Electronic Supplementary Information

Elucidation of the electron transfer environment in the MMOR FAD-binding domain from *Methylosinus sporium* 5

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1. Supplementary materials and methods

1.1. General materials and chemicals

All chemicals, including nitrate minimal salt (NMS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). MMOH was expressed and purified using *Methylosinus sporium* strain 5 (ATCC35069) purchased from the American Type Culture Collection (ATCC). BL21 (DE3) and pET30a(+) cells were purchased from EMD Millipore. DH5α(DE3) cells were purchased from New England Biolabs (USA). Synthetic nucleotides for the expression and purification of MMOB and MMORs were purchased and sequenced by Cosmogenetech. Propylene (99.5%) and methane gas (99.9%) were purchased from Hankook Gases. Protein purification was performed using an ATKA Pure 25 L Fast Protein Liquid Chromatography System (GE Healthcare Life Science). Optical absorption was measured using a UV-visible spectrometer (Agilent Technologies, Cary 60). Cells and proteins were harvested by centrifugation using a Beckman Coulter (Allegra X-15R) and Hanil Science (Combi 514R).

1.2. Expression and purification of MMOH and MMOB from *Methylosinus sporium* 5

MMOH was expressed and purified through *Methylosinus sporium* 5 in a copper-limited NMS medium at 37°C in a 5 L incubator to achieve an OD_{600} of 12-16 with a methane:air (v/v) ratio of 1:5, and the pH was adjusted to 7.0 with 100 mM NaOH or HCl. Cultures of *M. sporium* 5 were harvested by centrifugation (11,300 × *g*, 20 min at 4°C). MMOH was lysed through sonication and purified using a three-step process (DEAE, S200, and Q-Sepharose) to yield more than 95% purity and stored at -88°C for subsequent experiments.

1.3. Expression and purification of MMOB

A synthetic nucleotide, *mmoB*, in a pUC18 vector (*pUC18-mmoB*, Cosmosgene Tech) was amplified in DH5α (DE3). Amplified *mmoB* was digested with NdeI and HindIII

and cloned into pET30a(+) to generate *pET30a-mmoB,* which was then transformed into BL21 (DE3, Novagen). These transformed bacteria were cultured in LB medium containing 50 μg/mL kanamycin at 37°C until the mid-log phase. Lysis and two-step purification, including Q-sepharose and S75, were performed as reported.

1.4. Expression and purification of synthetic and mutated MMOR

Synthetic and mutated *mmoC* (Cosmogene Tech) were independently constructed using oligoprimers in the pUC18 plasmid by PCR as described below. These oligonucleotides *pET30a-mmoC* and mutated *pET30a-mmoCs* were transformed into BL21 (DE3) cells to express MMORs under the control of isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25°C and 200 rpm before harvesting. MMORs were purified by two-step purification using DEAE and Q-Sepharose, resulting in more than 95% purity. Ferrozine assays were performed and FAD concentrations were measured based on previous studies.

Primer	Sequence
MMOR forward primer	5' CGCATATGTACCAGATCG 3'
MMOR reverse primer	5' ATAAGCTTCAGCCGCTCG 3'
MMOR S159A forward primer	5' CGCGCCGCGCCTATTCCATGGCCTCG 3'
MMOR S159A reverse primer	5' CCATGGAATAGGCGCGGCGCGTATGCGTGCC 3'
MMOR Y160A forward primer	5' TCGGTCGCGGAGGATGGT 3'
MMOR Y160A reverse primer	5' GGCCATGGAAGCGGAGCGGCGC 3'
MMOR Y160F forward primer	5' CCGCTCCTTCTCCATGGCCTC 3'
MMOR Y160F reverse primer	5' CGCGTATGCGTGCCGGGAATTTC 3'
MMOR S161A forward primer	5' GGTCGCGGAGGATGGTCG 3'
MMOR S161A reverse primer	5' GAGGCCATGGCATAGGAGCGG 3'
MMOR S184A forward primer	5' GCGCACGCAGGCGAGCGT 3'
MMOR S184A reverse primer	5' AGATAATTGGCGAAAGCGCCGTCCGGCAG 3'

