Supporting Information

for

Dioxygen Activation at Non-Heme Diiron Centers: Characterization of Intermediates in a Mutant Form of Toluene/*o*-Xylene Monooxygenase Hydroxylase

Leslie J. Murray,[1] Ricardo García-Serres,[2] Sunil Naik,[2] Boi Hanh Huynh,[2,]* and Stephen J. Lippard[1,]*

[1]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and [2]Department of Physics, Emory University, Atlanta, Georgia 30322

E-mail: lippard@mit.edu and vhuynh@physics.emory.edu

Experimental

General Considerations. Plasmids containing the genes for the toluene/*o*-xylene monooxygenase components were supplied by Professor Alberto Di Donato. Heterologous expression and preparation of the ToMO component proteins were carried out as described elsewhere[1] and these conditions were used to prepare the ToMOH I100W mutant protein. The iron content ranged from 4.2 to 4.6 per ToMOH dimer for wild-type and I100W samples. ToMOH samples, both I100W and wild-type, enriched with ^{57}Fe for Mössbauer spectroscopy were obtained by expression in LeMaster's Media[2] containing $^{57}FeCl_3$, which was prepared by dissolving ^{57}Fe powder (96.7% isotopic purity, AMT Ltd.) in concentrated hydrochloric acid. All other reagents were purchased from Aldrich Chemical Company.

Site Directed Mutagenesis of the ToMOH α Subunit. The mutation I100W was introduced by site directed mutagenesis on the parent pET-22b(+)/touBEA plasmid with an MJ Research MiniCycler using DNA Polymerase pfU Turbo, dNTPs, and reaction buffer (Stratagene) according to the manufacturer's protocol. The oligonucleotide sequences used for the primers were as follows: 5′-caacttcacttcggagcgTGGgcacttgaagaat-acg-3′, and 5′-cgtattcttcaagtgcCCAcgctccgaagtgaagttg-3′, where the former is the sense and the latter the anti-sense primer. PCR products were transformed into *E. coli* XL-1 Blue Supercompetent cells as outlined by the manufacturer (Strategene) and grown overnight on LB-Agar plates containing ampicillin (300 μg/mL). Five colonies from each plate were picked and grown in 5 mL cultures (LB media, 300 μg ampicillin /mL) for 20 h. Cells were pelleted at 3500 rpm for 15 min and the plasmids were isolated with a Qiagen Mini-Prep kit. Isolated plasmids were submitted for sequencing in the forward and reverse directions.

Stopped-Flow UV/Visible Experiments for the Reaction of $I100W_{red}$:3 ToMOD with O_2. For the SF experiments, the HiTech DX2 stopped flow unit was made anaerobic by washing with sodium dithionite solutions (in excess of 4 mM). The sodium dithionite solution was allowed to stand in the

instrument syringes and lines for at least 15 min to ensure complete scavenging of dioxygen. The instrument was then flushed extensively with anaerobic buffer, 25 mM MOPS pH 7.0, immediately prior to use. All protein solutions were made anaerobic by cycles of vacuum degassing and purging with nitrogen. Samples were loaded either into tonometers or Hamilton gas-tight sample lock syringes in an anaerobic chamber. The I100W ToMOH variant (120 μM) was reduced in the presence of ToMOD (360 μM) and methyl viologen (120 μM) in 25 mM MOPS pH 7.0 with excess sodium dithionite in the anaerobic chamber. The mixture was allowed to stand for 20 min and then dialyzed twice for one h against anaerobic 25 mM MOPS pH 7.0 buffer. The reduced hydroxylase and coupling protein were then transferred to a Hamilton gas-tight syringe, removed from the anaerobic chamber, placed on to the stopped-flow instrument, and pushed versus oxygenated 25 mM MOPS pH 7.0 buffer. Single wavelength data monitored 500 nm in photomultiplier tube mode (Figure S1, inset) and multi-wavelength diode array data (Figure S1) were collected at 4.0 ± 0.1 °C.

Rapid-Freeze Quench EPR and Mössbauer Sample Preparation. Samples for EPR and Mössbauer spectroscopy were prepared and the spectra recorded with instrumentation previously described.[3,4] Diiron(II) I100W ToMOH was prepared as described above for the stopped-flow UV/visible experiments except at higher protein concentrations. The concentrations of I100W:3ToMOD were 396 μM for EPR and 590 μM for Mössbauer samples, after mixing against O_2-saturated buffer. EPR spectra of the g = 2.00 signal were recorded at 30 K with the following parameters: power = 0.02 mW; frequency = 9.65 GHz; modulation frequency = 100 kHz; modulation amplitude = 5 G; gain = 6.3 x 10^4. EPR spectra of the g = 16 signal were recorded at 8 K with the following parameters in parallel mode: power = 20 mW; frequency = 9.39 GHz; modulation frequency = 100 kHz; modulation amplitude = 10 G; gain = 6.3 x 10^4. Mössbauer spectra were recorded at 4.2 K with an applied magnetic field of either 50 mT or 8 T parallel to the γ-irradiation.

