrane-bound methane monoxygenases (MMOs)\(^{1}\).**

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\(^{3}\) The abbreviations used are: MMO, methane monoxygenase; sMMO, soluble methane monoxygenase; MMOH, hydroxylase component of sMMO; MMOR, coupling protein of sMMO; MMOR, reductase component of sMMO; FAD, flavin adenine dinucleotide; FNR, ferredoxin; MMO enzymes (4–6). The sMMO proteins from two methanotrophic bacteria, *Methylococcus capsulatus* (Bath) (7, 8) and *Methylosinus trichosporium* OB3b (9, 10), have been studied in great detail (11–19). Both systems require three components: a dimeric (αβ)\(_2\) hydroxylase (MMOH, 251 kDa) with dinuclear, carboxylate-bridged iron centers, where dioxygen activation and methane hydroxylation occur; a regulatory protein (MMOB, 15.9 kDa); and a reductase (MMOR, 38.5 kDa) that transfers electrons from NADH to the MMOH diiron sites, thereby priming the hydroxylase for reaction with dioxygen. The N-terminal domain of MMOR, which houses a [2Fe-2S] cluster, shares sequence (20) spectroscopic (16, 21–24), and structural (25) properties with ferredoxins of plants, cyanobacteria, and archaea. A flavin adenine dinucleotide (FAD) cofactor located in the C-terminal portion of MMOR accepts two electrons from NADH. These electrons are passed sequentially from the reduced FAD moiety through the MMOR [2Fe-2S] center into the hydroxylase active sites by means of a series of intra- and intermolecular electron transfer reactions (16, 24, 26, 27).

MMOR is a member of a class of modular flavoprotein electron transferases, also known as the ferredoxin:NAD\(^+\) oxidoreductase (FNR) family. These proteins contain a flavin domain that transfers electrons between a nicotinamide dinucleotide and a one-electron carrier domain, which may be linked or dissociable (28–30). Because the electron carrier domain can be attached to either the N- or C-terminal end of the core flavin/NAD(P) unit, this domain appears to be an independent modular element. In support of this designation, the crystal structure of *Burkholderia cepacia* phthalate dioxygenase reductase (PDR), an FNR family member with C-terminal electron carrier connectivity, shows distinct flavin mononucleotide (FMN), NADH binding, and [2Fe-2S] domains (Fig. 1) (30). To examine the modular nature of MMOR and facilitate characterization of this complex protein, the ferredoxin (MMOR-Fd) and FAD/NADH (MMOR-FAD) domains were expressed as separate polypeptides (31). NMR studies reveal that
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Both MMOR-Fd (25) and MMOR-FAD form well defined structures in solution. In nearly every respect examined, the individual MMOR domains retain biochemical properties essential to those of the corresponding portions of full-length MMOR. Without a covalent tether between the domains, however, the MMOR-Fd and MMOR-FAD proteins are unable to form a complex suitable for rapid interdomain electron transfer, because \( k = 1500 \text{ m}^{-1} \text{s}^{-1} \) at 25 °C, compared with 90 s\(^{-1}\) at 4 °C for internal electron transfer between cofactors in MMOR (31). This interdomain electron transfer reaction represents the second step in the biological conveyance of electrons from NADH to MMOH.

To explore further the modularity of the sMMO reductase, MMOR constructs were prepared in which the wild-type arrangement of [2Fe-2S] and FAD/NADH domains is reversed to reproduce the connectivity of PDR. The goal for this project was to determine the extent to which the rapid interdomain electron transfer of wild-type MMOR could be restored by connecting the domain proteins in a non-native fashion employed in a related protein. In this work, four linker sequences, encompassing a variety of chemical and geometric properties, were employed to tether the reversed domains; the spectroscopic, redox, and electron transfer properties of these reversed-domain MMOR constructs were determined. Through such domain engineering, the relative contributions of domain orientation, affinity, and proximity to interdomain electron transfer may be delineated.

EXPERIMENTAL PROCEDURES

**MMOR Reversed-domain Construct Design**—To define the Fd and FAD/NADH domain boundaries of *M. capsulatus* (Bath) MMOR and B. cepacia PDR, sections of MMOR were aligned with the full-length PDR protein sequence (PDB code 2P1A) by using ClustalW (32). In addition, the approximate MMOR domain sequences (residues 1–114, Fd domain; residues 89–348, FAD/NADH domain) were compared with and aligned with other protein sequences in the non-redundant National Center for Biotechnology Information (NCBI) protein data bases with the PSI-BLAST program (33). The final domain definition technique involved examination of the PDR secondary structural features to ensure that the selected domain linker region was not the terminus of an existing \( \alpha \)-helical or \( \beta \)-sheet structure. Once the domain boundaries were set, the PDR and reversed-domain MMOR sequences were aligned. A short 7-amino acid linker sequence between the domains was selected based on lower homology and lack of defined secondary structure in that region.

Four different linker sequences were designed for the reversed-domain MMOR mutants. The first two 7-residue linkers were chosen with the principle of minimal perturbation. One linker was selected as the sequence in the PDR interdomain region and another as the MMOR sequence in that part. A third 7-residue linker was designed to incorporate a factor X\(_n\) protease cleavage site, which would allow the domains to be disconnected if desired. Finally, to investigate the importance of domain proximity in the MMOR intramolecular electron transfer reactions, a longer 14-residue linker was generated with the program LINKER (34). The primary amino acid sequences and basic properties of the four MMOR reversed-domain constructs (MMOR-RD) are displayed in Table I.

**DNA Primer Synthesis and Purification**—All of the primers used for cloning and DNA sequencing were designed manually and synthesized on an Applied Biosystems model 392 DNA/RNA synthesizer. Each primer was purified by polyacrylamide gel electrophoresis on a 12% denaturing polyacrylamide gel, extracted from the gel slices by using a crush and soak method, and then precipitated to remove most of the salt added during extraction. The DNA pellets were suspended in appropriate quantities of deionized distilled water and stored at −20 °C.