1.5. Crystallization, data collection and structure determination

The initial crystallization conditions were tested with crystallization kits, such as Crystal Screen 1 and 2, Index, Salt from Hampton Research (California, USA), and Wizard Classic 1 and 2 from Regaku Reagents (Washington, USA) in 96-well MRC Crystallization Plates (Molecular Dimensions). A total of 1.6 μL mixture of the MMOC-FAD domain (10 mg/mL) in 10 mM HEPES (pH 7.0) and an equal volume of crystallization solution were equilibrated against 70 μL reservoir solution at 22°C. Clustered rectangular rod-shaped crystals were obtained in the G12 condition (0.1 M Na-phosphate-citric acid pH 4.2, 10% PEG3000 and 0.2 M NaCl) of Wizard Classic 1 and 2 crystallization kit. Crystals were prepared using only the crystallization solution obtained from the company. For the native data collection, a single crystal was cryoprotected with the reservoir solution supplemented with 30% glycerol and flash-frozen directly in a -173°C nitrogen stream using a nylon loop. A dataset consisting of 360 frames with 1° oscillation at a wavelength of 0.9793 was collected on an ADSC Q270 CCD detector at beamline 7A of the Pohang Light Source II (PLS II) at the Pohang Accelerator Laboratory (PAL, Pohang, Korea). For the preparation of the heavy atom derivative, a crystal was soaked for 10 min in a cryo-solution containing 10 mM $AuCl_2$ and then flash-frozen in a -173°C nitrogen stream. A single anomalous dispersion (SAD) dataset was collected using the same scheme as the native data but at a wavelength of 1.03926. The data were indexed and scaled using the HKL-2000 software package.[1]

Au atoms were identified and density modification was performed using the PHENIX package.[2] The electron density and the initial model after the Autosol step were further improved by the AutoBuild task in the PHENIX.[2, 3] Manual model building of missing parts and subsequent refinement were performed several times with Coot[4] and Refmac[5], respectively, using native data at a resolution of 1.5 Å. The refinement statistics are presented in Table 1. The atomic coordinates and structure factors were deposited in the Protein Data Bank using the accession code 6L2U (www.rcsb.org/pdb).

1.6. Measurements of binding affinities between MMOH and MMORs

The binding affinities between MMOH and MMORs were monitored using a spectrofluorometer (JASCO, FP-8300) through tryptophan (Trp) quenching at 282 nm (λ_{ex}) and 336 nm (λ_{em}). One equivalent of MMOH (2.56×10^{-10} mol) in 25 mM MOPS, 50 mM NaCl, 1 mM DTT, and pH 6.5 in cuvette (JASCO, J/3 type material Q) was titrated by MMORs to saturation, i.e., approximately 110 equivalents. The 1:2 binding (MMOH:MMOR) curve was fitted to measure the binding affinities. Origin®2018 (OriginLab) and Mathematica (Wolfram) were used for curve fitting.

A represents MMOH with two binding sites, and B indicates MMORs. [B] and $[B]_f$ indicate the concentrations of MMOH-bound MMOR and free-MMORs, respectively.

$$K_{d1}: AB \rightleftharpoons A + B \Longrightarrow K_{d1} = \frac{[A]_f[B]_f}{[AB]} \Longrightarrow [AB] = \frac{[A]_f[B]_f}{K_{d1}}$$

$$K_{d2}: AB_2 \rightleftharpoons AB + B \Longrightarrow K_{d2} = \frac{[AB][B]_f}{[AB_2]} \Longrightarrow [AB_2] = \frac{[AB][B]_f}{K_{d2}}$$

One equivalent of MMOH (0.32 μM) can be expressed. $[A]_t$ and $[B]_t$ represent the concentration of MMOH and MMOB

$$0.32\ \mu M = [A]_t = [A]_f + [AB] + [AB_2]$$

$[A]_t$ and $[B]_t$ can be replaced by the following equations:

$$[B]_t = [B]_f + [AB] + 2[AB_2]$$

Fluorescence intensities were measured through Trp quenching, which can be defined as equation-①.

$$F.I. = (\alpha_1[A]_f) + (\alpha_2[AB]) + (\alpha_3[AB_2])(\alpha_1 \gg \alpha_2, \alpha_3)$$

$$= \left(\alpha_1 + \alpha_2\left(\frac{[B]_f}{K_{d1}}\right) + \alpha_3\left(\frac{{[B]_f}^2}{(K_{d1}K_{d2})}\right)\right)\left(\frac{[A]_t}{1 + \left(\frac{[B]_f}{K_{d1}}\right) + \left(\frac{{[B]_f}^2}{K_{d1}K_{d2}}\right)}\right) \cdots ①$$

α_1 represents the fluorescence intensity of free protein A, and α_2 indicates the fluorescence intensity of protein A and 1 equiv. of protein B are bound. α_3 is the fluorescence intensity of protein A, and 2 equiv. of protein B are bound. The 1:2 binding model is curve fitted using equation-①.

