Supporting Information for

Active Site Threonine Facilitates Proton Transfer During Dioxygen Activation at the Diiron Center of Toluene/*o*-Xylene Monooxygenase Hydroxylase

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General Considerations. Plasmids containing the genes for the toluene/*o*-xylene monooxygenase components were supplied by the laboratory of Professor Alberto Di Donato, Naples, Italy. The preparation of all ToMO components was described previously.[1] Heterologous expression and purification of four ToMO component proteins, the hydroxylase (ToMOH), Rieske (ToMOC), reductase (ToMOF), and regulatory (ToMOD) proteins, were carried out as described.[1,2] Identical conditions were used to prepare ToMOH T201 variants. Conversion of T201 into T201C, T201G, and T201V was performed by site-directed mutagenesis as described[2] using the parent pET22b(+)/touBEA plasmid with the following designed primers (5′-C GAA ACA GGC TTC GGT AAT ATG CAG TTT C-3′ and 5′-G AAA CTG CAT ATT ACC GAA GCC TGT TTC G-3′ for T201G; 5′-C GAA ACA GGC TTC GTT AAT ATG CAG TTT CTC G-3′ and 5′-C GAG AAA CTG CAT ATT AAC GAA GCC TGT TTC G-3′ for T201V; and 5′-C ATT CGA AAC AGGC TTC TGC AAT ATG CAG TTT CTC-3′ and 5′-GAG AAA CTG CAT ATT GCA GAA GCC TGT TTC GAA TG-3′ for T201C. The primers were obtained from Integrated DNA Technologies. Detailed procedures for iron assays,[1] specific activity determinations with phenol,[3] measurements of the regiospecificity of toluene and *o*-xylene oxidation,[2] and single-turnover assays[4] were described previously.

Crystallization, Data collection, Structure Determination, and Refinement. The crystallization conditions for ToMOH T201X enzymes followed previously published precedures.[5,6] X-ray diffraction data were collected at the SSRL on beam line 11-1 or 12-2. Phasing of ToMOH T201X data was accomplished by using EPMR and the 1.85 Å native ToMOH coordinates (PDB code 2INC) as described previously.[5,6]

Characterization of Oxygenated Intermediates in ToMOH T201G by Mössbauer Spectroscopy. The ^{57}Fe-enriched T201G ToMOH sample was prepared as described previously.[2] For

Mössbauer experiments, ^{57}Fe-enriched ToMOH T201G (500-800 μM) was reduced anaerobically by reacting the protein with excess sodium dithionite in the presence of an equimolar amount of methyl viologen for 30 min. The reduced protein was dialyzed against 1 L of 25 mM MOPS, pH 7.0 buffer for ~ 3 hr, anaerobically. Following dialysis, the regulatory protein was added to the reduced hydroxylase. The solution was transferred into a Mössbauer cup and rapidly frozen in liquid N_2. To prepare an oxygenated sample of the T201G variant, a solution of T201G $ToMOH_{red}$:D was manually mixed with an equal volume of O_2-saturated buffer at 4 ^{o}C for 15 s and the reaction was quenched by rapidly spraying the solution into liquid nitrogen. As a control experiment, we also carried out the reaction using the T201S variant, as described previously.[2] The experimental conditions for Mössbauer experiments were as previously reported.[2]

Characterization of Oxygenated Intermediates in ToMOH T201X (X=S, C, G, V) by Optical Spectroscopy. An optical band characteristic of the oxygenated intermediate in ToMOH T201 variants was monitored by using a HiTech DX2 stopped-flow spectrophotometer. The drive syringes and flow lines of this instrument were made anaerobic by passage of at least 10 mL of anaerobic solution of 4 mM sodium dithionite in 25 mM MOPS, pH 7 buffer. The excess dithionite was removed by flushing the syringes with anaerobic buffer. T201X ToMOH protein was reduced anaerobically by reacting the protein with excess sodium dithionite in the presence of an equimolar amount of methyl viologen for 30 min. The reduced protein was dialyzed against 1 L of 25 mM MOPS, pH 7.0 buffer for ~ 3 hr, anaerobically. Following dialysis, the regulatory protein was added to the reduced hydroxylase. The solution was transferred to a tonometer and loaded into the anaerobic stopped flow instrument. This solution was mixed against an equal volume of O_2-saturated 25 mM MOPS, pH 7.0 buffer. The temperature was thermostatted using