**Construction of pTrc-Fd1 and pTrc-Fd2 Plasmids**—A four-step procedure was devised for constructing the four MMOR-RD expression vectors, as depicted in Fig. 2. The mmo genes, which code for all five components of the MMO system, are contained on a 5.5-kb section of pCH4 (35). Two variations (Fd1 and Fd2) of the Fd portion of the mmoC gene, which encodes MMOR, were amplified from pCH4 with *Flu* Turbo DNA polymerase (Strategene, La Jolla, CA) by using the primers LKFB10 (Fd1; 5'-CTGGGATCCACACATATACCGGCGGTG-3') or LKFB20 (Fd2; 5'-AGTGATCTCCGAGTGTAGCTGGGAGGCGGTG-3') and LKRB10 (5'-GGATCCGGATCCCTCTCAGAGCTTCCGCGGTG-3') and FdRH10 (5'-CCTCTCAAAACAGCTTGATCCGCTCAATGGTGATATAG-3'). The resulting 313-bp (Fd1) and 341-bp (Fd2) fragments were flanked by BamHI and HindIII sites (bold), which were introduced by LKFB10/LKFB20 and FdRH10, respectively. Each mmoC-Fd polymerase chain reaction (PCR) product was isolated with the QIAQuick PCR purification kit (Qiagen, Inc., Valencia, CA) and ligated with T4 DNA ligase (New England Biolabs) to yield the pTrc-Fd1 and pTrc-Fd2 plasmids. The plasmids were introduced into *E. coli* by the following procedures. The DNA was isolated from the reactions (QIAquick PCR purification kit) and then digested with HindIII (New England Biolabs). By using the same two-step procedure, the high copy number plasmid pTrc99A (Amersham Biosciences) was digested with BamHI and HindIII. The pTrc99A digest was cloned and DNA sequencing were designed manually and synthesized on an Applied Biosystems model 392 DNA/RNA synthesizer. Each primer was purified by polyacrylamide gel electrophoresis on a 12% denaturing polyacrylamide gel, extracted from the gel slices by using a crush and soak method, and then precipitated to remove most of the salt added during extraction. The DNA pellets were suspended in appropriate quantities of deionized distilled water and stored at −20 °C.

**Construction of pTrc-RD1m, pTrc-RD2m, pTrc-RD3m, and pTrc-RD4**—To introduce different linker regions into the MMOR-RD constructs, four versions (FAD1, FAD2, FAD3, and FAD4) of the FAD/NADH region of mmoC were PCR-amplified from pCH4 with the primers FNYTCAAGACGGTATGCGCTATCATATA (5'-GGATCCGGATCCTCTCAGAGCTTCCGGCGGTG-3') and LKRB10 (5'-GTAGGATCCGGATCCTCGTGTCTGGTGTCGCGGTGATTTTCCGAGAAGACCCTTGTCG-3') and LKRB10 (5'-GGATCCGGATCCTCTCAGAGCTTCCGGCGGTG-3'). The amplified DNA was digested with HindIII and ligated with T4 DNA ligase (New England Biolabs). The ligated DNA was then transformed into supercompetent *Escherichia coli* XL1-Gold cells (Stratagene), which were selected on Luria-Bertani (LB)-ampicillin (Ap; 100 \( \mu \)g/ml) agar plates. Positive clones were identified by restriction digests of DNA isolated from plasmid miniprep (36). The sequences of the mmoC-Fd inserts were verified by DNA sequencing (MIT Biopolymers Laboratory).
TTCCGAGAAGACCTGCTGC, or LRKB40 (FAD4: 5'-GGTGAAGTCGACCGCTACCAAGCGGGATATTTTTCGAAGAAGACCTGTCG). The 802-bp (FAD1, FAD2, and FAD3) and 795-bp (FAD4) products were flanked by EcoRI and BamHI sites (bold), which were introduced by FNFE10 and LRKB10/LRKB20/LRKB30/LRKB40, respectively. The underline A in LRKB10, LRKB20, and LRKB30, which was required to incorporate the BamHI site, added a point mutation in the PMOR sequence that was corrected by site-directed mutagenesis in the next stage of the cloning process. After isolating the amplified DNA from the PCR reactions, all four PCR products were digested with the restriction enzymes EcoRI (New England Biolabs) and BamHI. The enzymes were heat-inactivated, and the DNA was purified with the QiAquick PCR purification kit. The plasmids pTrc-Fd1 and pTrc-Fd2 were digested with the same restriction enzymes, treated with AP, and then ligated with mgod-FAD digests to yield pTrc-RD1m (pTrc-Fd1 + FAD1), pTrc-RD2m (pTrc-Fd1 + FAD2), pTrc-RD3m (pTrc-Fd1 + FAD3), and pTrc-RD4 (pTrc-Fd2 + FAD4) (Fig. 2). The ligation products were transformed into supercompetent E. coli XL1-Gold cells, and potential positive clones were selected on LB-Ap (100 µg/ml) agar plates. Colonies with ampicillin resistance were analyzed further by restriction mapping of DNA isolated from plasmid minipreps and DNA sequencing (MIT Biopolymers Laboratory).

Site-directed Mutagenesis to Yield pTrc-RD1, pTrc-RD2, and pTrc-RD3 Expression Vectors—Site-directed mutagenesis (GGATCC → GGGTCC) was performed to remove the point mutation required to introduce the BamHI site into the RD1, RD2, and RD3 reversed-domain constructs. Mutagenesis of pTrc-RD1m, pTrc-RD2m, and pTrc-RD3m was accomplished by using the QuikChange kit (Stratagene) and following the instructions from the manufacturer. The following primers and their reverse complements were used in the Pfu Turbo DNA polymerase-catalyzed PCR mutagenesis reaction (altered bases underlined: 5'-CCACACGAAATGCACCGGTGCTCAACTACAAAGC (RD1), 5'-CGGCACGACACGCACGGTCCACTACACGG (RD2), and 5'-CCATAGGGTGGTCGTCCACTACACGG (RD3)). Subsequent isolation and DNA sequencing (MIT Biopolymers Laboratory) of pTrc-RD1, pTrc-RD2, and pTrc-RD3 (Fig. 2) confirmed that the gene sequences contained only the desired mutations.

Construction of pRED-RD1, pRED-RD2, pRED-RD3, and pRED-RD4 Expression Vectors—The final step in the reversed-domain MMOR cloning scheme (Fig. 2) involved transferring the RD1, RD2, RD3, and RD4 genes from the pTrc-RD plasmids into pKK223-3 (Amerham Biosciences), the expression vector used for the recombinant MMOR, MMOR-Fd, and MMOR-FAD expression systems (24, 31). The plasmids pKK223-3, pTrc-RD1, pTrc-RD2, pTrc-RD3, and pTrc-RD4 were digested with EcoRI and HindIII. The mmoC-RD digest was separated from pTrc99A by resolution on a 1% agarose-ethidium bromide gel and then extracted from the gel with a QIAEX II kit (Qiagen). The pKK223–3 digest was treated with AP to prevent recircularization in the ligation reaction, the AP was inactivated, and the plasmid digest was isolated with a QIAquick PCR purification kit. T4 DNA ligase was then used to ligate each mmoC-RD digest into the digested pKK223–3 vector. The resulting plasmid solutions were transformed into supercompetent E. coli XL1-Gold cells, and pKK223–3-containing colonies were selected on LB-Ap (100 µg/ml) agar plates. Colonies containing pRED-RD constructs were identified by restriction mapping of DNA obtained from plasmid minipreps.