K_{d1} and K_{d2} are obtained by equation-② based on fluorescence intensity versus $[B]_t$ using equation-①.

$${[B]_f}^3 + \left((2[A]_t + K_{d2} - [B]_t){[B]_f}^2\right) + \left((K_{d2}[A]_t + (K_{d1}K_{d2}) - (K_{d2}[B]_t))[B]_f\right) - [B]_t = 0 \cdots ②$$

1.7. Measurements of specific enzyme activity

The binding affinities between MMOH (1.0 μM), MMOB (2.2 μM), and MMOR (0.5 or 1 μM) were incubated in a buffer (25 mM MOPS, 1 mM DTT, and 10 mM NaCl) bubbled with propylene gas for 20 min. The mixture was incubated at 25°C for 5 min, and steady-state kinetics were measured using a Cary 60 UV-visible spectrometer with the addition of excess NADH (340 nm, extinction coefficient = 6,220 M^{-1} cm^{-1}). Products were detected using a gas chromatography system (Thermo Fisher Scientific, Trace™ 1310) using an Agilent PLOT-Q stationary column. Experiments were performed at least thrice to obtain the average ± standard deviation.

2. Supplementary Tables

Table S1. Data collection and refinement statistics for MMOR-FAD domain

Statistics	Native	Au derivative
Data collection		
Space group	P212121	P212121
Cell dimensions (Å)		
a, b, c (Å)	34.59, 59.87, 94.50	34.11, 59.97, 96.66
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	30.0 - 1.5 (1.53 - 1.50)	30.0 - 2.0 (2.03 - 2.0)
R_{merge}[a] (%)	7.8 (26.1)	10.9 (58.1)
$CC_{1/2}$[b]	0.996 (0.988)	0.997 (0.966)
I/σ (I)	61.9 (14.7)	46.1 (7.0)
Completeness (%)	99.8 (100.0)	99.9 (100.0)
Redundancy	14.0 (14.3)	11.4 (11.3)
Wilson B factor (Å^2)	10.27	30.8
Structure refinement		
Resolution (Å)	30.0 - 1.5	
Reflections, total	30514	
R_{work}[c] / R_{free}	16.1/20.0	
No. atoms, protein/water	1700/170	
R.m.s.deviation		
Bond lengths (Å)	0.008	
Angles (°)	1.419	
Average B-factor (Å^2)	16.82	
Protein	15.85	
Ramachandran plot (%)		
Favored region	98.0	
Allowed region	2	

The numbers in parentheses are statistics from the highest resolution shell.

[a] $R_{merge} = \Sigma |I_{obs} - I_{avg}| / I_{obs}$, where I_{obs} is the observed intensity of individual reflection and I_{avg} is the average over symmetry equivalents. [b] (Karplus and Diederichs, 2012)[6]

[c] $R_{work} = \Sigma ||F_o| - |F_c|| / \Sigma |F_o|$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively. *The* R_{free} was calculated using 5% of the data.

Table S2. Data collection and refinement statistics for MMOR-FAD domain. All experiments were performed at least three times (average ± std).

Protein	Ratio of FAD : protein
MMOR	1.0 ± 0.0
S159A	1.6 ± 0.1
Y160A	0.4 ± 0.3
S161A	2.1 ± 0.1
S184A	1.7 ± 0.0
MMOR-Fd	0.0

Table S3. Measurements of specific enzyme activities in MMOR and mutated versions of MMOR. All experiments were performed at least three times (average ± std).

Protein	H:B:R=1:2:0.5	H:B:R=1:2:1
MMOR	648.4 ± 22.8	771.0 ± 32.4
S159A	313.2 ± 18.7	462.4 ± 20.9
Y160A	2.6 ± 2.0	3.1 ± 2.6
S161A	308.5 ± 18.8	568.9 ± 16.9
S184A	373.2 ± 9.7	627.3 ± 7.1

Table S4. Ferrozine assay to measure the ratio of iron:MMOR.

