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Mechanistic Studies of Reactions of Peroxodiiron(III) Intermediates in T201 Variants of Toluene/o-Xylene Monooxygenase Hydroxylase†

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Abstract

Site-directed mutagenesis studies of a strictly conserved T201 residue in the active site of toluene/o-xylene monooxygenase hydroxylase (ToMOH) revealed that a single mutation can facilitate kinetic isolation of two distinctive peroxodiiron(III) species, designated T201peroxo and ToMOHperoxo, during dioxygen activation. Previously we characterized both oxygenated intermediates by UV-vis and Mössbauer spectroscopy, proposed structures from DFT and QM/MM computational studies, and elucidated chemical steps involved in dioxygen activation through the kinetic studies of T201peroxo formation. In the current study, we investigated the kinetics of T201peroxo decay to explore the reaction mechanism of the oxygenated intermediates following O2 activation. The decay rates of T201peroxo were monitored in the absence and presence of external (phenol) or internal (tryptophan residue in an I100W variant) substrates under pre-steady-state conditions. Three possible reaction pathways were evaluated and the results demonstrate that T201peroxo is on the pathway of arene oxidation and appears to be in equilibrium with ToMOHperoxo.

Bacterial multicomponent monooxygenases (BMMs) are capable of activating dioxygen and catalyzing selective organic substrate oxidation (1, 2). The BMMs contain carboxylate-bridged non-heme diiron centers, now a common motif in metalloenzymes (3) including ribonucleotide reductases (4), desaturases (5), myo-inositol oxygenase (6), human deoxypentose hydroxylase (7), amine oxygenase (8), and a recently characterized enzyme on the ubiquinone biosynthesis pathway (9). Studies of BMMs have mainly focused on soluble methane monooxygenase hydroxylase (sMMOH), revealing two peroxodiiron(III) (P* and P or Hperoxo) and a diiron(IV) species (Q) that are generated during dioxygen activation in the presence of a regulatory protein (MMOB) (10–15). Recently, we have been investigating the toluene/o-xylene monooxygenase hydroxylase (ToMOH) component of

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SUPPORTING INFORMATION AVAILABLE. Table S1 and Figure S1–S2. This material is available free of charge via the Internet at http://pubs.acs.org.

1Abbreviations: BMMs, bacterial multicomponent monooxygenases; DFT, density functional theory; EPR, electron paramagnetic resonance; kdecay, decay rate constant; kform, formation rate constant; KIE, kinetic isotope effect; KSIE, kinetic solvent isotope effect; sMMOB, regulatory protein of soluble methane monooxygenase; sMMOH, hydroxylase component of soluble methane monooxygenase; MOPS, 3-(N-morpholino)propanesulfonic acid; P or Hperoxo, second peroxodiiron intermediate of sMMOH; P*, first peroxodiiron intermediate of sMMOH; Q, di(μ-oxo)diiron(IV) intermediate of sMMOH; QM/MM, quantum mechanics/molecular mechanics; RFQ, rapid freeze quench; T201peroxo, Peroxodiiron(III) intermediates observed in T201 variants; T201peroxo*, an intermediate generated in the decomposition pathway of T201peroxo; ToMO, toluene/o-xylene monooxygenase; ToMOD, regulatory protein of ToMO; ToMOH, hydroxylase component of ToMO; ToMOHred, resting state of ToMOH; ToMOHredD, reduced ToMOH; ToMOHredD, reduced ToMOH in complex with ToMOD; ToMOHperoxo, diiron(III) intermediate observed in the wild-type ToMOH enzyme.
toluene/o-xylene monooxygenase (ToMO), which evolved from an ancestor similar to that of sMMOH (16, 17). Because the two hydroxylases share very similar diiron active site structures (18), it seemed plausible that their dioxygen activation mechanisms might proceed through analogous peroxodiiiron(III)- and Q-type intermediates. Pre-steady-state studies of dioxygen activation by ToMOH in the presence of its cognate regulatory protein ToMOD (hereafter ToMOHredD), however, revealed that this enzyme system generates a previously unprecedented diiron(III) intermediate, ToMOHperoxo (Chart 1A) (19). Moreover, no evidence for formation of a Q-like species has yet been identified in ToMOH reactions. These results imply that ToMOH has a different O2 activation profile from that of sMMOH, oxidizing its substrates via different intermediates.

Pre-steady-state studies of dioxygen activation in ToMOH are hampered by the absence of an optical band in the ToMOHperoxo intermediate. By perturbing the active site structure through the generation of Ser, Cys, and Gly variants of ToMOH T201, however, a residue strictly conserved and located close to the diiron centers in all BMMs, we discovered a novel intermediate, T201peroxo. This species forms in addition to ToMOHperoxo in these variants (20, 21). T201peroxo exhibits UV-vis and Mössbauer spectra similar to those of Hperoxo in sMMOH (Chart 1B), and its optical feature allowed us to obtain kinetic parameters of its formation by stopped-flow spectroscopy in the T201 variants under a variety of reaction conditions. Formation rates of T201peroxo in the T201S variant are proportional to the concentration of O2, a result that allowed us to determine the likely pathway by which dioxygen accesses the active site diiron center (22). DFT and QM/MM calculations, revealed how the conformation of the side chain at T201 site perturbs the energetics of two oxygenated species ToMOHperoxo and T201peroxo (23). Our studies further suggested that that proton transfer to either the peroxo unit or an adjacent shifting carboxylate ligand (E231) during dioxygen activation can determine the geometry of oxygenated dii-iron(III) intermediates as either ToMOHperoxo or T201peroxo, respectively (Chart 1B) (21).