Expression Trials for the Reversed-domain MMOR Proteins—The pTrc99A, pTrc-RD1, pTrc-RD2, pTrc-RD3, pTrc-RD4, pKK223-3, pRED-RD1, pRED-RD2, pRED-RD3, and pRED-RD4 plasmids (Fig. 2) were transformed into E. coli JM105 cells for expression trials. The 10 clones were grown to saturation in 1 ml LB-Ap (100 µg/ml) cultures. At an A660 of 0.6, the cultures were supplemented with 80 µM FeSO4, and 1.0 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Samples (1.0 ml) were collected from each culture just before induction, 15 min after induction, and then at 60-min intervals for the following 5 h. To separate the soluble and insoluble fractions of the MMOR-RD time-course expression samples, BugBuster protein extraction reagent (Novagen, Inc., Madison, WI) was used according to the instructions from the manufacturer. In brief, each cell pellet was thawed and suspended in 100 µl of BugBuster solution to which DNaSe had been added. The samples were gently agitated at room temperature for ~12 min, centrifuged for 20 min at 10,000 × g to separate the soluble and insoluble cellular components, and decanted into clean tubes. The pellets were washed with 100 µl of 25 mM potassium phosphate (pH 7.0) and then suspended in 100 µl of the same buffer. The samples were analyzed by SDS-PAGE on 12% polyacrylamide gels.

Expression and Purification of MMOR-RD Proteins—A JM105/pRED-RD1 clone was grown to saturation in 100 ml of LB-Ap (100 µg/ml) medium at 37 °C with 200 rpm shaking. To each of six 1-liter quantities of LB-Ap (100 µg/ml) medium were added 10 ml of 10 mM Fe(NH4)2(SO4)2·6H2O in 100 mM sodium citrate (pH 7.0) and 10 ml of the saturated JM105/pRED-RD1 starter culture. When the A660 reached ~0.6, the cultures were supplemented with 80 µM FeSO4, added as 2 ml of 40 mM FeSO4·7H2O in 12.5 mM H2SO4/FeCl3 of cell culture, and induction was started by adding IPTG to a final concentration of 1.0 mM. After 4 h, the cells were collected by centrifugation, drained briefly, and then suspended in 100 ml of cold cracking buffer (25 mM MOPS (pH 7.0), 1.0 mM dithiothreitol (DTT), 5.0 mM MgCl2, and 200 µM Fe(NH4)2(SO4)2·6H2O) containing DNaSe (1.5 units/ml) and 1 mM Pefabloc SC (Roche Molecular Biochemicals). The cells were sonicated on ice with three 2-min pulses at 40% output (Branson Sonifier model 450 equipped with a 3/4-inch horn) and then centrifuged for 40 min at 95,000 × g to separate the soluble and insoluble materials. The soluble cell extract was filtered through 0.2-µm pore diameter membranes before loading onto a DEAE Sepharose CL-6B (Amersham Biosciences) column (2.6 × 15 cm) equilibrated in buffer A (25 mM MOPS (pH 7.0), 1.0 mM DTT, and 50 mM NaCl). After a 120-ml wash with buffer A, column with buffer B (25 mM MOPS (pH 7.0), 1.0 mM DTT, and 500 mM NaCl). Fractions containing the RD1 protein were identified by SDS-PAGE and UV-visible spectroscopy, pooled, and concentrated by ultrafiltration. This crude RD1 protein solution was diluted 5-fold with 25 mM MOPS (pH 7.0), 2 mM DTT and applied to a 5'-AMP Sepharose (Sigma) column (2.6 × 18 cm) equilibrated with buffer A. After washing the column with buffer A to remove protein contaminants, pure MMOR-RD1 was eluted with buffer B containing 0.8 mM NaDIH. Fractions containing the MMOR-RD1 protein (PDR linker), as identified by color and SDS-PAGE, were pooled and exchanged into 25 mM MOPS (pH 7.0), 2 mM DTT with a Biogel P6 desalting column (Bio-Rad). The MMOR-RD2 (MMOR linker), MMOR-RD3 (factor X a site), and MMOR-RD4 (long linker) proteins were expressed from JM105/pRED-RD2, JM105/pRED-RD3, and JM105/pTrc-RD4, respectively, and purified by following the same protocol.

Purification of MMOH, MMOB, and MMOR—MMOR was isolated from M. capsulatus (Bath) by using a slightly modified published protocol (16). Pure hydroxylase contained 3.9–4.0 mol of iron/mmol of protein, as determined by ferrozine iron assays (37, 38). MMOH exhibited a specific activity of 250–300 nmol min⁻¹ mg⁻¹ of MMOH for the conversion of propylene to propylene oxide at 45 °C. MMOB was expressed from a recombinant E. coli system (39) and purified as described previously (16). Recombinant MMOB was expressed and purified as published (24), except that the iron additions were performed as indicated above for saturation systems.

Basic Characterization of MMOR-RD Constructs—To assess the purity of the reversed-domain proteins, SDS-PAGE and native PAGE gels were run for the four MMOR-RD constructs with wild-type MMOR (MMOR-wt) included for comparison. The MMOR-RD proteins in 25 mM
MOPS (pH 7.0). 0.3 mM DTT were submitted to the MIT Biopolymers Laboratory for analysis by electro spray ionization mass spectrometry (ESI-MS). To remove cofactors that separated from the proteins upon treatment with formic acid, ammonium acetate, and acetoni trene, HPLC purification was necessary. The protein component was analyzed with a Sciex model API 365 triple-stage mass spectrometer. Ion assays were carried out to determine whether the MMOR-wt extinction coefficient (ε₂₈₀ = 30,800 (M protein)⁻¹ cm⁻¹) (16) was altered significantly in the MMOR-RD proteins.

**Optical Spectra of Oxidized and Reduced MMOR-RD Proteins—**All UV-visible spectra were obtained with a Hewlett Packard 8453 diode array spectrophotometer. To examine the MMOR-RD optical spectra in various oxidation states, anaerobic reductive titrations were performed. A concentration of 40 µM MMOR (RD1, RD2, RD3, or RD4) in 25 mM potassium phosphate (pH 7.0) was transferred to a sealed quartz cuvette and made anaerobic by 12 cycles of vacuum gas exchange with O₂-free N₂. Aliquots of 2 mM sodium dithionite were added with a gas-tight Hamilton titrating syringe. After each addition, optical spectra (300–900 nm) were collected until no further changes were observed (generally less than 5 min), and the final spectrum was saved for analysis. This process was repeated until the protein was reduced completely. The temperature was maintained at 25 °C with a circulating water bath.