a circulating water bath. Data were collected using either a PMT (photomultiplier tube) for halogen lamp illumination or a diode array detector for a xenon arc lamp. To vary the dioxygen concentration, nitrogen-saturated buffer was added to the dioxygen-saturated buffer to dilute the concentration of dioxygen. Buffers with varied pH values in the range of pH 6.5 - 8.0 were prepared with 25 mM MOPS buffer by adjusting the amount of NaOH/HCl solution. For KSIE measurements, deuterium oxide (99.9%) was purchased from Cambridge Isotope Laboratories or Icon Isotopes and used to prepare 25 mM MOPS buffer, pL 7.0. The pL value was adjusted by adding an appropriate amount of NaOD solution (Aldrich). Upon varying reaction conditions, including temperature and pH, no distinguishable differences in the optical feature of $T201_{peroxo}$ were observed. The time-dependent absorbance data were analyzed by the software packages Kinetic Studio (TgK Scientific) and Origin 6.1 (OriginLab Corporation). For the reaction of the T201C variant, the best fit was obtained with a three-exponential function, consisting of one formation and two consecutive decay processes corresponding to $ToMOH_{red} \rightarrow T201_{peroxo} \rightarrow T201^*_{peroxo} \rightarrow ToMOH_{ox}$, where $T201^*_{peroxo}$ and $ToMOH_{ox}$ are the sequential decay products of $T201_{peroxo}$ (eq 1). For simplicity, each component, $ToMOH_{red}$, $T201_{peroxo}$, $T201^*_{peroxo}$, and $ToMOH_{ox}$, and their

$$\begin{aligned}\text{Abs} = [A]_0 *(\varepsilon_D +((\varepsilon_A - \varepsilon_D)*\exp(-k_1*x))+((\varepsilon_B - \varepsilon_D)*k_1*1/(k_2-k_1)*(\exp(-k_1*x)-\exp(-k_2*x)))+((\varepsilon_c-\varepsilon_D)\\ *k_1*k_2*1/(k_2- k_1)*(1/(k_3- k_1)*(\exp(-k_1*x)-\exp(-k_3*x))-1/(k_3- k_2)*(\exp(-k_2*x)-\exp(-k_3*x))))) \quad (1)\end{aligned}$$

conversion rate constants are represented as A, B, C, and D, and as k_1, k_2, and k_3 respectively. The initial enzyme concentration was defined as $[A]_o$. For dioxygen activation with the T201G variant, a two-exponential function derived from $ToMOH_{red} \rightarrow T201_{peroxo} \rightarrow ToMOH_{ox}$ gave the

best fit (eq 2). For simplicity, $ToMOH_{red}$, $T201_{peroxo}$, and $ToMOH_{ox}$ and rate constants for the conversion are abbreviated as A, B, and C and k_1 and k_2, respectively.

$$Abs = \varepsilon_c*[A]_0 +(\varepsilon_A-\varepsilon_c)*[A]_0 *\exp(-k_1*x)+(\varepsilon_B-\varepsilon_c)*[A]_0*k_1/(k_2-k_1)*(\exp(-k_1*x)-\exp(-k_2*x)) \quad (2)$$

Table S1. X-ray data collection, phase determination, and refinement statistics of T201X variants (X= S, G, C, V).

	T201S	T201G	T201C	T201V
Data Collection				
Beamline	SSRL 11-1	SSRL 11-1	SSRL 12-2	SSRL 11-1
Wavelength (Å)	0.979	0.979	0.979	0.979
Space Group	$P3_121$	$P3_121$	$P3_121$	$P3_121$
Unit cell dimensions (Å)	182.8 x 182.8 x 68.9	183.0 x 183.0 x 68.7	183.249x 183.249 x 68.128	182.9 x 182.9 x 68.6
Resolution range (Å)	50.0 - 2.90	50.0 - 2.10	50-2.4	50.0 - 1.90
Total Reflections	223666	1151797	1539056	1801358
Unique Reflections	29616	74572	48523	98258
Completeness (%)[a]	100 (99.9)	96.7 (92.9)	99.2 (96.8)	95.5 (84.6)
I/s(I)[a]	22.7 (7.7)	30.8 (3.8)	11.3 (4.2)	36.8 (2.9)
R_{sym} (%)[a,b]	10.4 (31.2)	10.3 (56.0)	9.3 (48.3)	9.8 (66.0)
Refinement				
R_{cryst} (%)[c]	18.4	22.3	18.4	20.6
R_{free} (%)[d]	22.5	23.5	24.3	21.8
Average B-value (Å^2)	19.6	35.6	55.5	33.9
r.m.s deviation bond length (Å)	0.008	0.007	0.031	0.006
r.m.s deviation bond angles (°)	1.54	1.36	2.39	1.36
No. Protein Atoms	7337	7335	7370	7338
No. Non-Protein Atoms	159	248	164	699
Fe Atoms	2	2	2	2
Water Molecules	101	196	152	597
PEG-400 Fragments	2	2	1	4
Glycerol Molecules	3	2	0	4
RMSD to Wild-Type ToMOH	0.170	0.136	0.178	0.114
PDB Code	3N1Z	3N1Y	3N1X	3N20