Although accurate stopped-flow kinetic parameters for the formation and decay of ToMOHperoxo could not be measured in the T201S variant, time-dependent Mössbauer spectra obtained from freeze-quench investigations of its reaction with O2 in the presence of ToMOD revealed values comparable to those previously obtained with the wild-type enzyme (20). The data suggested that T201peroxo and ToMOHperoxo are generated by separate pathways (19, 20), with T201peroxo forming more rapidly than To-MOHperoxo, their respective rate constants being kform = 85 ± 11 s−1 vs. ~ 26 s−1 at 4 °C, pH 7.0. Moreover, the decay rate constant of T201peroxo at this temperature (2.9 s−1) is much less than kform of ToMOHperoxo (~ 26 s−1), further consistent with T201peroxo and ToMOHperoxo forming by separate pathways during di-oxygen activation.

To further probe the properties of T201peroxo and ToMOHperoxo in T201 variants of ToMOH, we explored in the present study three plausible scenarios: (i) that T201peroxo and ToMOHperoxo are formed and react consecutively; (ii) that T201peroxo and ToMOHperoxo form and react independently; (iii) that T201peroxo and ToMOHperoxo are in equilibrium, with one dominating subsequent reactivity. From the kinetics of the reaction of T201peroxo with arene substrates, following by monitoring changes in its optical spectrum, we were able to evaluate these three working models for formation and decay of T201peroxo and ToMOHperoxo and to identify the arene-oxidizing intermediate(s). Aromatic hydroxylation by T201peroxo was monitored in the three T201 variants, T201S, T201C, and T201G, previously determined to form the intermediate, although with distinct kinetics. These experiments were conducted with the use of phenol as an external substrate or by converting I100, a residue in close proximity to the active site, into tryptophan as an internal substrate. In addition to the kinetic studies of T201peroxo, the amount of the oxidized phenol or I100W
was quantitated in the three T201 variants to further explore the role of T201 during catalysis.

EXPERIMENTAL PROCEDURES

General Considerations

Plasmids containing the genes for expressing toluene/o-xylene monooxygenase components were supplied by the laboratory of Professor Alberto Di Donato, Naples, Italy. All ToMO components and ToMOH T201X mutants (X= S, C, G) were prepared as described previously (20, 24). I100W/T201X double mutants (X= S, C, G, V) were obtained by using the pET22b (+)/touBEA T201X vector with I100W primers (5′-CAA CTT CAC TTC GGA GCG TGG GCA CTT GAA GAA TAC G-3′ and 5′-CGA TTC TTC AAG TGC CCA CGC TCC GAA GTG AAG TTG-3′). DNA sequences were confirmed by the MIT-BioPolymers Laboratory. Vectors were transformed into E. coli strain BL21(DE3) cells for protein expression. Cell growth and protein purification procedures were as same as for the wild type enzyme. An iron assay was performed, as described previously (19, 24).

Kinetic Studies of Oxygenated Intermediates in T201X/I100W (X= S, G, C, V)

UV-vis spectra of T201peroxo (20, 21) and I100W-radical species (25) were monitored as described. Optical bands originating from T201peroxo and the I100W-radical were 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 or T201X/I100W ToMOH proteins were reduced anaerobically by reacting the protein with excess sodium dithionite in the presence of an equimolar amount of methyl viologen for 10 min. The reduced protein (ToMOHred) was dialyzed against 1 L of 25 mM MOPS, pH 7.0 buffer for ~ 3 hr, anaerobically. Following dialysis, the regulatory protein (ToMOD) was added to the reduced ToMOH. The solution was transferred to a tonometer and loaded into the anaerobic stopped flow instrument. This solution was rapidly mixed against an equal volume of O2-saturated 25 mM MOPS, pH 7.0 buffer. The temperature was thermostatted at 4 °C using a circulating water bath. Time-dependent optical changes at wavelengths corresponding to the formation and decay of T201peroxo (675 nm) and to the I100W-radical species (500 nm) were collected using a PMT (photomultiplier tube) following halogen lamp illumination or a diode-array with a xenon lamp. Data were analyzed by the software packages Kinetic Studio (TgK Scientific) and Origin 6.1 (OriginLab Corporation) as described previously (15, 20, 26). For the T201S and T201G ToMOH variants, an analytical function derived from a model ToMOHred → T201peroxo → ToMOHox was applied to obtain formation and decay rate constants for T201peroxo. For the T201C variant, an analytical function corresponding to the model ToMOHred → T201peroxo → T201peroxo* → ToMOHox was derived to measure the formation and two decay rate constants of T201peroxo. In the T201X/I100W variants, the formation and decay of the T201peroxo and I100W-radical species were fit to analytical functions derived for To-MOHred → T201peroxo → ToMOHox and I100W-radical precursor → I100W-radical → oxidized product of I100W models, respectively.