Protein	Ratio of Fe:protein
MMOR	1.9 ± 0.0
S159A	1.7 ± 0.1
Y160A	2.2 ± 0.1
S161A	2.1 ± 0.1
S184A	1.7 ± 0.1
MMOR-Fd	2.0 ± 0.0

Table S5. Measurements of iron and FAD in MMOR-Y160F.

Protein	Fe	FAD
MMOR	2.0 ± 0.0	1.0 ± 0.0
MMOR-Y160F	2.0 ± 0.1	2.0 ± 0.0

All experiments were performed at least three times.

Table S6. Measurement of specific enzyme activity of MMOR-Y160F.

Protein	H:B:R=1:2:0.5	H:B:R=1:2:1
MMOR	648.4 ± 22.8	771.0 ± 32.4
MMOR-Y160F	4.6 ± 2.0	27.2 ± 12.3

All experiments were performed at least three times.

Supplementary Figures

Fig. S1 Solution structures of MMOR domains from *Methylococcus capsulatus* Bath (a) The structure of the ferredoxin binding domain of MMOR (MMOR-Fd, PDB: 1JQ4) was elucidated using nuclear magnetic resonance (NMR). Reddish brown spheres represent Fe ions and yellow sphere represent sulfur atoms from [2Fe-2S] cluster. (b) MMOR-FAD/NADH binding domain as reported by NMR (PDB: 1TVC). A cofactor, FAD, is located between MMOR-FAD and MMOR-NADH binding domains.

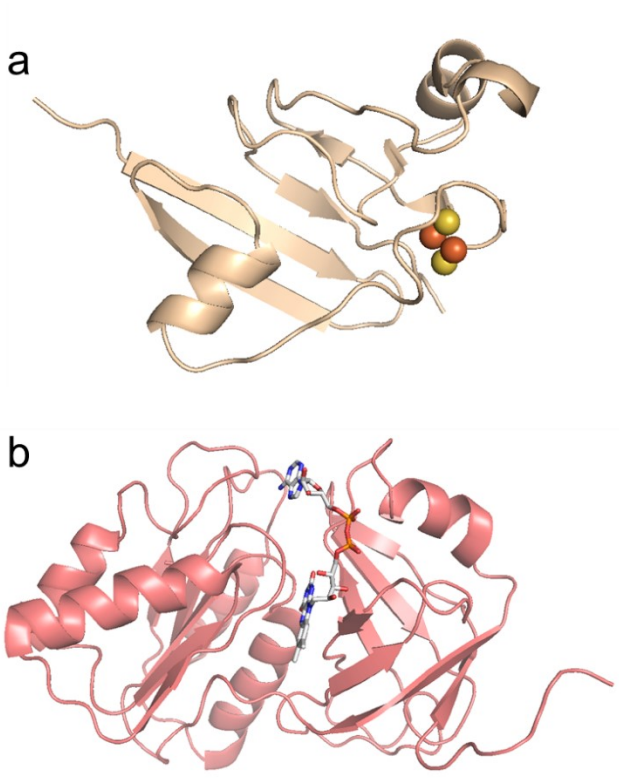


Fig. S2 Sequence alignment of MMOR. The sequence from *Methylosinus sporium* 5 aligned to other type II methanotrophs. The sequence identity and similarity are presented as percentage with gene accession number. Each colored alphabet represents identity (black), mismatch (red), and similarity (green). Bold letters indicate the targets of mutagenesis in this study. The highlighted regions represent ferredoxin (pale green), FAD (pale blue) and NADH (pale orange) domains.