[a]Values in parentheses are for the highest resolution shell. [b]$R_{sym} = \Sigma_i\Sigma_{hkl}|I_i(hkl) - \langle I(hkl)\rangle|/ \Sigma_{hkl}\langle I(hkl)\rangle$, where $I_i(hkl)$ is the ith measured diffraction intensity and $\langle I(hkl)\rangle$ is the mean intensity for the Miller index (hkl). [c]$R_{cryst} = \Sigma_{hkl}||F_o(hkl)| - |F_c(hkl)||/ \Sigma_{hkl}|F_o(hkl)|$. [d]$R_{free} = R_{cryst}$ for a test set of reflections (5% in each case).

Table S2. Product distribution of ToMOH T201X variants in toluene and *o*-xylene oxidation (X= S, G, C, V).

toluene oxidation					
	Wild type	T201S	T201C	T201G	T201V
o-cresol (%)	30 (2)	31 (1)	32 (1)	59 (1)	18 (1)
m-cresol (%)	21 (1)	16 (0)	27 (1)	13 (1)	32 (1)
p-cresol (%)	47 (1)	51 (1)	36 (1)	25 (1)	37 (1)
benzyl alcohol (%)	2 (1)	2 (1)	4 (1)	3 (1)	12 (1)
o-xylene oxidation					
	Wild type	T201S	T201C	T201G	T201V
2,3-dimethyl phenol (%)	19	36	32	64	15
3,4-dimethyl phenol (%)	81	64	68	36	85
2-methyl benzyl alcohol (%)	Not detected	Not detected	Not detected	Not detected	Not detected

Figure S1. Active site coordination and geometry in (A) ToMOH T201G, (B) T201S, (C) T201V, (D) T201C, (E) wild type (PDB Code 2INC). Iron atoms are displayed as green spheres and solvent and oxygen ligands derived from PEG are shown as cyan spheres. Amino acid side chains are represented as sticks in grey (carbon), blue (nitrogen), and red (oxygen). Interatomic distances were represented in angstroms.

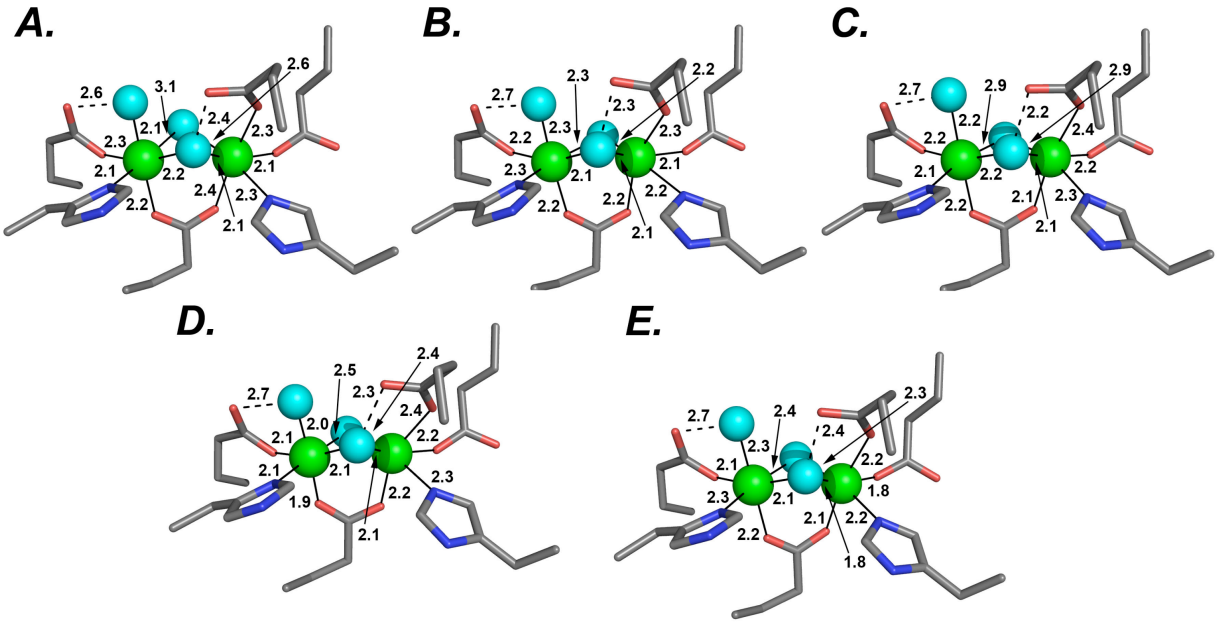


Figure S2. Michaelis-Menten kinetics of ToMOH wild-type and T201X variants for phenol oxidation.