Kinetics of an Oxygenated Intermediate in the Reactions with Arene Substrates

The arene substrate phenol was dissolved in dioxygen-saturated buffer and the solution was rapidly mixed with ToMOHredD in a single-mixing stopped flow spectrophotometer at 4 °C. Time-dependent optical changes arising from the formation and decay of T201peroxo were then analyzed, as described previously (21).
For a kinetic isotope effect (KIE) measurement, phenol-2,3,4,5,6-\(d_5\) (hereafter phenol-\(d_5\)) (98 atom % D) purchased from Sigma-Aldrich was used without further purification. For kinetic solvent isotope effect (KSIE) measurements, deuterium oxide (99.9 atom % D) purchased from Cambridge Isotope Laboratories or Icon Isotopes was used to prepare 25 mM MOPS buffer, pH 7.0. The pH value was adjusted by adding an appropriate amount of NaOD solution (Aldrich). Dioxygen activation of ToMOH was monitored at 5 °C, which was thermostatted using a circulating water bath.

RESULTS AND DISCUSSION

Decay of T201\(_{\text{peroxo}}\) in the Presence of Aromatic Substrates

To determine whether T201\(_{\text{peroxo}}\) is kinetically competent to hydroxylate aromatic substrates, its formation and decay rates were monitored in the presence of phenol. Phenol was selected as substrate because the natural substrates, toluene and \(o\)-xylene, are less soluble in water. In addition, ToMO displays a high steady state activity with phenol (19). To observe its effects on the decay rate of T201\(_{\text{peroxo}}\), phenol was dissolved in dioxygen-saturated buffer and rapidly mixed with the same volume of a solution containing reduced T201X ToMOH and ToMOD. Changes in absorbance at 675 nm, corresponding to formation and decay of T201\(_{\text{peroxo}}\), were monitored using stopped-flow optical spectroscopy (Figure 1). The rate constants were then obtained by the fit to an analytical function derived from a model involving two consecutive, irreversible steps.

In the reaction of phenol and dioxygen with the T201S variant of ToMOH\(_{\text{redD}}\), the formation rate of T201\(_{\text{peroxo}}\) was unaltered over a 0 – 25 mM range of phenol concentrations (Figure 2A). This result indicates that the substrate does not alter the mechanism of dioxygen activation to form T201\(_{\text{peroxo}}\). The presence of phenol, however, accelerated the decay of T201\(_{\text{peroxo}}\), thereby diminishing the accumulation of this intermediate (Figure 1A). Because the decay rate of T201\(_{\text{peroxo}}\) in the presence of phenol is much faster than the rate of catechol production measured under steady-state conditions (20), it is apparent that T201\(_{\text{peroxo}}\) is kinetically competent for, and probably on the pathway to, phenol oxidation. A plot of \(k_{\text{decay}}\) versus phenol concentration yielded a linear relationship, from which we could derive a second-order rate constant of 0.18 ± 0.02 s\(^{-1}\) mM\(^{-1}\) (Figure 2A).

T201\(_{\text{peroxo}}\) is also observed during dioxygen activation of the T201C and T201G variants of ToMOH. T201\(_{\text{peroxo}}\) reacts in a similar manner with phenol in the T201C and T201G ToMOH variants. In the absence of arene substrate, T201\(_{\text{peroxo}}\) in T201C variant decays by two consecutive processes, T201\(_{\text{peroxo}}\) → T201\(_{\text{peroxo}}^*\) → ToMOH\(_{\text{ox}}\). Two decay rate constants, \(k_{\text{decay1}}\) and \(k_{\text{decay2}}\), corresponding to the conversion of T201\(_{\text{peroxo}}\) to T201\(_{\text{peroxo}}^*\) and of T201\(_{\text{peroxo}}^*\) to ToMOH\(_{\text{ox}}\), were obtained. The change in the absorbance of T201\(_{\text{peroxo}}\) at 675 nm in T201C variant was recorded in the absence and presence of arene substrate (Figure 1B). As described above for the T201S variant, formation rate of T201\(_{\text{peroxo}}\) was independent of phenol concentration. Less T201\(_{\text{peroxo}}\) accumulated when the concentration of phenol was increased due to acceleration of its decay rate. A plot of the first derived decay rate constant (\(k_{\text{decay1}}\)) versus phenol concentration yielded a linear relationship (Figure 2B) and was fit (eq 1) to the processes depicted in Scheme 1, resulting in \(k_{\text{sat}}\) and \(K_d\) of 9 ± 2 s\(^{-1}\) and 10 ± 8 mM, respectively. The second decay process (\(k_{\text{decay2}}\)) was slightly perturbed by the presence of phenol, but with no clear dependence on its concentration, suggesting that T201\(_{\text{peroxo}}^*\) may not react directly with substrate.

\[
k_{\text{obs}} = k_0 + \frac{k_{\text{sat}} \left[ S \right]}{K_d + \left[ S \right]}
\]

(1)
To study further the arene-oxidizing mechanism of T201$_{\text{peroxo}}$-phenol-$d_5$ was used in the reaction of T201C ToMOH$_{\text{red}D}$ with dioxygen. Arene oxidation typically involves a hybridization change from sp$^2$ to sp$^3$ at a phenol carbon atom and, accordingly, an inverse kinetic isotope effect, KIE < 1, was observed (27). In this manner, the decay rate of T201$_{\text{peroxo}}$ arising from the use of phenol-$h_5$ versus phenol-$d_5$ and the resulting KIE can signal kinetic coupling to reaction with phenol. An inverse KIE of 0.82 ± 0.05, listed in Table 1, was derived, indicating that the decay of T201$_{\text{peroxo}}$ is kinetically linked to the arene oxidation. T201$_{\text{peroxo}}$ presumably attacks the arene ring in an electrophilic manner to generate an arene-oxide species (19). No KIE was observed for the following decay rate, T201$_{\text{peroxo}}^*$ → ToMOH$_{\text{ox}}$. As suggested above, T201$_{\text{peroxo}}^*$ is probably not involved in arene oxidation.