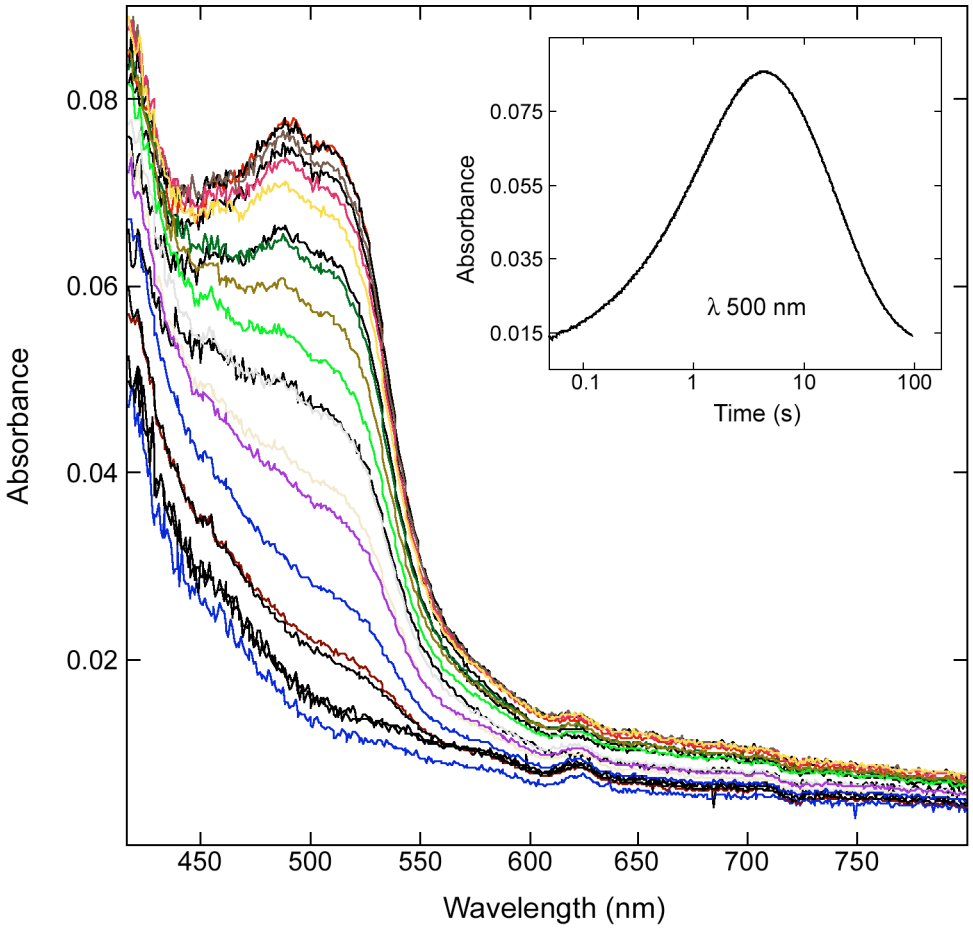

Figure S1. Stopped-flow UV/visible spectra for the reaction of I100W$_{red}$:3 ToMOD with O_2-saturated buffer. Multi-wavelength data reveal the formation and decay of a transient species with an absorbance maximum at 500 nm. No other absorbance bands are observed during the reaction. The single wavelength data at 500 nm (inset) were fit to a three component sequential kinetic model and the calculated rate constants and the molar extinction coefficient for the intermediate are 0.804 ± 0.001 s^{-1} (formation), 0.054 ± 0.002 s^{-1} (decay), and 1.5 ± 0.2 x 10^3 $M^{-1}cm^{-1}$ respectively.

References

1. Sazinsky, M. H.; Bard, J.; Di Donato, A.; Lippard, S. J., *J. Biol. Chem.* **2004,** 279, 30600-30610.
2. LeMaster, D. M.; Richards, F. M., *Biochemistry* **1985,** 24, 7263-7268.
3. Baldwin, J.; Krebs, C.; Ley, B. A.; Edmondson, D. E.; Huynh, B. H.; Bollinger, J. M., Jr., *J. Am. Chem. Soc.* **2000,** 122, 12195-12206.
4. Krebs, C.; Chen, S.; Baldwin, J.; Ley, B. A.; Patel, U.; Edmonsdon, D. E.; Huynh, B. H.; Bollinger, J. M., Jr., *J. Am. Chem. Soc.* **2000,** 122, 12207-12219.