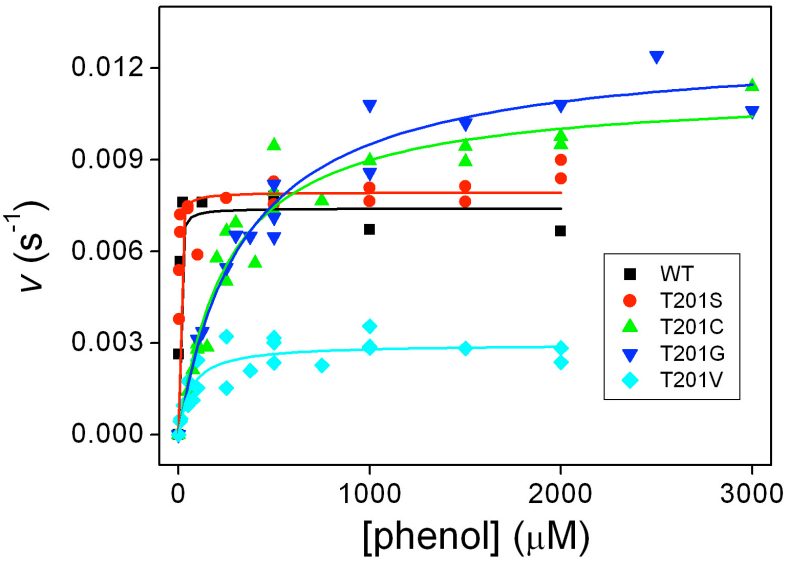
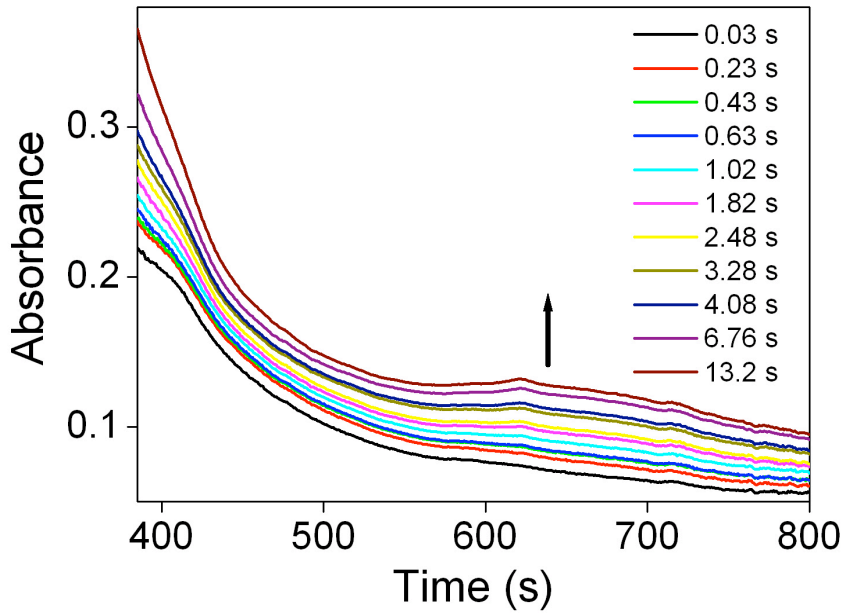
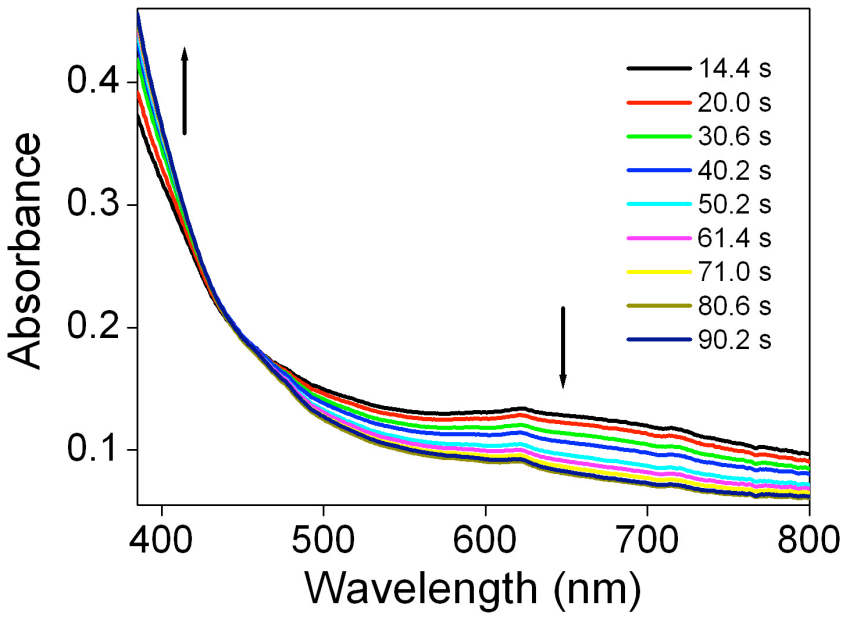