Reaction of T201G ToMOH$_{\text{red}D}$ with a solution of phenol in dioxygen-saturated buffer also led to the formation of T201$_{\text{peroxo}}$ (Figure 1C). The kinetic behavior of this reaction could not be monitored, however, because the oxidized product, catechol, binds to the diiron core, forming a Fe(III)-catecholate species with characteristic strong, interfering optical bands between 600–1020 nm that mask the optical feature of T201$_{\text{peroxo}}$ (28). Binding of catechol to the resting state enzyme after single turnover was also observed for the wild-type and T201S and T201C variants of ToMOH, but the decay rates of T201$_{\text{peroxo}}$ were much faster than the formation rates of Fe(III)-catecholate species so that optical changes at 675 nm, corresponding to the decay of T201$_{\text{peroxo}}$, were not obscured as they were for the T201G variant (data not shown).

**Kinetic Solvent Isotope Effect in the Decay of T201$_{\text{peroxo}}$ in the Absence and Presence of Arene Substrates**

A kinetic solvent isotope effect (KSIE) can be measured when deuterium oxide (D$_2$O) is used instead of H$_2$O as the solvent (29). A proposed chemical mechanism for T201$_{\text{peroxo}}$ decay in the absence of arene substrate is protonation of the hydroperoxo unit and subsequent release of hydrogen peroxide (19, 21). If T201$_{\text{peroxo}}$ requires a proton(s) in the decomposition pathway and the proton translocation step is coupled to decay of T201$_{\text{peroxo}}$, a KSIE ≥ 1 will be observed. The KSIE derived from the decay rates of T201$_{\text{peroxo}}$ in the absence of arene substrate, however, can be altered depending on whether or not the reaction is linked to arene oxidation, because a typical KIE for aromatic hydroxylation differs from that for proton translocation, as described above. We therefore derived KSIE values from the decay rates of T201$_{\text{peroxo}}$ in the absence and presence of phenol to distinguish the kinetically prevailing chemical step in T201$_{\text{peroxo}}$ decay and to provide an additional assessment of our previous conclusion that the more enhanced decay of T201$_{\text{peroxo}}$ upon addition of phenol is an indication of its kinetic competence.

To measure the KSIE for the decay of T201$_{\text{peroxo}}$, the reaction of T201C ToMOH$_{\text{red}D}$ with dioxy-gen was monitored in H$_2$O or D$_2$O buffer at 5 °C. Two consecutive decay rates of T201$_{\text{peroxo}}$ were observed in D$_2$O buffer, as previously seen in H$_2$O buffer. The $k_{\text{decay}1}$ and $k_{\text{decay}2}$ values measured in the two buffers returned KSIE$_{\text{decay}1} = 3.4 ± 0.3$ and KSIE$_{\text{decay}2} = 6.5 ± 0.1$ (Table 2). These large KSIE values for the decay process can imply multiple protons in a decay pathway (30), which presumably, for T201$_{\text{peroxo}}$, involve protonation of the peroxo moiety and release of H$_2$O$_2$. KSIE$_{\text{decay}}$ values ≥ 1 were observed for the T201S and T201G variants, 1.40 ± 0.11 and 10.6 ± 1.6, respectively (data not shown). KSIE$_{\text{decay}}$ values obtained from the decay rates of T201$_{\text{peroxo}}$ increase in the order T201S<T201C<T201G, possibly because of their increasingly poor ability to facilitate proton transfer during the decomposition of T201$_{\text{peroxo}}$.

Different KSIE$_{\text{decay}}$ results were obtained when experiments were conducted in the presence of phenol. KSIE studies in the presence of phenol were conducted only with T201C but not...
the T201S and T201G variants. Although the $K_{\text{SIE}}$ decay in T201S $\geq 1$, it is not large enough to permit an accurate value to be obtained in the presence of phenol. For the T201G variant, formation of the optical bands of Fe(III)-catecholate, as described previously, made it impossible to measure the $K_{\text{SIE}}$ decay. A solution of T201C ToMOH$_{\text{peroxo}}$D in either H$_2$O or D$_2$O buffer was therefore mixed with dioxygen-saturated buffer containing 10 mM phenol. Decay rates of T201$_{\text{peroxo}}$ in each buffer were measured, yielding $K_{\text{SIE}}$ decay values of 1.15 ± 0.06 and 2.1 ± 0.2 for two successive steps, respectively (Table 3). The dramatically reduced $K_{\text{SIE}}$ value for the first decay step compared to that determined in the absence of phenol, 3.4 ± 0.3, indicates that decay of T201$_{\text{peroxo}}$ is no longer entirely dependent on solvent protons but primarily involves an interaction of the peroxodiiron(III) intermediate with phenol. Therefore, the significant decrease in $K_{\text{SIE}}$ values due to introduction of the arene substrate supports the conclusion that T201$_{\text{peroxo}}$ is kinetically competent to hydroxylate phenol. The $K_{\text{SIE}}$ decay result for the second decay process ($k_{\text{decay}2}$) is also considerably perturbed by addition of phenol. Because T201$_{\text{peroxo}*}$ does not probably react with phenol, as discussed above, the presence of phenol must trigger as yet unidentified reactions, for examples conformational changes, that contribute to the decay of T201$_{\text{peroxo}*}$.

