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Characterization of a Peroxodiiron(III) Intermediate in the T201S Variant of Toluene/\textit{o}-Xylene Monooxygenase Hydroxylase from \textit{Pseudomonas} sp. OX1

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Abstract

We report the observation of a novel intermediate in the reaction of a reduced toluene/\textit{o}-xylene monooxygenase hydroxylase (ToMOH\textsubscript{red}) T201S variant, in the presence of a regulatory protein (ToMOD), with dioxygen. This species is the first oxygenated intermediate with an optical band in any toluene monooxygenase. The UV-Vis and Mössbauer spectroscopic properties of the intermediate allowing us to assign it as a peroxodiiron(III) species, T201S\textsubscript{peroxo}, similar to H\textsubscript{peroxo} in methane monooxygenase. Although T201S generates T201S\textsubscript{peroxo} in addition to optically transparent ToMOH\textsubscript{peroxo}, previously observed in wild type ToMOH, this conservative variant is catalytically active in steady state catalysis and single turnover experiments, and displays the same regiospecificity for toluene and slightly different regiospecificity for \textit{o}-xylene oxidation.

Carboxylate-bridged diiron centers are common active site motifs in enzymes, performing a variety of dioxygen-dependent functions, including hydrocarbon oxidation.\textsuperscript{1,–4} The diiron centers in these enzymes have similar primary coordination spheres,\textsuperscript{5–7} suggesting that the protein scaffold tunes their chemical reactivity to favor a specific reaction pathway. Previous construction of variants of RNR-R2\textsuperscript{8} and ToMOH demonstrated that changes in the secondary and tertiary coordination spheres can divert the reaction pathway from electron abstraction to aromatic hydroxylation and vice versa.\textsuperscript{9,10} For these variants, however, the chemistry of the diiron(II) cluster with dioxygen was unchanged, and the oxygenated intermediates reacted with nearby amino acid residues. Here, we provide the first direct evidence of that a single conservative amino-acid mutation in BMMs partially bifurcates the oxygenation reaction to generate a diiron(III) peroxo intermediate not observed in the native system.

Recently, a transient diiron(III) intermediate in ToMOH, ToMOH\textsubscript{peroxo}, was identified by Mössbauer spectroscopy and shown to be kinetically competent for arene oxidation.\textsuperscript{11} Although this enzyme is similar to sMMOH both in sequence\textsuperscript{12} and active site structure,\textsuperscript{13} the spectroscopic properties of ToMOH\textsubscript{peroxo} were unexpected. Whereas H\textsubscript{peroxo} in sMMOH displays an optical band at \textasciitilde 720 nm ($\varepsilon_{720} = \text{~2000 cm}^{-1} \text{M}^{-1}$)\textsuperscript{14,15} and exhibits Mössbauer parameters of $\delta = 0.66 \text{ mm/s}$, $\Delta E_Q = 1.51 \text{ mm/s}$,\textsuperscript{16} ToMOH\textsubscript{peroxo} has no optical bands in the visible region and Mössbauer parameters of $\delta = 0.54 \text{ mm/s}$ and $\Delta E_Q = 0.67 \text{ mm/s}$.\textsuperscript{11} No other transient intermediate, like Q, was observed in ToMOH. These results suggest that
ToMOH$_{\text{peroxo}}$ has a structure distinct from that of any other peroxodiiron(III) intermediate in carboxylate-bridged diiron proteins, including sMMOH, Δ9D, and RNR-R2 variants. Here we report the observation of a novel peroxodiiron(III) intermediate in a variant of ToMOH (T201S$_{\text{peroxo}}$) having spectroscopic properties similar to those of H$_{\text{peroxo}}$ in sMMOH. The T201S variant (hereafter T201S) was originally prepared to investigate the role of a strictly conserved threonine residue near the carboxylate-bridged diiron active site on catalysis. During the course of our study, a structure of the T4moHD complex was reported revealing T201 to be involved in a novel hydrogen-bonding network terminating at a water molecule. Analysis revealed that T201S is even more efficient than wild type enzyme in aromatic hydroxylation. A Michaelis-Menten kinetic analysis revealed $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values for T201S of 0.08 ± 0.03 s$^{-1}$ and $2.9 ± 0.2$ µM$^{-1}$s$^{-1}$, respectively. This result indicates that T201S is even more efficient than wild type enzyme in aromatic hydroxylation. A Michaelis-Menten kinetic analysis revealed $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values for T201S of 0.08 ± 0.03 s$^{-1}$ and $2.9 ± 0.2$ µM$^{-1}$s$^{-1}$, respectively. This result indicates that T201S is even more efficient than wild type enzyme in aromatic hydroxylation.

When investigating pre-steady-state dioxygen activation at the reduced diiron(II) center of T201S by stopped-flow spectrophotometry, we observed a new transient intermediate (T201S$_{\text{peroxo}}$) with a broad absorption band at $\lambda_{\text{max}}$~650 nm (Figure 1 and Figure S1) and $\Delta \lambda_{\text{max}}$~700 nm (Figure S2). This feature provides optical spectroscopic evidence for an oxygenated intermediate in toluene monooxygenase. A typical kinetic trace of the growth and decay of the transient intermediate is provided in the inset, together with the fit. The absorbance at 675 nm maximizes at ~40 ms after dioxygen is mixed at 4.0 ± 0.1 °C. Fitting the time-dependent absorption spectra to a ToMOH$_{\text{red}}$→T201S$_{\text{peroxo}}$→diiron(III) product model yielded $k_{\text{form}} = 85 ± 11$ s$^{-1}$ and $k_{\text{decay}} = 2.9 ± 0.2$ s$^{-1}$. The intermediate is only observed in the presence of the regulatory protein (ToMOD), which is consistent with previous reports on oxygenated intermediates in BMMs such as H$_{\text{peroxo}}$ or Q in sMMOH, or ToMOH$_{\text{peroxo}}$ in wild type or 1100W ToMOH.