Figure S3. Change in absorption of UV-vis spectrum of the reaction of ToMOH$_{red}$D T201X mixed with dioxygen-saturated buffer (X=C, G), [ToMOH T201X] = ~150 µM, [ToMOD] = 450 µM in 25 mM MOPS, pH 7.0 at 4.0 ± 0.1 °C. (A) Formation of T201$_{peroxo}$ in ToMOH T201G at various reaction times. (B) Decay of T201$_{peroxo}$ in ToMOH T201G (C) Decay of T201$_{peroxo}$ in ToMOH T201C at various reaction times.

(A)



(B)



(C)

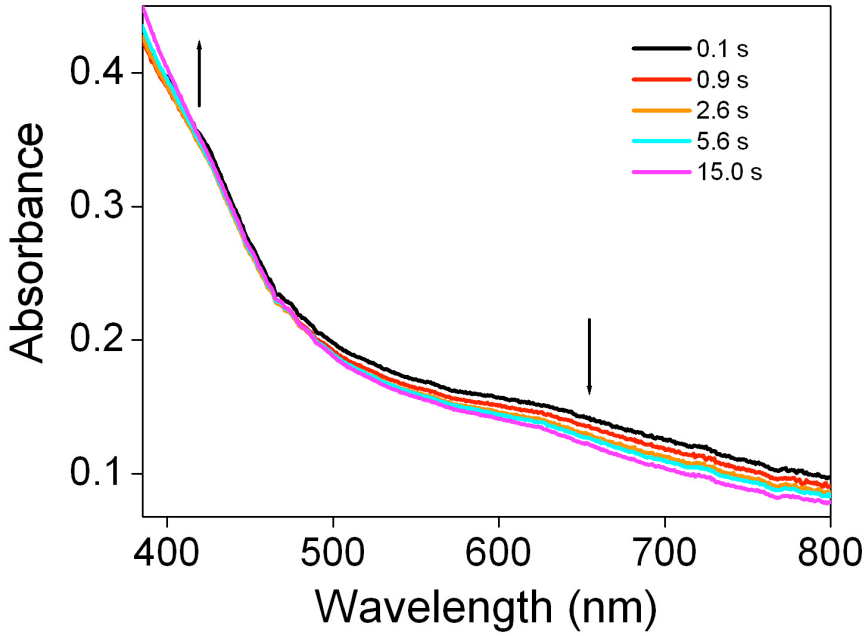

0.1 s
0.9 s
2.6 s
5.6 s
15.0 s
Absorbance
0.4
0.3
0.2
0.1
400
500
600
700
800
Wavelength (nm)