Studies of T201X/I100W Variants (X = G, C, S, V)

As reported previously, I100 is located near the diiron active site where it helps to form a hydrophobic pocket (18). When I100 residue was mutated to I100W, the indole ring of the installed tryptophan approached the iron atoms, with Fe···C distances ranging from 6.0 to 11.9 Å (31). Addition of dioxygen to the reduced form of I100W ToMOH and To-MOD demonstrated that the variant activates O$_2$ at a rate similar to that observed for the wild type enzyme. The decay rate of ToMOH$_{\text{peroxo}}$ was accelerated, however, because the tryptophan residue serves as a substrate closely positioned near the active site, reacting with ToMOH$_{\text{peroxo}}$ to form diiron(III,IV) and I100W-radical species (31). The generation of the I100W-radical from ToMOH$_{\text{peroxo}}$ was nearly quantitative, and time-dependent RFQ/ Mössbauer, EPR, and UV-vis spectroscopic studies revealed that the ToMOH$_{\text{peroxo}}$ decay rate corresponds to the formation rate of the I100W-radical species. Based on these findings, T201X/I100W double mutants were prepared to examine the effect of the tryptophan residue on the decay rate of T201$_{\text{peroxo}}$ as well as the formation rate of the anticipated I100W-radical. I100W/T201X variants of ToMOH, prepared as described previously (X= S, G, C, V), all contained ~ 4 iron atoms/protein. When reduced T201S/ I100W ToMOH and ToMOD were mixed with dioxygen-saturated buffer in the stopped-flow spectrophotometer at 4 °C, changes in the optical spectra at 675 nm and 500 nm originating from T201$_{\text{peroxo}}$ and I100W radical species, respectively, were observed (Figure 3 and Supporting Information Figure S1). From the data we computed formation and decay rates of T201$_{\text{peroxo}}$ and the I100W-radical species by fitting the optical changes to a function representing two consecutive, irreversible processes (Table 4). Both the formation and decay rate constants of T201$_{\text{peroxo}}$ and I100W-radical species in T201S/I100W were greatly perturbed compared to their values in T201S or I100W single variants (32). An accelerated decay rate of T201$_{\text{peroxo}}$ in T201S/I100W, compared to that in the T201S variant, implies that T201$_{\text{peroxo}}$ is on the reaction pathway of I100W oxidation, as discussed above for phenol oxidation. Three mechanisms were proposed to account for these data, as portrayed in Scheme 2. The increased T201$_{\text{peroxo}}$ decay rate rules out mechanism A in Scheme 2, whereby T201$_{\text{peroxo}}$ irreversibly decomposes to ToMOH$_{\text{peroxo}}$. If mechanism A were operative, only the decay rate of ToMOH$_{\text{peroxo}}$, but not that of T201$_{\text{peroxo}}$, would be accelerated by introduction of the arene substrate, tryptophan.

Both formation and decay of the I100W radical species are well fit by a single exponential function (Figure 3 and Table 4), which indicates that there is only one kinetically observable precursor, either T201$_{\text{peroxo}}$ or ToMOH$_{\text{peroxo}}$, but not both. These data therefore exclude
mechanism B in Scheme 2. The decay rate constant for T201\textsubscript{peroxo}, 14.9 ± 0.4 s\textsuperscript{−1}, however, is much larger than the formation rate constant of the I100W radical species, 2.8 ± 0.1 s\textsuperscript{−1}, indicating that T201\textsubscript{peroxo} does not directly convert to the I100W radical species. These results indicate that an additional species, presumably ToMOH\textsubscript{peroxo}, exists along the decay reaction pathway.

A third possible mechanism that we considered is one in which T201\textsubscript{peroxo} and ToMOH\textsubscript{peroxo} are in equilibrium with one another (Mechanism C, Scheme 2). This possibility is supported by the observed kinetic properties of the T201\textsubscript{peroxo} and I100W-radical intermediates in the T201S, I100W, and T201S/I100W variants. Although equilibrium between T201\textsubscript{peroxo} and ToMOH\textsubscript{peroxo} could potentially have complicated the kinetic analysis of T201\textsubscript{peroxo} decay, a single exponential function gave satisfactory estimates of the decay rate constants of T201\textsubscript{peroxo} in both the T201S and T201S/I100W variants (Table 4). This result indicates that the rate constant for conversion of T201\textsubscript{peroxo} to ToMOH\textsubscript{peroxo} (k\textsubscript{1}) is much faster than the reverse rate constant (k\textsuperscript{−1}), Scheme 2C. The rapid forward rate presumably conveys kinetic competence to T201\textsubscript{peroxo} in arene oxidation, although this intermediate does not directly react with the substrate. Our observation that T201\textsubscript{peroxo} appears to be kinetically competent in arene oxidation is essentially due to its rapid conversion to ToMOH\textsubscript{peroxo} through the chemical equilibrium of Mechanism C in the presence of arene substrates.