Previous studies of the individual MMOR-FAD domain revealed that its optical spectrum is quite sensitive to buffer composition, whereas the optical properties of MMOR-FAD domain remained unchanged by varying pH or ionic strength (31). Therefore, it was assumed that the [2Fe-2S] cofactors in the MMOR-RD constructs have optical spectra identical to that of MMOR-Fd. To determine component spectra for the fully oxidized MMOR-RD flavin domains (FAD_o), a scaled oxidized MMOR-Fd (Fd_o) spectrum was subtracted from the initial titration spectrum such that the absorbance above 550 nm was zero in each calculated spectrum. In the same manner, fully reduced component spectra (FAD_r) were obtained by subtracting appropriately scaled reduced MMOR-Fd (Fd_r) spectra from the fully reduced MMOR-RD spectra.

**Relative Redox Potential Determinations—**The differences between the three reductase equilibrium midpoint potentials were determined for each MMOR-RD construct by analyzing reductive titration data. In addition to the FAD_o and FAD_r component spectra calculated for the MMOR-RD constructs (see above), the Fd_o, Fd_r, and FAD_o component spectra determined previously for the separate MMOR domains (31) were employed. For each redox couple (Fd/Fd_r, FAD/FAD_r, and FAD/FAD_o), a difference spectrum, ∆λ(λ), was generated by subtracting the extinction coefficients of the oxidized species from those of the reduced species.

First, dilution corrections were applied to each spectrum in a titration data set. A difference spectrum for each titration point was calculated with linear combinations of component difference spectra (KaleidaGraph, version 3.51; Synergy Software, Reading, PA) as shown in Equation 1.

\[ \Delta \lambda(\lambda) = \sum \lambda_r(\lambda) \Delta \lambda_c \]  

(Eq. 1)

The concentration differences, ∆λ_c, returned by fitting with Equation 1 were readily converted to species concentrations by comparison to total cofactor concentrations. Relative MMOR-RD redox potentials (∆E° values) were calculated with modified Nernst equations. Average ∆E° values were computed by including only those data for which all four relevant species were present at greater than 10% of the total cofactor concentrations.

Redox Potential Determinations for MMOR-RD Constructs—Because many of the MMOR-RD ∆E° values were significantly altered from those of MMOR-wt, absolute values for the redox potentials were determined. Anaerobic reductive titrations of the MMOR proteins were repeated exactly as described except for the inclusion of one or more redox-active indicator dyes with known midpoint potentials to permit calculation of the solution potential throughout the titrations. Dyes used for MMOR-RD potential determination included anthraquinone-2-sulfonate (AQ2S; E° = −226 mV), phenosafranine (E° = −235 mV), safranine O (E° = −289 mV; RD3 only), and anthraquinone-2,6-disulfonate (AQ2,6S; E° = −184 mV; RD2 only) in combination with phenosafranine (30%). The AQ2S concentration was 10 µM for AQ2S and 20 µM for AQ2S and AQ2,6S and 10 µM for safranine and safranine O. The MMOR-RD concentration was 30 µM in all titrations including redox dyes.

The titration data were converted to difference spectra as described and fitted with linear combinations of component difference spectra for the following redox couples: (dyne/Fd_o, FAD/FAD_r, and FAD/FAD_o). Modified Nernst equations were used to calculate the solution potential and MMOR-RD midpoint potentials for each titration point. Only those data for which the solution potential was within 40 mV of both the indicator potential and approximate MMOR-RD potential were included in average ∆E° calculations.

**Steady-state Dye Reduction Activity Assays—**The ability of the MMOR-RD constructs to transfer electrons from NADH to redox-active dyes was investigated with steady-state assays. Reductase (83 nm), dichlorophenolindophenol (DCPIP, Sigma; 47 µM, E° = +217 mV), and 25 mM MOPS (pH 7.0) were combined in a masked cuvette. NADH (160 µM) was introduced to initiate the reaction, and DCPIP reduction was monitored at 604 nm (ε_604 = 22,000 M⁻¹ cm⁻¹) with a 1-s integration time. The assays were repeated with K_Fe(CN)_6 (92 µM, E° = +360 mV) in place of DCPIP. Reduction of [Fe(CN)_6]³⁻ was observed at 420 nm (ε_420 = 1020 M⁻¹ cm⁻¹) for 60 s. Duplicate samples were analyzed for each MMOR-RD construct as well as wild-type MMOR. The temperature was held constant at 15 °C with a circulating water bath for all assays. The absorbance versus time data were fit with lines in the spectrophotometer software to obtain rate constants for the dye reduction reactions. Control experiments provided background rates for electron transfer from NADH to the redox-active dyes in the absence of reductase. These low rates were used to correct the measured values.

**Steady-state MMOR Activity Assays—**To determine the competence of the MMOR-RD constructs for binding and transferring electrons to the hydroxylase, steady-state MMOR assays were performed. In a quartz cuvette, 1 µM MMOH, 1 µM MOB, 0.8 mM propylene, and 25 mM MOPS (pH 7.0) were mixed. The reductase concentration was varied from 0 to 3.2 µM; nine different concentrations were assayed. After adding NADH (160 µM) to initiate the propylene epoxidation reaction, the cuvette was sealed and NADH consumption was monitored at 340 nm (ε_340 = 6220 M⁻¹ cm⁻¹) for 60 s. The temperature was maintained at 25 °C with a circulating water bath. Kinetic traces were fit with lines in the spectrophotometer software. Plots of activity versus [MMOR] were constructed and fit with a hyperbola plus a line (MMOR-wt) or a line (MMOR-RD constructs) in the least-squares sense.

In addition, steady-state activity assays were carried out with nitrobenzene as the substrate. MMOH (1 µM), MOB (2 µM), reductase (0.4 µM or 2 µM), and nitrobenzene (1 mM), were combined in 25 mM MOPS (pH 7.7). After adding NADH (150 µM), p-nitrophenol formation was monitored at 404 nm (ε_404 = 15,000 M⁻¹ cm⁻¹) for 18 h. The temperature was held constant at 25 °C. Rates were determined by fitting the kinetic traces with lines in the spectrophotometer software. Upon complete oxidation of NADH, a visible spectrum was collected for each reaction.