Figure S4. 4.2-K/53-mT Mössbauer spectra of oxygen activation in T201 variants of ToMOH. Left panel: (A) Spectrum of an anaerobic solution of T201S $ToMOH_{red}D$ ([^{57}Fe]/[ToMOH] = 3.5 ± 0.1, 0.5 mM of ToMOH and 1.5 mM of ToMOD), which was allowed to react at 4 °C with an equal volume of an O_2-saturated buffer solution for 45 ms using an Update Instruments rapid freeze-quench apparatus (vertical bars). The spectrum of an anaerobic control sample is overlaid as a solid line. (B) The experimental difference spectrum (vertical bars) fitted to four quadrupole doublets with the following parameters: $\delta(1) = 0.65$ mm/s and $\Delta E_Q(1) = 1.49$ mm/s (18 % of total intensity, blue line); $\delta(2) = 0.53$ mm/s and $\Delta E_Q(2) = 0.66$ mm/s (11 % of total intensity, red line); $\delta(3) = 1.26$ mm/s and $\Delta E_Q(3) = 3.46$ mm/s (-8 % of total intensity, orange line); and $\delta(4) = 1.23$ mm/s and $\Delta E_Q(4) = 2.40$ mm/s (-17 % of total intensity, green line). The total simulation is shown as a black line. The parameters of quadrupole doublets 1 and 2 are within the experimental uncertainty (±0.03 mm/s) identical to those published for $T201_{peroxo}$ and $ToMOH_{peroxo}$.[2] The parameters of quadrupole doublets 3 and 4 are typical of high-spin Fe(II) and are assigned to the reactant complex.
Right panel: (A) Spectrum of an anaerobic solution of T201G $ToMOH_{red}D$ ([^{57}Fe]/[ToMOH] = 3.9 ± 0.1, 0.6 mM of ToMOH and 1.8 mM ToMOD), which was mixed at 4°C with an equal volume of an O_2-saturated buffer solution for ca. 15 s before manual freezing of the sample (vertical bars). The spectrum of an anaerobic control sample is overlaid as a solid line and is scaled to account for 63% of the total intensity of the spectrum of the 15-s sample. (B) Removal of the features of the anaerobic complex results in the "reference spectrum" of the diiron(III/III) complexes in T201G $ToMOH_{red}D$ after a 15-s reaction time (vertical bars). Comparison of this spectrum to experimental reference spectra of $T201_{peroxo}$ (blue line) and $ToMOH_{peroxo}$ (red line), which were prepared by removal of the respective three other quadrupole doublets from the difference spectrum described in the left panel, reveals that ~10% of each species is present in the 15 s sample of the T201G $ToMOH_{red}$:D complex. In addition, there is additional broad absorption observed (purple bracket), which emanates from a quadrupole doublet with parameters [$\delta \approx 0.5$ mm/s and $\Delta E_Q \approx 1.1$ mm/s] typical of high-spin Fe(III) and is assigned to one or more additional diiron(III/III) complexes.

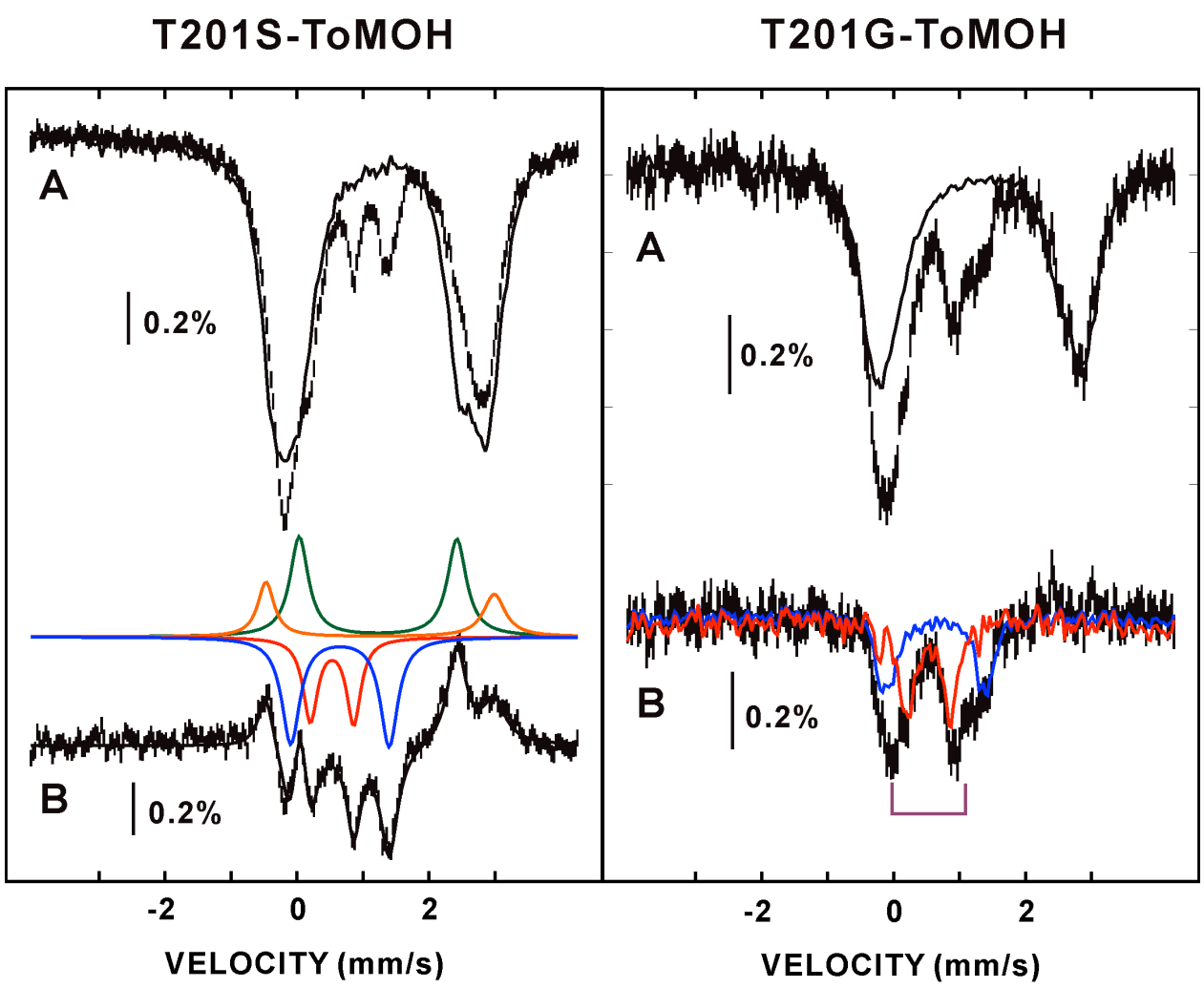
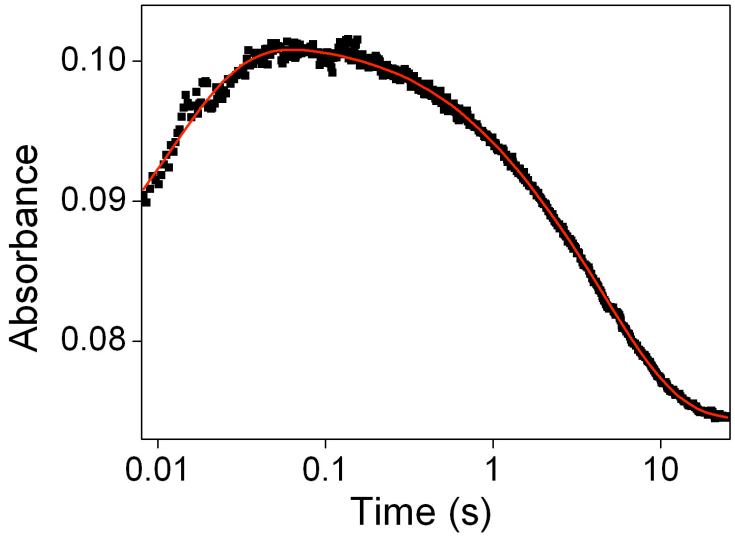