A possible mechanism for the interconversion of ToMOH\textsubscript{peroxo} and T201\textsubscript{peroxo} is suggested in Scheme 3. A hydrogen-bonded proton on the hydroperoxo unit of ToMOH\textsubscript{peroxo} or on an oxygen atom of the adjacent glutamate (E231) in T201\textsubscript{peroxo} can easily shift between the two positions, as illustrated in the scheme. Such proton translocation can trigger reorganization of peroxo unit, altering the geometry of oxygenated diiron(III) species. The relative energetics of the two geometries, 1,1-hydroperoxodiiron(III) and 1,2-peroxodiiiron(III), computed by QM/MM methods (23), indicated the energy difference between ToMOH\textsubscript{peroxo} and T201\textsubscript{peroxo} to be relatively small, as ~2.63 kcal/mol, and the occurrence of both intermediate species is therefore energetically plausible.

Introduction of I100W has a similar effect on the kinetic properties of T201\textsubscript{peroxo} in the other T201 variants. For T201C/I100W, formation of T201\textsubscript{peroxo} and the I100W radical species were monitored during the reaction of T201C/I100W ToMOH\textsubscript{red}D with O\textsubscript{2}, as described for the T201S/I100W variant. Formation and decay of T201\textsubscript{peroxo} and the I100W-radical species in T201C/I100W monitored at 675 nm and 500 nm, respectively (Figure S2) and time-dependent traces were fit to a consecutive two exponential function without inclusion of T201\textsubscript{peroxo}\textsuperscript{*} in the decomposition pathway of T201\textsubscript{peroxo} to ToMOH\textsubscript{ox} (Table S1). Given that a consecutive three-exponential function was required to fit the trace of T201\textsubscript{peroxo} in the T201C single variant, the rapid decay of T201\textsubscript{peroxo} in the reaction with I100W residue does not proceed through T201\textsubscript{peroxo}\textsuperscript{*}. Acceleration of the decay rate of T201\textsubscript{peroxo} in T201C/I100W relative to the value in the T201C variant (k\textsubscript{decay1}) again supports a mechanism in which T201\textsubscript{peroxo} is on the pathway of aromatic hydroxylation through equilibrium with ToMOH\textsubscript{peroxo} (Scheme 2C).

Both formation and decay rates of the I100W radical species in T201C/I100W are accelerated by comparison to those of I100W and T201S/I100W variants (Table S1). These kinetic characteristics of the I100W radical species in T201C/I100W can be perturbed if the local environment near the active sites is significantly altered. Previous studies with the I100W variant of ToMOH revealed that the formation and decay rates of I100W-radical species are pH-dependent (31). The rates are accelerated at high pH values, suggesting that deprotonation at tryptophan residue during formation and decay of I100W-radical species is preceded by a fast oxidation. Therefore, the acceleration of the formation and decay rates of
I100W-radical species in T201C/I100W relative to rates in the I100W or T201S/I100W variants possibly reflects local pH changes near the active site, induced by the T201C mutation. Because T201C is a poorer residue than T201 or T201S for promoting proton transfer, the T201C variant may experience an increase in local pH at the active site.

The T201G/I100W variant also generated the I100W-radical species during dioxygen activation (Table S1 and Figure S2). No T21_perox species could be observed, however, probably because it reacts rapidly with I100W and does not accumulate to a detectable level.

Finally, dioxygen activation by the T201V/I100W variant was investigated (Table S1 and Figure S2). As expected from the T201V single variant study (21), T201_perox was not detected in T201V/I100W. Possibly proton translocation, required for to generate T201_perox, is too slow; alternatively, formation of I100W-radical is too rapid to allow the intermediate to build up. Another possibility is that the T201V variant energetically disfavors formation of T201_perox (21, 23). Reaction of T201V/I100W ToMOH_{red}D with dioxygen also generated the I100W-radical species, possibly because the pectroscopically silent species, ToMOH_perox, formed and reacted with the tryptophan residue. The kinetic properties of the I100W-radical species in T201V/I100W are slightly different from those of I100W but rather similar to those of T201C/I100W, indicating that the local pH at the active site might similarly be perturbed owing to the presence of the hydrophobic valine side chain.

Quantification of I100W Radical Species in T201X/I100W Double Variants

The wild-type ToMOH displays half-sites reactivity, in which only one of the two diiron centers in the dimeric hydroxylase undergoes productive reactivity during single-turnover experiments. The other site can be reduced to the diiron(II) state but does not simultaneously form ToMOH_perox upon introduction of O2 (19). Half-sites reactivity is also consistently observed in reactions with phenol, whereby 50% catechol per diiron sites form during single turnover experiments (20, 33). In T201S, ~50% phenol oxidation was similarly measured during single-turnover, indicating that the serine variant retains half-sites reactivity (33). This property seems to be conserved in other T201 variants. Reactions of T201G ToMOH_{red}D with dioxygen generated oxygenated intermediates at approximately half of the diiron centers. If half-sites reactivity is operative in all the T201G/C/V variants, approximately half of the enzyme present in solution would be able to oxidize phenol in single turnover experiments, but the yields were consistently lower than that value. This result contrasts with our findings for the wild-type and T201S variant. These findings indicate that decay of peroxodiiron(III) species is not tightly coupled to oxidation of hydrocarbon substrate when a hydroxyl group is lacking at position 201, presumably leading to release of hydrogen peroxide, rather than catechol, as an uncoupling product (34).