**MMOR-Reactions with NADH—**Interdomain electron transfer rates for the MMOR-RD constructs were determined in stopped-flow experiments. A Hi-Tech Scientific SF-61 DXZ double-mixing stopped-flow spectrophotometer configured in either single-wavelength photomultiplier or multiwavelength diode array mode was used for all data collection. The stopped-flow apparatus was made anaerobic by flushing the flow cell and syringes first with a solution of ~5 mM sodium dithionite and then with anaerobic buffer to remove the dithionite. Oxidized MMOR (wild-type, RD1, RD2, RD3, or RD4) in 25 mM MOPS (pH 7.0) was made anaerobic by 10–12 cycles of vacuum gas exchange with O₂-free N₂. Anaerobic NADH solutions were prepared by bubbling with N₂ for at least 15 min. After mixing, concentrations of MMOB and NADH were 20 and 200 µM, respectively. The reaction of reductase with NADH was monitored at 458, 625, and 725 nm in single-wavelength monitoring and at five points (512) were collected in 1–500 ms time-intervals at atmospheric time base. The experiment was repeated with the stopped-flow configured for diode array mode. For each 1.5–300-s shot, 160 spectra (350–800 nm) were recorded on a log time scale. The temperature was maintained at 4 °C with a constant temperature circulating water bath for all experiments. Single-wavelength kinetic traces were fit with a hyperbola to three to five exponential decays with the presence of a constant offset, version 3.04 (Hi-Tech Limited, Salisbury, England). Diode array data analysis was performed with Spectfit, version 3.0.16 (Spectrum Software Associates, Chapel Hill, NC).

**RESULTS**

**Expression of MMOR-RD Constructs—**Eight plasmids (pTrc-RD and pRED-RD; see “Experimental Procedures” and Fig. 2) were prepared for expressing the engineered reversed-domain MMOR constructs in E. coli hosts. In each of the initial expression tests, there was a time-dependent build-up of pro-
tein of the appropriate molecular weight in the soluble cell extract. The recombinant MMOR-RD proteins represented the major soluble protein in all of the systems. Only in JM105/pRED-RD4 cells did a significant amount of recombinant protein accumulate in the insoluble fraction. Therefore, the JM105/pTrc-RD4 expression system, for which inclusion bodies were not observed, was used to express the MMOR-RD4 (long linker) construct.

Initial Characterization of MMOR-RD Constructs—All four MMOR-RD proteins were expressed and purified in good yield (Table II). On SDS-PAGE and native PAGE gels, the reversed-domain reductases migrated like wild-type MMOR. The molecular weights of the apoproteins determined by ESI-MS matched closely the expected values. Each MMOR-RD construct contained nearly stoichiometric iron (Table II) when the MMOR-wt extinction coefficient (\( \varepsilon_{458/20,800} \) cm\(^{-1} \) mol\(^{-1} \)) was used to calculate protein concentrations. Only MMOR-RD2, with 1.86 mol of iron/mol of protein, was slightly iron-depleted. Although extinction coefficients for the reversed-domain constructs were not determined directly by amino acid analysis, the good agreement between the measured iron concentrations and calculated protein concentrations demonstrated that the MMOR-wt extinction coefficient was relatively unchanged in the domain-swapped mutants. Upon storage of samples at 4 °C, the [2Fe-2S] clusters in the MMOR-RD proteins were significantly less stable than the same cofactor in MMOR-wt, as revealed by accelerated loss of the pinkish-brown ferredoxin color in the mutants.

MMOR-RD Optical Spectra—Optical spectra recorded for the oxidized MMOR-RD proteins could be fit reasonably well with linear combinations of the oxidized MMOR-Fd (Fd\(_{ox}\)) and MMOR-FAD (FAD\(_{ox}\)) component spectra (31). An extremely good fit to the MMOR-RD2 spectrum was obtained (Fig. 3), suggesting that the RD2 [2Fe-2S] and flavin cofactors are in environments essentially identical to those of wild-type MMOR. Small but significant deviations between the combined component spectra and the RD1, RD3, and RD4 visible spectra were noted. By subtracting the [2Fe-2S] contributions from the fully oxidized and fully reduced MMOR-RD optical spectra, FAD\(_{ox}\) and FAD\(_{sq}\) component spectra were generated (Fig. 4). In both cases, the RD2 spectra were identical to those of wild-type MMOR, whereas the RD1 and, to a greater extent, RD3 and RD4 spectra displayed differences, indicating slight perturbation of the flavin site with respect to that of MMOR-wt.

Redox Potentials of MMOR-RD Constructs—Relative redox potentials of each reversed-domain reductase were determined with reductive titrations (Table III). Many of the \( E^\circ \) values were significantly altered compared with those measured for MMOR (24) or the individual MMOR domains (31). In particular, the FAD\(_{ox/sq}\) and FAD\(_{ox/sq}\) difference potentials of RD1, RD3, and RD4 were considerably lower ( \(-30 \text{ mV} \) ), suggesting decreased FAD\(_{sq/sq}\) potentials in these mutants. A key limitation of this method is the difficulty of accurately measuring \( E^\circ \) values for widely separated potentials, because there are few solution potentials at which significant amounts of all four relevant species are present.

Therefore, a series of reductive titrations including indicator dyes was performed to establish absolute values for the MMOR-RD redox potentials. Fig. 5 shows selected difference spectra obtained from a titration of MMOR-RD2 with the indicator AQ2S. Calculated midpoint potentials for the three redox couples of each protein are displayed in Table IV. The relative
and absolute redox potential measurements are generally in good agreement because the mean deviation between the directly measured relative redox potentials and those calculated from the absolute values is only 6.8 mV. The FADox/sq potentials of RD1, RD3, and RD4 are 32, 44, and 33 mV, respectively, lower than that of MMOR-wt (24). In addition, a 27-mV decrease in the FADsq/hq potential was measured for the RD3 construct. All four MMOR-RD proteins have moderately lower [2Fe-2S] ox/red potentials (9–18 mV) compared with wild-type MMOR.

Steady-state Dye Reduction Activity—The reversed-domain reductases were able to transfer electrons from NADH to re-

**TABLE II**

Properties of MMOR and MMOR-RD constructs

| Reductase Linker | Yield | Mass, measured (Da) | Fe content (mol/mol protein) | λmax (pH 7.0, 25 °C) | A458/A394 (FADox) |
|------------------|-------|---------------------|-----------------------------|---------------------|-------------------|
| MMOR NA          | 15b   | 38,547 ± 4b         | 2.05 ± 0.05b               | 337, 395, 465       | 1.33              |
| RD1 PDR          | 25    | 38,413 ± 4e         | 1.94 ± 0.02                | 337, 398, 458       | 1.25              |
| RD2 MMOR         | 23    | 38,409 ± 4e         | 1.86 ± 0.05                | 337, 397, 464       | 1.35              |
| RD3 X site       | 19    | 38,484 ± 4e         | 1.98 ± 0.11                | 339, 398, 463       | 1.11              |
| RD4 long         | 13    | 38,985 ± 4f         | 1.97 ± 0.02                | 339, 396, 458       | 1.14              |

a NA, not applicable.
b As reported in Ref. 24.
c Expected 38,415 Da for apoprotein.
d Expected 38,410 Da for apoprotein.