T201S-ToMOH
T201G-ToMOH
A
0.2%
B
0.2%
A
0.2%
B
0.2%
-2
0
2
VELOCITY (mm/s)
-2
0
2
VELOCITY (mm/s)

Figure S5. Time-resolved stopped-flow absorption spectra at 675 nm in the reaction of T201X $ToMOH_{red}$:D (~150 μM) at 4 °C with O_2 saturated buffer. (A) T201C (B) T201G variants. The formation and decay trace of $T201_{peroxo}$ (black dots) was fitted as described in the experimental section (red solid line). The best fit in T201C enzyme gave the following parameters as $\varepsilon_{ToMOHred}$ = 527 ± 4 $cm^{-1}M^{-1}$, $\varepsilon_{T201peroxo}$ = 663.1 ± 0.3 $cm^{-1}M^{-1}$, $\varepsilon_{T201peroxo*}$ = 609 ± 3 $cm^{-1}M^{-1}$, $\varepsilon_{ToMOHox}$ = 486.3 ± 0.5 $cm^{-1}M^{-1}$, k_1 = 82 ± 2 s^{-1}, k_2 = 1.2 ± 0.1 s^{-1}, k_3 = 0.204 ± 0.004 s^{-1} when the initial concentration of the enzyme, $[ToMOH_{red}]_{0,}$ set to 0.00015 M (R^2 = 0.998), was assumed to be equal to the concentration of $T201_{peroxo}$ generated in the reaction. The best fit in T201G variant gave the following parameters as $\varepsilon_{ToMOHred}$ = 593.0 ± 0.4 $cm^{-1}M^{-1}$, $\varepsilon_{T201peroxo}$ = 826 ± 2 $cm^{-1}M^{-1}$, $\varepsilon_{ToMOHox}$ = 558 ± 3 $cm^{-1}M^{-1}$, k_1 = 0.128 ± 0.002 s^{-1}, k_2 = 0.0209 ± 0.0007 s^{-1} when $[ToMOH_{red}]_{0,}$ was set to 0.00014 M (R^2 = 0.996). The lower value of $\varepsilon_{T201peroxo}$ than we have determined from the UV-vis and Mössbauer spectra (~1500 $cm^{-1}M^{-1}$) was resulted from the partitioning of $ToMOH_{red}$ in that not all of $ToMOH_{red}$ was converted to $T201_{peroxo}$ species.