Single-turnover yields from the reaction of the internal I100W substrate were also determined for T201X/I100W double variants. The extinction coefficient of the I100W radical species at 500 nm is $\varepsilon_{500\text{ nm}} \approx 1500 \text{ cm}^{-1} \text{ M}^{-1}$, based on Mössbauer and stopped-flow UV-vis data (31). Using this value, we were able to quantitate the amount of I100W-radical species generated in reactions of reduced T201X/I100W ToMOH and ToMOD with dioxygen. The I100W radical species produced was measured by taking into account of the individual formation and decay rate constants corresponding to the time-dependent spectral changes at 500 nm, as described previously (Table 5) (21). In I100W and T201S/I100W variants, ~50% of the I100W-radical species formed per diiron sites which is consistent with half-sites reactivity and ~50% of single turnover yields during phenol oxidation (20). By contrast, ~30% of I100W-radical species per diiron sites were generated in the T201C/G/V variants, even though ~50% of the diiron sites presumably reacted with dioxygen. The uncoupling chemistry in T201C/G/V variants is consistent with the single-turnover yields of these species during phenol oxidation. In addition, the results agree with the steady state.

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kinetics showing ~70–150-fold lower $k_{cat}/K_M$ values for the T201C/G/V variants compared to the wild type and T201S enzymes. These results further support the notion that the hydroxyl group at position 201 site is necessary for efficient hydrocarbon oxidation (35).

**Comparisons of Oxygenated Intermediates and Their Reactivities in ToMOH and sMMOH**

Reaction of sMMOH with dioxygen consecutively generates three oxygenated intermediates, $P^*$, $P$ or $H_{\text{peroxo}}$, and $Q$, and the latter two species are capable of oxidizing hydrocarbon substrates (26, 36–38). $H_{\text{peroxo}}$ and $Q$ react with substrates through different mechanisms, with $H_{\text{peroxo}}$ preferring more electron-rich substrates, such as propylene, ethyl vinyl ether, and diethyl ether, and operating by a two-electron transfer process. In contrast, $Q$ favors one-electron transfer chemistry for methane oxidation (39). The promiscuous reactivity of sMMOH, therefore, can be attributed, at least in part, to the divergent reactions catalyzed by the two distinctive intermediates.

In contrast, ToMOH has evolved specifically to perform aromatic hydroxylation. A long and wide hydrophobic channel, ~ 6–10 Å by ~ 30–35 Å, is present only in toluene monooxygenases, where it most likely serves as the pathway for arene substrate access/product egress (18, 40). This structural feature may explain how ToMOH developed specificity for aromatic substrates. To perform aromatic hydroxylation, a high-valent oxygenated species like $Q$ is unnecessary, because one-electron oxidation or C–H homolysis for arenes is not thermodynamically favorable owing to their high redox potentials and large C–H bond dissociation energies (41). An alternative mechanism is most likely operative in ToMOH. Oxidation of the arene can occur via two-electron transfer from substrates to an electrophilic oxidant, such as $H_{\text{peroxo}}$ in sMMOH, the analogous species proposed for amine oxygenase (42, 43), and a hydroperoxoiron(III) intermediate in cytochrome P450 monooxygenase (41). The ToMOH$_{\text{peroxo}}$ intermediate in ToMOH therefore most likely shares the electronic and geometric structures of hydroperoxoiron(III) or peroxodiiron(III) species rather than those of $Q$ in sMMOH.

A mechanism for the oxidation of arenes by ToMOH$_{\text{peroxo}}$ is proposed in Scheme 4. As previously reported (19), a Hammett plot for the oxidation of para-substituted phenols in ToMOH has a negative slope, consistent with the electrophilic character of ToMOH$_{\text{peroxo}}$. Electrophilic attack of the hydroperoxo unit in ToMOH$_{\text{peroxo}}$ on the arene ring can initiate the oxidation, followed by formation of an arene-oxide species, weakly bound to the diiron center. Addition of water and rearrangement can provide the arene product and return the resting state diiron site. A similar reaction with an arene substrate might be possible for T201$_{\text{peroxo}}$, although not kinetically feasible due to fast conversion to To-MOH$_{\text{peroxo}}$. Without such conversion, T201$_{\text{peroxo}}$ might participate in futile side reactions, becoming protonated with subsequent O–O bond cleavage and formation of a Q-type product with undesired reactivity.

**CONCLUDING REMARKS**

The present kinetic studies of T201$_{\text{peroxo}}$ in the absence and presence of external (phenol) or internal (tryptophan, as I100W) substrates clearly demonstrate that T201$_{\text{peroxo}}$ is kinetically on the reaction pathway of arene oxidation. Kinetic solvent isotope effects in the reaction of T201$_{\text{peroxo}}$ with phenol confirm this kinetic competence. Three reaction models were considered to account for the measured kinetics of T201$_{\text{peroxo}}$ and I100W-radical species formation and substrate reactivity. The only one that accounts for all of the experimental results requires that T201$_{\text{peroxo}}$ be in the equilibrium with ToMOH$_{\text{peroxo}}$. Acceleration in the decay rate of T201$_{\text{peroxo}}$ in the presence of arene substrates is therefore ascribed to the rapid conversion of T201$_{\text{peroxo}}$ to ToMOH$_{\text{peroxo}}$, the latter being the reactive species in arene oxidation.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


32. Dramatic changes due to the I100W residue in T201perox formation rate constants will be described separately.


34. We attempted to quantitate the concentration of H2O2 in this reaction, but the catalase activity of ToMO led to an inaccurate measurement of H2O2 produced during single-turnover experiments.