**TABLE III**

Relative redox potentials for MMOR-RD constructs

Table shows differences between MMOR redox potentials measured at pH 7.0 and 25 °C. FADox/sq, FADox/FADsq redox couple; Fdox/red, [2Fe-2S]ox/[2Fe-2S]red redox couple; FADsq/hq, FADsq/FADhq redox couple. Boldface values deviate significantly from those of the MMOR domains.

| Protein | FADox/sq | Fdox/red | FADsq/hq |
|---------|----------|----------|----------|
| MMOR   | 33 ± 21  | 57 ± 29  | 90 ± 22  |
| Domains| 33 ± 3   | 61 ± 5   | 94 ± 6   |
| RD1    | 4 ± 6    | 58 ± 7   | 62 ± 13  |
| RD2    | 45 ± 2   | 64 ± 1   | 109 ± 4  |
| RD3    | 9 ± 6    | 53 ± 8   | 62 ± 14  |
| RD4    | 19 ± 3   | 48 ± 8   | 67 ± 11  |

a As reported in Ref. 24.
b Separate MMOR domain proteins; as reported in Ref. 31.

Fig. 3. Optical spectrum of pure oxidized MMOR-RD2 (squares, solid line) fit with a linear combination (squares, dashed line) of oxidized MMOR-Fd (Fd ox; triangles) and oxidized MMOR-FAD (FADox; circles) component spectra determined previously for the separated MMOR domains (31).

Fig. 4. Flavin component spectra in the fully oxidized (A) and fully reduced (B) oxidation states calculated by subtracting appropriately scaled MMOR-Fd spectra (31) from experimentally determined reductase spectra for MMOR-wt (heavy line), MMOR-RD1 (squares), MMOR-RD2 (diamonds), MMOR-RD3 (triangles), and MMOR-RD4 (circles).

Fig. 5. Difference spectra for the reductive titration of a 1-ml mixture of 30 μM MMOR-RD2 and 40 μM anthraquinone-2-sulfonate by dithionite at pH 7.0 and 25 °C. Spectra 1–6 correspond to the addition of 10, 15, 25, 45, 70, and 80 μl of ~2 mM dithionite, respectively. Fits (dashed lines) are superimposed on the difference spectra.

and absolute redox potential measurements are generally in good agreement because the mean deviation between the directly measured relative redox potentials and those calculated from the absolute values is only 6.8 mV. The FADox/sq potentials of RD1, RD3, and RD4 are 32, 44, and 33 mV, respectively, lower than that of MMOR-wt (24). In addition, a 27-mV decrease in the FADsq/hq potential was measured for the RD3 construct. All four MMOR-RD proteins have moderately lower [2Fe-2S]ox/red potentials (9–18 mV) compared with wild-type MMOR.

Steady-state Dye Reduction Activity—The reversed-domain reductases were able to transfer electrons from NADH to re-
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**TABLE IV**
Absolute redox potentials for MMOR-RD constructs

| Protein   | FAD<sub>ox</sub>-FAD<sub>red</sub> | Δ | Fd<sub>ox</sub>-Fd<sub>red</sub> | Δ | FAD<sub>eq</sub>-FAD<sub>red</sub> | Δ |
|-----------|---------------------------------|---|-------------------------------|---|---------------------------------|---|
| MMOR<sup>a</sup> | −176 ± 7 | NA<sup>c</sup> | −209 ± 14 | NA | −266 ± 15 | NA |
| Domains<sup>d</sup> | −172 ± 2 | NA | −205 ± 1 | NA | −268 ± 4 | NA |
| RD1       | −208 ± 1 | −32 | −219 ± 2 | −10 | −269 ± 5 | −3 |
| RD2       | −166 ± 5 | +10 | −218 ± 7 | −9 | −270 ± 7 | −4 |
| RD3       | −220 ± 4 | −44 | −227 ± 5 | −18 | −293 ± 6 | −27 |
| RD4       | −209 ± 4 | −33 | −221 ± 3 | −12 | −272 ± 4 | −6 |

<sup>a</sup> FAD<sub>ox</sub>-FAD<sub>red</sub> redox couple; Fd<sub>ox</sub>-Fd<sub>red</sub> redox couple; FAD<sub>eq</sub>-FAD<sub>red</sub> redox couple. Boldface values deviate significantly from those of the MMOR domains.

<sup>b</sup> As reported in Ref. 24.

<sup>c</sup> NA, not applicable.

<sup>d</sup> Separate MMOR domain proteins; as reported in Ref. 31.

**TABLE V**
Steady-state activities for MMOR-RD proteins

| Protein | DCPIP reduction<sup>a</sup> | Relative activity | [Fe(CN)<sub>6</sub>]<sup>3-</sup> reduction<sup>a</sup> | Relative activity | Oxidase activity<sup>a</sup> | Relative activity |
|---------|----------------------------|-------------------|---------------------------------|-------------------|-------------------------|------------------|
| MMOR    | 7.9 ± 0.2                  | 1.00              | 9.8 ± 0.6                        | 1.00              | 0.095 ± 0.013           | 1.00             |
| RD1     | 7.5 ± 0.1                  | 0.95              | 9.8 ± 0.5                        | 0.99              | 0.377 ± 0.004           | 4.00             |
| RD2     | 5.5 ± 0.3                  | 0.70              | 11.4 ± 0.3                       | 1.16              | 0.052 ± 0.009           | 0.53             |
| RD3     | 8.2 ± 0.3                  | 1.05              | 1.9 ± 0.1                        | 0.19              | 0.619 ± 0.009           | 6.59             |
| RD4     | 9.6 ± 0.3                  | 1.22              | 18.0 ± 1.1                       | 1.99              | 0.710 ± 0.004           | 7.55             |

<sup>a</sup> Measured at 15 °C.

<sup>b</sup> Measured at 25 °C; sMMO propylene assay in the presence of 1 μM MMOH and 1 μM MMOB.

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Fig. 6. Dependence of the steady-state sMMO reaction with propylene at 25 °C on reductase concentration. Samples were air-saturated and contained 1 μM MMOH, 1 μM MMOB, 160 μM NADH, and 0.8 mM propylene. Activity curves were fit with a hyperbola plus a line (MMOR-wt, open triangles) or a line (MMOR-RD constructs: MMOR-RD1, squares; MMOR-RD2, diamonds; MMOR-RD3, triangles; MMOR-RD4, circles).

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Assays with ferricyanide revealed enhanced activity for the RD4 (93%) and RD2 (16%) constructs and substantially decreased activity for RD3 (19% of wild type).