(A)



(B)

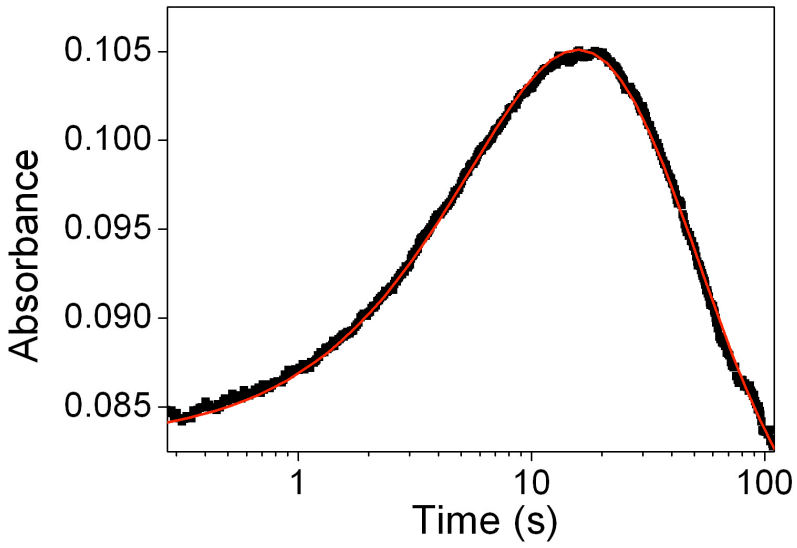
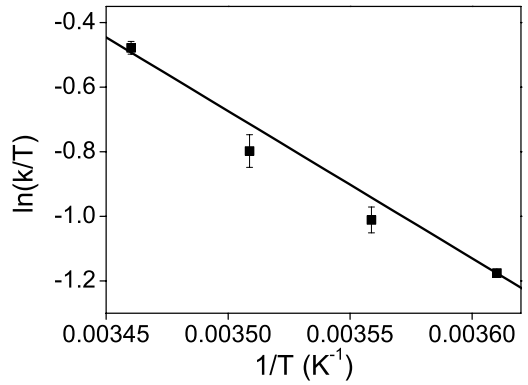

0.105
0.100
0.095
0.090
0.085
Absorbance
1
10
100
Time (s)

Figure S6. Eyring plot for the formation rates of $T201_{peroxo}$ species in T201 variants. (A) T201S, (B) T201C, and (C) T201G. Time-resolved stopped-flow absorption spectra at 675 nm in the reaction of T201X $ToMOH_{red}$:3D (~150 μM) with O_2 saturated buffer were monitored at 4-20 ^{o}C. Data in T201S and T201G variants were linearly fit to measure the activation parameters.

(A)



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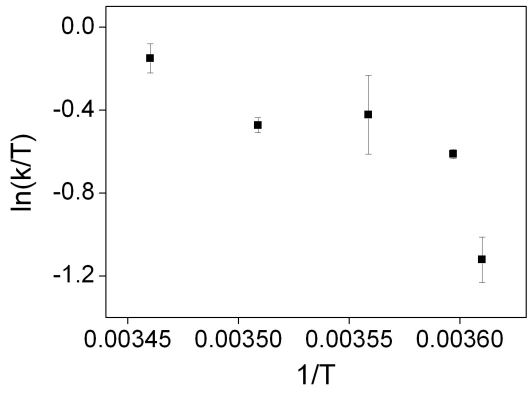
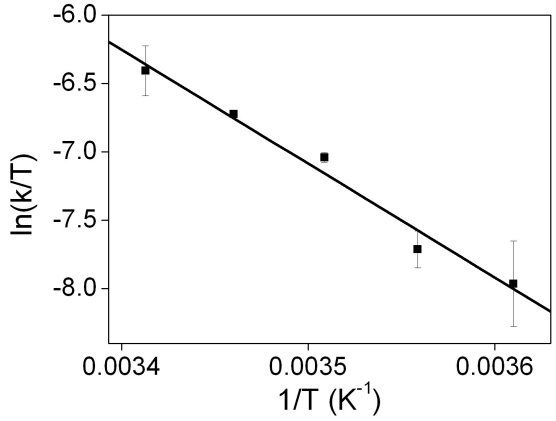


(C)



Reference

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