Figure 1. Trace of T201peroxo at 675 nm in the absence and presence of phenol in T201 variants of To-MOH. (A) T201S (B) T201C (C) T201G.
Figure 2.
Plots of T201_{peroxo} formation and decay rate constants versus phenol concentrations in the reaction of T201X ToMOH_{red}D with phenol in dioxygen-saturated buffer. (A) T201S (B) T201C Formation and decay rates are represented with black squares and red circles, respectively. For T201C variant, second decay rate constants are shown as navy diamonds. Formation and decay rate constants are plotted with either a linear or a saturation function.
Figure 3.
Time-dependent optical changes in the reaction of T201S/I100W ToMOH<sub>red</sub>D with dioxygen at 675 nm (black squares) and 500 nm (red circles) and fit to a function representing two consecutive, irreversible processes. Fitting results are represented as blue lines in spectra and with residuals shown below.
Scheme 1.
Scheme 2.
Reaction Models for the Formation and Decay of $\text{T201}_{\text{peroxo}}$ during Dioxygen Activation of $\text{T201X/I100W ToMOH}$. 
Scheme 3.
Proposed Mechanism for the Interconversion of $T201_{\text{peroxo}}$ and $\text{ToMOH}_{\text{peroxo}}$. 

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Scheme 4.
Proposed Mechanism of Aromatic Hydroxylation by ToMOH$_{peroxo}$ and T201$_{peroxo}$ in T201 Variants of ToMOH.
Chart 1.
Dioxygen Chemistry in (A) Wild-Type ToMOH and (B) the T201S Variant of ToMOH at 4 °C, pH 7.
Table 1
Consecutive Decay Kinetic Constants for T201$_{\text{peroxo}}$ in the Reaction of T201C ToMOH$_{\text{red}}$D with Phenol in Dioxygen-Saturated Buffer.

<table>
<thead>
<tr>
<th></th>
<th>phenol-$d_5$</th>
<th>phenol-$d_5$</th>
<th>KIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{decay1}}$ (s$^{-1}$)</td>
<td>7.2 ± 0.3</td>
<td>8.7 ± 0.3</td>
<td>0.82 ± 0.05</td>
</tr>
<tr>
<td>$k_{\text{decay2}}$ (s$^{-1}$)</td>
<td>2.2 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>
### Table 2

Consecutive Decay Rate Constants and KSIE Values for T201_{peroxo} in the Reaction of T201C ToMOH_{red}D with Dioxygen in H$_2$O or D$_2$O Buffer.

<table>
<thead>
<tr>
<th></th>
<th>H$_2$O</th>
<th>D$_2$O</th>
<th>KSIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{decay1}}$ (s$^{-1}$)</td>
<td>1.9 ± 0.1</td>
<td>0.557 ± 0.007</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>$k_{\text{decay2}}$ (s$^{-1}$)</td>
<td>0.241 ± 0.004</td>
<td>0.0382 ± 0.0001</td>
<td>6.5 ± 0.1</td>
</tr>
</tbody>
</table>

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Table 3
Consecutive Decay Rate Constants and KSIE Values for T201\textsubscript{peroxo} in the Reaction of T201C ToMOH\textsubscript{red}D with Dioxygen and 10 mM Phenol in H\textsubscript{2}O or D\textsubscript{2}O Buffer.

<table>
<thead>
<tr>
<th></th>
<th>H\textsubscript{2}O</th>
<th>D\textsubscript{2}O</th>
<th>KSIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{\text{decay}1} \text{ (s}^{-1}\text{)})</td>
<td>7.2 ± 0.3</td>
<td>6.26 ± 0.07</td>
<td>1.15 ± 0.06</td>
</tr>
<tr>
<td>(k_{\text{decay}2} \text{ (s}^{-1}\text{)})</td>
<td>2.2 ± 0.2</td>
<td>1.02 ± 0.03</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
</table>

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Table 4

Formation and Decay Rate Constants for T201_{peroxo} and W-radical Intermediates Generated During Dioxygen Activation of I100, T201S, and T201S/I100W ToMOH_{red}.

<table>
<thead>
<tr>
<th></th>
<th>I100W</th>
<th>T201S</th>
<th>T201S/I100W</th>
</tr>
</thead>
<tbody>
<tr>
<td>T201_{peroxo}</td>
<td>nd$^a$</td>
<td>$k_{\text{form}} = 88 \pm 7 \text{ s}^{-1}$</td>
<td>$k_{\text{form}} = 55 \pm 14 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>I100W-radical</td>
<td>$b k_{\text{form}} = 0.804 \pm 0.001 \text{ s}^{-1}$</td>
<td>$b k_{\text{decay}} = 0.054 \pm 0.002 \text{ s}^{-1}$</td>
<td>nd$^a$</td>
</tr>
</tbody>
</table>

$^a$Not determined.

$^b$Taken from ref. 31.
Table 5

Quantification of I100W–Radical Species Generated in T201X/I100W ToMOH.

<table>
<thead>
<tr>
<th></th>
<th>Coupling (%)</th>
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<tbody>
<tr>
<td>I100W</td>
<td>55 ± 1%</td>
</tr>
<tr>
<td>T201S/I100W</td>
<td>49 ± 1%</td>
</tr>
<tr>
<td>T201C/I100W</td>
<td>36 ± 3%</td>
</tr>
<tr>
<td>T201G/I100W</td>
<td>38 ± 1%</td>
</tr>
<tr>
<td>T201V/I100W</td>
<td>31 ± 2%</td>
</tr>
</tbody>
</table>

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