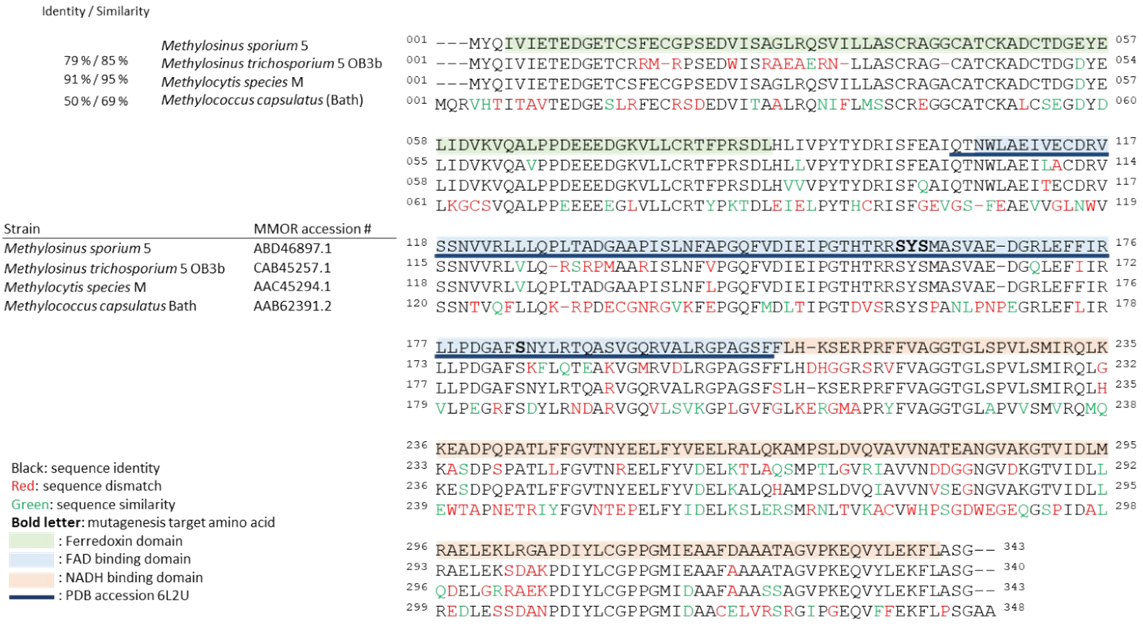


Fig. S3 Expression and purification of synthetic and mutated MMOR. Purified MMORs were identified using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

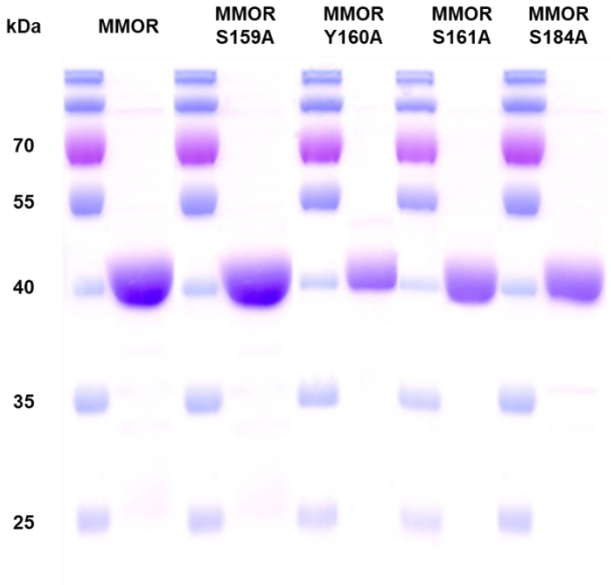


Fig. S4 UV-visible spectra of MMORs purified from *Methylosinus sporium* 5.

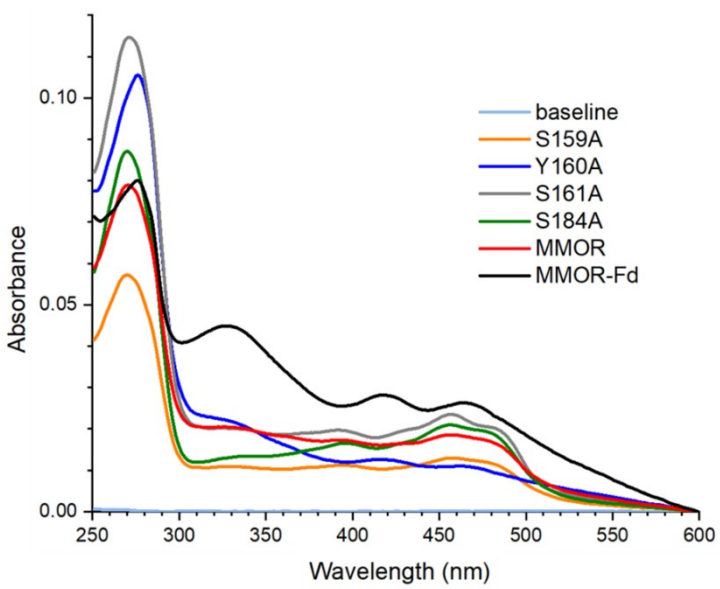


Fig. S5 Tryptophan quenching results of MMOH by the addition of MMORs. Fluorescence intensities were measured of MMOH by titration of (a) MMOR in oxidized state, (b) MMOR-S159A, (c) MMOR-S161A, and (d) MMOR-S184A.