Steady-state sMMO Activity—To investigate electron transfer to MMOH, assays were performed for the steady-state sMMO reaction with propylene at 25 °C at various reductase concentrations. As shown in Fig. 6, none of the four MMOR-RD proteins behaved like wild-type MMOR in this experiment. For MMOR-wt, the NADH consumption versus [MMOR] plot could be fit with a hyperbolic function representing NADH oxidation coupled to propylene oxide formation (sMMO activity) plus a line representing dioxygen reduction to hydrogen peroxide by excess reductase (oxidase activity). All four MMOR-RD NADH consumption curves could be fit extremely well without the hyperbolic function, suggesting that electron transfer to the hydroxylase was negligible. The oxidase activities of the RD1, RD3, and RD4 reductases were 4–8 times higher than that measured for MMOR-wt (Table V). The RD2 construct, however, reduced dioxygen to peroxide at only half the wild-type rate. When nitrobenzene was used as a substrate, p-nitrophenol formation was observed with MMOR-wt but not for any of the reactions including MMOR-RD constructs, confirming that the reversed-domain reductases are not competent for electron transfer to the hydroxylase.

Kinetics of MMOR-RD Reduction with NADH—The reaction of the MMOR-RD constructs with NADH was studied by stopped-flow UV-visible spectroscopy. Fig. 7 (A and B) shows typical data sets collected for the reduction of MMOR-wt and MMOR-RD4, respectively, with NADH at pH 7.0 and 4 °C. The kinetic steps for this reaction, which were determined previously for wild-type MMOR (16, 24) and MMOR-FAD (31), are depicted in Fig. 8. Spectra corresponding to four or five intermediates were resolved with Speclt software by using a sequential four-component (RD4) or five-component (wt, RD1, RD2, and RD3) model (Fig. 7, C and D). When NADH binds to MMOB, species CT1, which is characterized by a charge transfer interaction between the NADH nicotinamide ring and the FAD isovaloxazine moiety (λ<sub>max</sub> ~ 575 nm), is formed. Hydride transfer from NADH to the oxidized flavin (FAD<sub>ox</sub>) yields intermediate CT2, which exhibits a lower-energy charge transfer band (λ<sub>max</sub> ~ 740 nm) between the reduced FA (FAD<sub>red</sub>) and NAD<sup>+</sup> cofactors. Upon NAD<sup>+</sup> release, species CT2 decays to form intermediate HQ, which has FAD<sub>eq</sub> and [2Fe-2S]<sub>ox</sub> cofactors. Interdomain electron transfer produces the final species, SQ, with a spectrum characteristic of FAD<sub>eq</sub> and [2Fe-2S]<sub>red</sub>. In MMOR-wt, ~65% of the total electron transfer from FAD<sub>eq</sub> to the oxidized [2Fe-2S] cluster occurs simultaneously with NAD<sup>+</sup> release.

Single-wavelength stopped-flow data were collected at 458,
625, and 725 nm for each of the MMOR-RD proteins, as well as MMOR-wt (Fig. 9). The kinetic traces were fit with sums of three to five exponential decays. CT1 formation is observed as an increase in $A_{625}$; CT2 formation produces an increase in $A_{725}$. Subsequent decreases in both $A_{625}$ and $A_{725}$ correspond to CT2 decay/HQ formation. Intramolecular electron transfer to form SQ yields an increase in $A_{625}$ and a decrease in $A_{725}$. All four transitions are marked by decreases in $A_{458}$. A fifth step describing a slow process, such as reaction with excess NADH to form three-electron reduced MMOR, was required for data fitting at long time points. The rate constants determined for the five reductases are summarized in Table VI.

**DISCUSSION**

**MMOR-RD Constructs**—All four MMOR-RD constructs were prepared in good yield and with nearly full cofactor occupancy. The optical spectra of the oxidized RD1, RD3, and RD4 reductases show changes in the flavin component with respect to the MMOR-wt spectrum (Fig. 4). The altered MMOR-RD FAD$_{ox}$ spectra, which exhibit increased extinction coefficients at 394 nm, resemble free FMN more closely than does the wild-type FAD$_{ox}$ spectrum. Therefore, this optical change may correspond to an increase in solvent accessibility to the flavin cofactor in the RD1, RD3, and RD4 constructs. In fact, the largest optical changes are observed for RD3 (long linker) and RD4 (negatively charged linker); the RD1 (uncharged polar linker) spectrum is moderately perturbed. Only RD2, with a largely hydrophobic linker, displays an unchanged optical spectrum. Thus, in the reversed-domain reductases, the linker sequence appears to influence the flavin environment either by direct contact or by structural perturbations that decrease hydrophobicity near the cofactor.

**MMOR-RD Redox Potentials**—Measurements of both relative and absolute redox potentials for the four MMOR-RD proteins revealed substantial decreases in many of the flavin potentials compared with those of MMOR-wt (24) and MMOR-FAD (31) (Tables III and IV). The FAD$_{ox}$/sq redox potential in the RD1, RD3, and RD4 constructs is lowered by 30–45 mV, a result that correlates well with the optical changes observed for these proteins. Like the 350–450 nm region of the FAD$_{ox}$ visible spectrum, the FAD$_{ox}$/sq potential seems to be modulated by the hydrophobicity of the flavin environment. The first residue of the linker sequence is probably the key determinant in these shifted flavin properties. Analysis of the MMOR-FAD NMR structure indicates that the side chains of Leu-246 (residues 342 and 343 in full-length MMOR) pack against the FAD cofactor. In MMOR-RD2 (MMOR linker), the Leu, Pro, and
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Steady-state MMOR-RD Activity—Altering the connectivity of the MMOR domains did not seriously disrupt steady-state electron transfer from NADH to redox-active dyes. As indicated by increased activities in both DCPIP and K$_3$Fe(CN)$_6$ assays, the long, flexible RD4 linker may afford better accessibility of substrates to the reductase cofactors. Conversely, the protected flavin cofactor of RD2 may prevent efficient interaction with DCPIP, resulting in a 30% decrease in diaphorase activity compared with MMOR-wt. The substantial rate decrease observed for MMOR-RD3 (19% of wild-type activity) in the ferrocyanide experiment is probably the result of repulsion between the three negatively charged residues in the RD3 linker sequence and the negatively charged [Fe(CN)$_6$]$^{3-}$ substrate.

Although the MMOR-RD constructs were predicted to retain activity in steady-state sMMO reactions, no evidence of productive electron transfer to MMOH was observed in propylene or nitrobenzene assays. Even with somewhat altered reductase redox potentials, the normal electron transfer pathway, NADH → FAD → [2Fe-2S] → MMOH, is thermodynamically favored in the MMOR-RD constructs. Therefore, either interdomain electron transfer is blocked in MMOR-RD proteins or the reversed-domain constructs do not make suitable complexes with MMOH. Attaching a His$_6$ tag to the N terminus of MMOR-Fd abolishes cross-linking to MMOH, which suggests that the N-terminal portion of the ferredoxin domain is involved in binding MMOH. If so, linking the 27-kDa MMOR-FAD domain at this position, as done in this work, would interfere with MMOH-MMOR complex formation.