To investigate further the spectroscopic properties of this intermediate, we performed Mössbauer studies with $^{57}$Fe-enriched T201S ToMOH enzyme. We present the Mössbauer spectra of reduced diiron(II) in the presence of ToMOD (Figure 2A), a rapid freeze-quenched sample at 45 ms (Figure 2B), and the diiron(III) product (Figure 2C). The spectra corresponding to the diiron(II) starting material and diiron(III) product of T201S ToMOH were respectively fit to (i) two quadrupole doublets having the same $\delta = 1.32$ mm/s, $\Delta E_Q = 2.32$ and 3.16 mm/s and (ii) $\delta = 0.50$ mm/s, $\Delta E_Q = 0.82$. Within experimental error, these parameters are indistinguishable from those of wild type ToMOH. When T201S ToMOH$_{\text{red}}$ was allowed to react with dioxygen (Figure 2B), we observed that only 50% of the diiron(II) sites react, whereas the rest becomes slowly oxidized to a diiron(III) species, suggesting half-sites reactivity as in wild type ToMOH and the small subunit of ribonucleotide reductase.

Upon reaction of T201S ToMOH$_{\text{red}}$ with dioxygen in the presence of ToMOD, two distinctive transient intermediates accumulate (Figure 2B). One of the intermediates displays Mössbauer parameters very similar to those of the diiron(III) intermediate in wild type ToMOH, with $\delta = 0.55$ mm/s and $\Delta E_Q = 0.70$ mm/s. This intermediate, ToMOH$_{\text{peroxo}}$, accounts for approximately 40% of total iron at 45 ms and fully decays by ~100 sec (Figure 2C), exhibiting...
kinetic behavior similar to that of ToMOH\textsubscript{peroxo} in the wild type enzyme. As in the wild type, ToMOH\textsubscript{peroxo} generated in T201S also has no optical band in the visible range.

The other intermediate displays Mössbauer parameters identical to those of H\textsubscript{peroxo} in sMMOH with $\delta = 0.67$ mm/s and $\Delta E_Q = 1.51$ mm/s, implying that structure of T201S\textsubscript{peroxo} is similar to the structure of H\textsubscript{peroxo} in sMMOH, but different from that of ToMOH\textsubscript{peroxo}. This intermediate accounts for 10% of total iron in the Mössbauer spectrum of the sample (Figure 2B), which allows us to estimate the molar extinction coefficient of T201S\textsubscript{peroxo} to be $\varepsilon_{675} \approx 1500$ cm$^{-1}$ M$^{-1}$. This value is within the range of those reported for peroxo-to-iron charge transfer bands in several diiron(III) peroxo enzyme intermediates\textsuperscript{14,15,17,18,27} as well as synthetic model complexes.\textsuperscript{28,29}\ The spectroscopic similarity, we assign this species as a peroxodiiron (III) intermediate. Moreover, the kinetic properties of the two discrete diiron(III) intermediates demonstrate that T201S\textsubscript{peroxo} is not a precursor of ToMOH\textsubscript{peroxo}, for which $k_{\text{form}} \approx 26$ s$^{-1}$ implying that T201S has an additional, alternative pathway of activating dioxygen.

In conclusion, we report the first observation of an oxygenated intermediate having an optical band in ToMOH, generated by a single, conservative mutation of the T201 residue. Although during dioxygen activation T201S generates T201S\textsubscript{peroxo} and ToMOH\textsubscript{peroxo}, the former being similar to MMOH\textsubscript{peroxo}, it behaves in a manner similar to the wild type enzyme in steady state activity and single turnover experiments. It also has regiospecificity comparable to that of the wild type enzyme in toluene and o-xylene oxidations. Time-resolved optical and rapid freeze-quench Mössbauer experiments strongly support the assignment of the oxygenated intermediate in ToMOH, T201S\textsubscript{peroxo}, as a peroxodiiron(III) species. Although further studies are required to define the mechanism of dioxygen activation and the role of the T201 residue in ToMOH, our results raise the interesting possibility that this single amino acid perturbs the thermodynamics of dioxygen activation in carboxylate-bridged non-heme diiron enzymes.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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8. Abbreviations: RNR-R2, *E. Coli* ribonucleotide reductase R2 subunit; ToMO, toluene/o-xylene monooxygenase; MMOH, methane monooxygenase hydroxylase; BMM, bacterial multicompartment monooxygenase; $\Delta 9D$, $\Delta 9$-desaturase; T4moH, toluene 4-monooxygenase.


Figure 1.
UV-Vis spectrum of the reaction of reduced ToMOH T201S in the presence of ToMOD mixed with dioxygen-saturated buffer. [ToMOH] = ~ 120 µM, [ToMOD] = 360 µM in 25 mM MOPS, pH 7.0 at 4.0 ± 0.1 °C. (Inset) The time-dependent absorption trace monitored at 675 nm is shown with the fit in the inset.
Mössbauer spectra of freeze-quenched samples from reaction of ToMOH$_{\text{red}}$T201S:3ToMOD with O$_2$. The spectra (vertical bars) are collected at 4.2 K in a 50-mT field parallel to the $\gamma$-beam. The three spectra correspond to (A) the diiron(II) starting material, (B) 45 ms after mixing with O$_2$, and (C) the diiron(III) product. The green, red, cyan and orange lines are simulated spectra of the unreacted diiron(II) protein, ToMOH$_{\text{peroxo}}$, T201S$_{\text{peroxo}}$, and diiron (III) product, respectively. The solid line overlaid with the experimental spectrum in B is the composite spectrum.