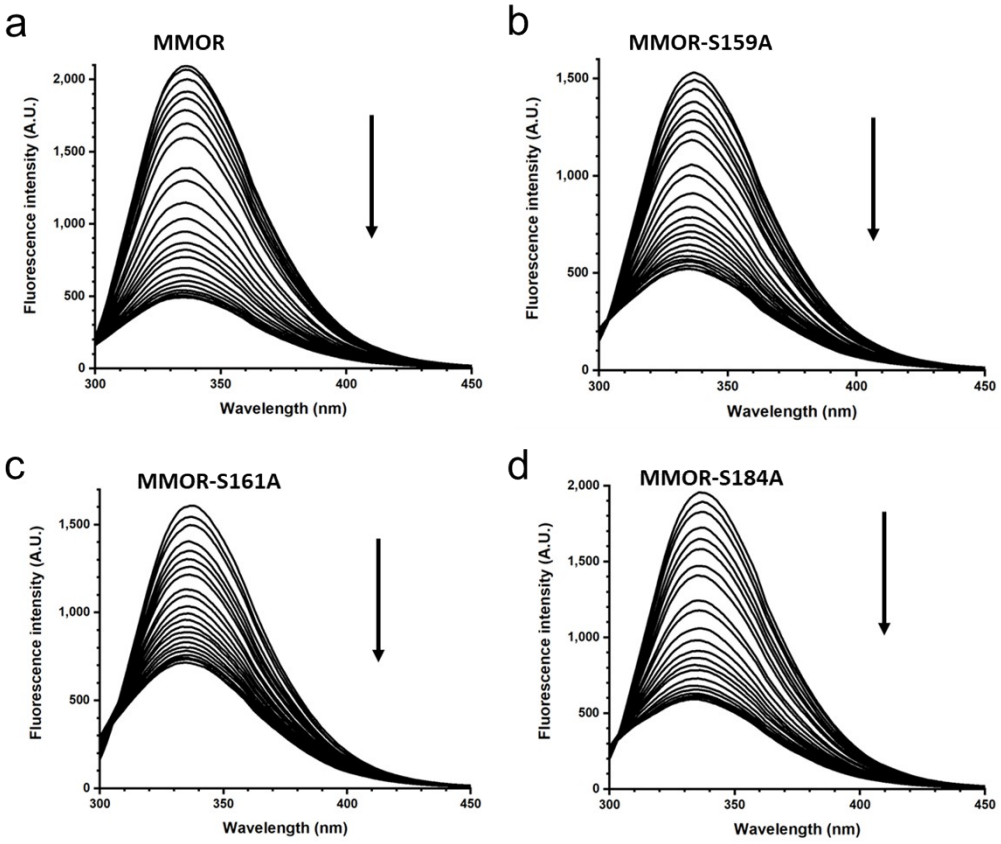


Fig. S6 Binding affinities including the first dissociation constant (K_{d1}) and second dissociation constant (K_{d2}) were determined by the curve fitting based on a binding ratio of 1:2. The fluorescence intensities were measured and fitted through Origin®2018 (OriginLab) and Mathematica (Wolfram).