In steady-state sMMO assays, the RD1, RD3, and RD4 reductases exhibited considerably elevated oxidase activities relative to that of wild-type MMOR (Table V). The MMOR-RD1 construct, with an uncharged polar linker, showed a 4-fold increase in dioxygen reduction. More hydrophilic (RD3) and longer (RD4) linkers afforded even larger (6-8-fold) enhancements. Again, this behavior can be explained by increased solvent/substrate accessibility to the reductase cofactors. On the other hand, the low oxidase activity measured for the MMOR-RD2 protein indicates that its cofactors are well isolated from solvent.

Reaction of MMOR-RD Constructs with NADH—Reduction of the MMOR-RD constructs with NADH at pH 7.0 and 4°C proceeds via the same kinetic steps described previously for wild-type MMOR (16, 24), except that NAD$^+$ release and interdomain electron transfer do not coincide (Fig. 8). By analogy to MMOR (24) and MMOR-FAD (31), NADH is presumed to bind rapidly to MMOR-RD, forming a Michaelis complex, MC1, with an optical spectrum identical to that of oxidized reductase. Next, a conformational rearrangement gives rise to the FAD-NADH charge transfer interaction of species CT1. This step occurs at 350–400 s$^{-1}$ in both MMOR-wt and MMOR-FAD, but for the latter protein, relatively weak binding to NADH ($K_d$ = 25 μM versus 3.8 μM for MMOR) reduces the observed rate constant to 290 ± 20 s$^{-1}$ in the presence of a 10-fold excess of NADH (31). All four MMOR-RD proteins exhibit reduced CT1 formation rates, ranging from 96 s$^{-1}$ for RD3 to 229 s$^{-1}$ for RD2 (Table VI). Because NADH concentration dependence experiments were not performed for the reversed-domain reductases, this decrease in the observed rate constant cannot be assigned definitively to either reduced affinity for NADH or a slower conformational change. It was noted during purification, however, that the MMOR-RD proteins, particularly the RD3 construct, did not bind well to the Sepharose 5’-AMP affinity column. This observation suggests that weak affinity for NADH is at least partly responsible for the depressed CT1 formation rates.

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3 D. A. Kopp and S. J. Lippard, unpublished results.
Hydride transfer from NADH to the flavin cofactor yields intermediate CT2, which is characterized by a charge transfer interaction between NAD$^+$ and FADH$. This electron transfer step proceeds at 180–190 s$^{-1}$ in MMOR-wt and MMOR-FAD, as well as MMOR-RD2. The other MMOR-RD proteins have nearly identical CT1 and CT2 formation rates, indicating that the rate of hydride transfer may be limited by generation of the initial charge transfer complex. Release of NAD$^+$ to form immediate HQ, which has FAD$_{\text{eq}}$ and [2Fe-2S]$_{\text{eq}}$ cofactors, occurs at 90 s$^{-1}$ in MMOR and MMOR-FAD. Rates ranging from 75 s$^{-1}$ (RD2) to 14 s$^{-1}$ (RD3) were observed for the reversed-domain constructs, reflecting varying affinities for NAD$^+$. In MMOR-wt, a fraction of intramolecular electron transfer from FAD$_{\text{eq}}$ to [2Fe-2S]$_{\text{eq}}$ (−65% of total) takes place upon NAD$^+$ release; no electron transfer was detected for any of the MMOR-RD constructs in this kinetic step.

The final step, interdomain electron transfer to yield species SQ with cofactors largely in the FAD$_{\text{eq}}$ and [2Fe-2S]$_{\text{eq}}$ oxidation states, proceeds at 25 s$^{-1}$ for wild-type MMOR, corresponding to 35% of the total observed intramolecular electron transfer. This reaction is significantly slower in all four MMOR-RD proteins. A rate constant of 0.9 s$^{-1}$ was measured for MMOR-RD4 (long linker), which represents a 28-fold decrease with respect to MMOR-wt. Participation of the histidine and tyrosine residues of the RD4 linker in electron transfer is possible, although rather unlikely. The other reversed-domain reductases exhibit far slower interdomain electron transfer rates: 0.036 s$^{-1}$ for RD1 (700-fold decrease), 0.0062 s$^{-1}$ for RD2 (4000-fold decrease), and 0.038 s$^{-1}$ for RD3 (660-fold decrease).

Electron transfer in the three extremely slow reactions may actually involve bimolecular processes. If this step were to occur by a second-order reaction in the RD1, RD2, and RD3 constructs, the sequestered flavin ring in MMOR-RD2 may contribute to the especially low interdomain electron transfer rate observed for this protein.

Regardless of the electron transfer pathway, none of the MMOR-RD constructs with a short, 7-residue linker sequence is able to transfer electrons efficiently between the flavin and [2Fe-2S] cofactors. The short linkers apparently do not allow the reductase domains to align properly for rapid electron transfer. Tethering the reductase domains with a long, flexible linker, however, afforded an engineered protein, MMOR-RD4, with a reasonably fast interdomain electron transfer rate (0.9 s$^{-1}$ at 4°C). This result suggests that the orientation of domains in MMOR may be different than that in PDR. There is precedence for such variable interdomain contacts among FNR family proteins. For instance, in the maize leaf ferredoxin (Fd)-FNR complex, the Fd protein is rotated ~180° about the [2Fe-2S]-flavin axis with respect to the PDR domain arrangement (40). Aligning the FAD/NADH domains of PDR with those of benzoyl 1,2-dioxynogense reductase from Acinetobacter sp. strain ADP1, an FNR family member with MMOR-like domain connectivity (Fig. 1), reveals that each ferredoxin domain occupies almost completely different three-dimensional space (41). Alternatively, a longer linker may be required in MMOR to accommodate the adenosine portion of FAD, which is absent from the FNR cofactor of PDR.

Conclusions—By reattaching the separated [2Fe-2S] and FAD domains of MMOR in a non-native configuration, interdomain electron transfer can be restored, at least in part, thereby demonstrating the truly modular nature of the FNR family proteins. The requirement of a long, flexible linker for efficient electron transfer between MMOR-RD cofactors provides the first information about domain orientation in full-length MMOR, the structure of which has not yet been determined. Changing Leu-343 (MMOR numbering) to glycine in three of the MMOR-RD constructs reveals a role for this residue in modulating the flavin optical spectrum. FAD$_{\text{eq}}$/FAD$_{\text{eq}}$ redox potential, and reactivity with electron acceptors. This work opens the road to a more systematic investigation of MMOR-RD proteins with long linker sequences to establish the roles of affinity, proximity, and orientation in the interdomain electron transfer reaction.

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