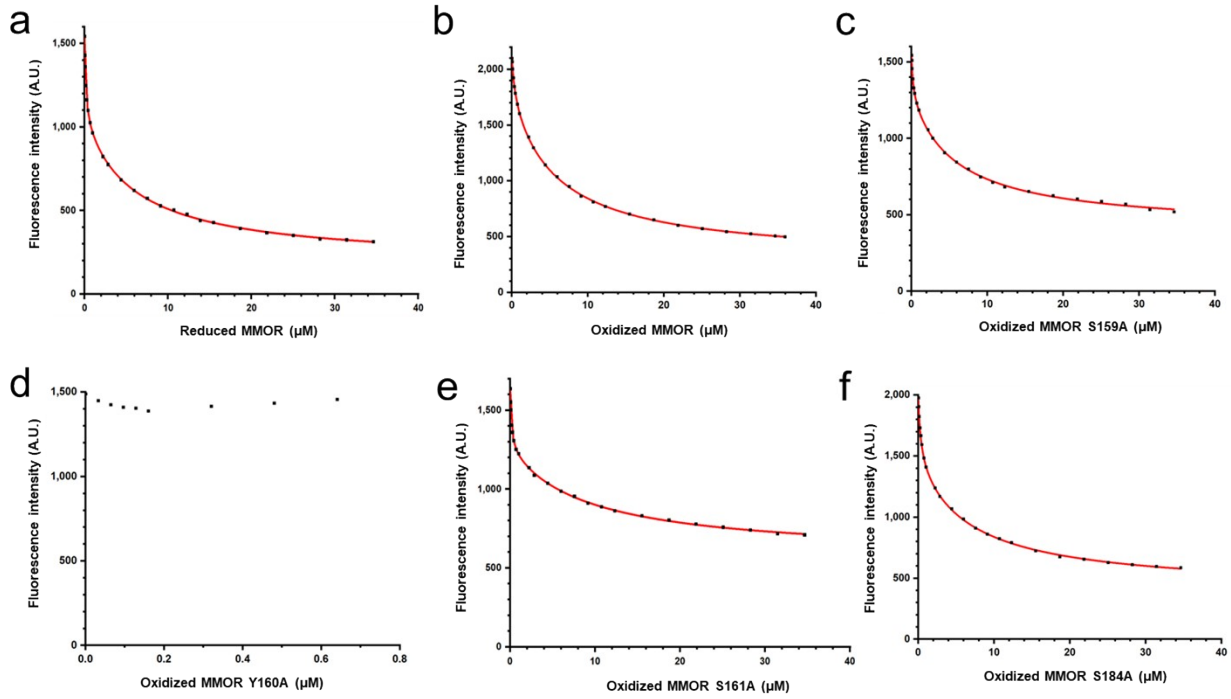
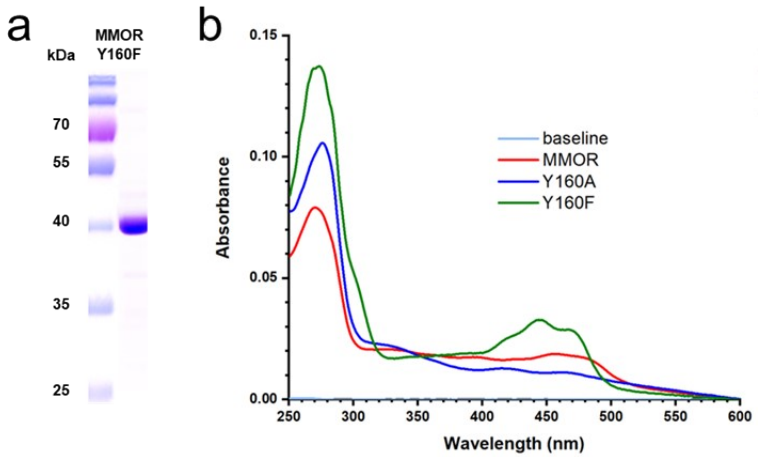


Fig. S7 Characterization of mutated MMOR-Y160F. (a) Purified MMOR-Y160F as detected in SDS-PAGE gel. (b) UV-visible spectra corresponding to MMOR (red), MMOR-Y160A (blue), and MMOR-Y160F (green).



REFERENCES

1. Z. Otwinowski and W. Minor, *Meth. Enzymol.*, 1997, **276**, 307-326.
2. P. D. Adams, P. V. Afonine, G. Bunkoczi, V. B. Chen, I. W. Davis, N. Echols, J. J. Headd, L. W. Hung, G. J. Kapral, R. W. Grosse-Kunstleve, A. J. McCoy, N. W. Moriarty, R. Oeffner, R. J. Read, D. C. Richardson, J. S. Richardson, T. C. Terwilliger and P. H. Zwart, *Acta Crystallogr D Biol Crystallogr*, 2010, **66**, 213-221.
3. T. C. Terwilliger, R. W. Grosse-Kunstleve, P. V. Afonine, N. W. Moriarty, P. H. Zwart, L. W. Hung, R. J. Read and P. D. Adams, *Acta Crystallogr D Biol Crystallogr*, 2008, **64**, 61-69.
4. P. Emsley and K. Cowtan, *Acta Crystallogr D Biol Crystallogr*, 2004, **60**, 2126-2132.
5. G. N. Murshudov, P. Skubak, A. A. Lebedev, N. S. Pannu, R. A. Steiner, R. A. Nicholls, M. D. Winn, F. Long and A. A. Vagin, *Acta Crystallogr D Biol Crystallogr*, 2011, **67**, 355-367.
6. P. A. Karplus and K. Diederichs, *Science*, 2012, **336**, 1030-1033.